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Genetic and environmental factors associated with the risk of cognitive decline and dementia

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Index

Chapter I

Introduction

Alzheimer's disease (AD) is a debilitating and degenerative dementia with a senile onset (over 65 years, but can occur even in the presenile age - before age 65). In 1906, Aloise Alzheimer, a German psychiatrist, identified and presented the first case of what became known as Alzheimer's disease in a fifty-year-old woman called Auguste D.

In this report, he underlined several cardinal features of the disorder that are currently observed in most patients: progressive memory impairment, disordered cognitive functions, altered behavior including paranoia, delusions, and loss of social appropriateness and a progressive decline in language function.

In the early stages of AD, the most commonly recognized symptom is inability to acquire new memories, such as difficulty in recalling recently observed facts. Gradually, bodily functions are lost, ultimately leading to death. Individual prognosis is difficult to assess, and the duration of the disease varies. AD develops for an indeterminate period of time before becoming clinically apparent, and it can progress undiagnosed for years. In fact, the cause and progression of Alzheimer's disease are not well understood. When AD is suspected, the diagnosis is usually confirmed by behavioral assessments and cognitive tests, often followed by a brain scan.

An internationally agreement upon standard of TEST for clinical diagnosis of AD includes a detailed history, functional measurement of decline such as instrumental activity of daily living scales, mini mental status examination tests (MMSE), Clinical Dementia Rating (CDR), Disability

Assessment for Dementia (DAD), neuropsychological evaluation, neurological and psychiatric examination.

The MMSE test, for example, allows to classify subjects with dementia according to categories of clinical severity and to the rate of cognitive decline. This test, developed by Folstein in 1975, is widely used, since it allows a semi quantitative evaluation of the degree of cognitive impairment (Folstein MF et al., 1975).

The cognitive evaluation obtained from these test is today accurate and reached up to 90% of the confirmed autopsy cases. AD must be differentiated from other causes of dementia: vascular dementia, dementia with Lewy bodies, Parkinson's disease with dementia, frontotemporal dementia, and reversible dementias.

An intermediate stage between normal ageing and dementia has long been recognized by several classification systems and these attempts have viewed the condition as either physiological ageing or the beginnings of a pathological process and has now defined as mild cognitive impairment (MCI) (DeCarli C, 2003).

Originally, MCI diagnosis required the presence of memory complaint (preferably corroborated by an informant), objective memory impairment for age, preserved general cognitive function, normal functional activities, and no dementia. The impact of MCI is different in a high functioning professional aged 55 to a retired 80 year old with few cognitive demands in their life.

Because not all individuals with MCI progress to clinical dementia, it is critical to identify risk factors and biomarkers for the development of dementia and AD in this cohort. Toward this end, Aggarwal et al. showed that possession of the ε4 variant of the allele for apolipoprotein E (APOE), a known risk factor for the development of AD in normal elderly individuals, is also associated with increased risk of developing AD in individuals with MCI (Aggarwal NT et al., 2005).

Epidemiology of Alzheimer's disease

AD is now a worldwide spread disease, as the incidence of AD increases with age, it is particularly important to consider the mean age of the population of interest. In the United States, Alzheimer's disease prevalence was estimated to be 1.6% in 2000 both overall and in the 65–74 age group, with the rate increasing to 19% in the 75–84 group and up to 42% in the older than 84 year group (Hebert LE et al., 2003).

Another study estimated that in 2006, 0.40% of the world population (absolute number 26.6 million, range 11.4–59.4 million) would be afflicted by AD, that the prevalence rate would triple and the absolute number would quadruple by 2050 (Brookmeyer R et al., 2007).

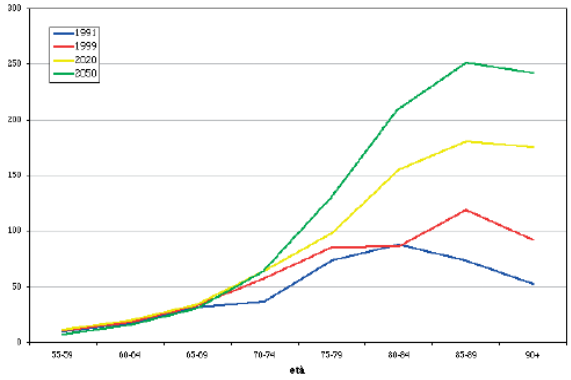

Figure 1: Estimated number of people with AD in Italy in 1991, 1999, 2020 and 2050

In Italy, AD affected subjects are between 800.000 and 1 million and unfortunately the number of new cases/year (incidence) is going to dramatically increase as a consequence of the progressive increase of the mean age and life expectancy in our population. In Fig 1 the increasing number of Italian subjects affected by Alzheimer's disease during the last 10 years and the projection of the incidence for the next 40 years are shown.

Pathogenesis of Alzheimer's Disease

Alzheimer's disease is a heterogeneous multifactorial and progressive neurodegenerative disease that affects specific areas of the brain. The neuro-pathological hallmarks of the disease are: neuritic senile plaques, neurofibrillary tangles, neuronal atrophy and cortical neurodegeneration (Terry RD, 1994) AD begins in the entorhinal cortex, a brain region that is near the hippocampus and has direct connections to it. Healthy neurons in this region begin to work less efficiently, lose their ability to communicate, and ultimately die. This process gradually spreads to the hippocampus, the brain region that plays a major role in learning and is involved in converting short-term memories to long-term memories. Finally, affected regions begin to atrophy**.**

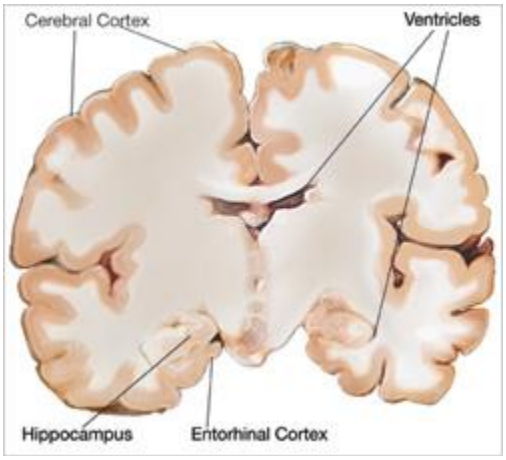

Figure 2: Specific areas of the brain mainly affected by AD

Senile Plaques

Senile plaques (Fig. 3), with diameters between 10 and 200µm, are extracellular deposits of amyloid in the gray matter of the brain. The deposits are associated with degenerative neural structures and an abundance of microglia and astrocytes (Mancardi GL et al., 1983; McGeer

PL et al., 1993). These plaque have been identified in several AD brain areas like hippocampus, amygdale and in brain cortex.

Beta amyloid peptide (Aβ) is the main component of senile plaques: Aβ is a short peptide of 40-42 amino acids and with a molecular weight of 4.2kDa. Moreover, amyloid filaments are able to be folded in a beta sheet structure (McLean C and Beureuthe K, 1997).

Aβ peptide derives from a precursor protein molecule named Amyloid precursor protein or APP that undergoes to an abnormal processing.

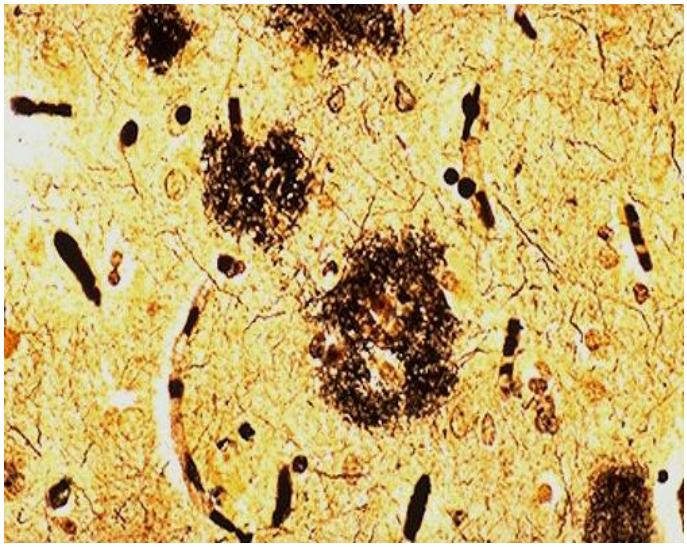

Figure 3: Senile plaques in affected AD brain

Senile plaques are surrounded also by other molecules present in minor amounts such as cytokines, apolipoprotein E and inflammatory molecules

Neurofibrillary tangles (NFT)

NFT (Fig. 4) are a major microscopic lesion of AD and are located primarily in large pyramidal neurons of Ammon's horn and the cerebral neocortex, although neurofibrillary pathology is also encountered in deep structures, including midbrain and pontine tegmentum, basal nucleus of Meynert, and hypothalamus (Braak H and Braak E, 1991).

Morphologically, NFT are classically described as consisting of numerous paired helical filaments (PHF), (Kidd M, 1963) which are composed of 2 axially opposed helical filaments with a diameter of 10 nm and a half-period of 80 nm (Wisniewski HM et al., 1964).

Healthy neurons have an internal "bone" performed by microtubular structures; these structures are mainly composed and stabilized by tau protein.

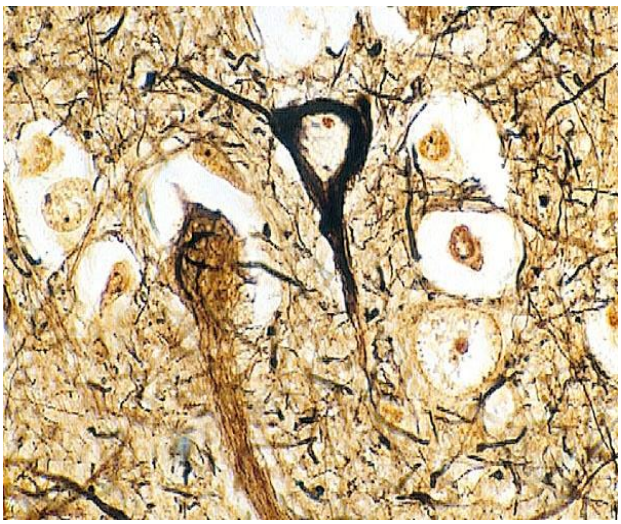

Figure 4: Neurofibrillary tangles in affected AD brain

In Alzheimer's disease, this protein is not in a normal conformation: tau protein results in an iper phosphorilated form and this implies a grater probability of self annealing of tau units (Buèe L et al., 2000). This abnormal annealing of tau protein causes a degeneration of microtubular structure and compromise axonal transport resulting in a very instable conditions where neurons are no longer able to communicate in an optimal manner.

Pathogenetic hypothesis

Alzheimer's disease is a very complex and a multifactorial disease in which intrinsic, genetic and environmental factor interact with each other and contribute to the onset of the disease.

A large number of hypothesis and theories have been proposed to explain all the processes and the mechanisms leading to the pathogenesis and the onset of the disease. For example amyloid hypothesis, inflammatory hypothesis, vascular hypothesis, viral hypothesis.

Amyloid hypothesis

Classical pathological features of AD are the presence of senile plaques and neurofibrillary tangles in SNC. The main component of senile plaques is the β amyloid peptide resulting from a proteolitic cut of the amyloid precursor protein (APP). APP is the Aβ peptide precursor and is a trans-membrane glycoprotein widely expressed (it is also present on platelets), produced by the endoplasmatic reticulum and involved in the neuronal and dendritic growth and synapses formation.

The metabolic cleavage of APP involves three different enzymes called α, β and γ secretase. The membrane protein α-secretase is the first enzyme that cleaves βAPP molecule between residues Lys687 and Leu688; in this way a small peptide remains anchored to the membrane (αCTF) and a soluble N-Ter peptide (sAPPα) is released in the extracellular compartment. Proteolitic

cleavage by α secretase prevents Aβ release and results in the so called non amyloidogenic pathway (Thinakaran G and Koo EH, 2008).

On the other hand, the combined and sequential cleavage on APP by β and γ secretases releases Aβ peptide composed of 40-42 aminoacids. β secretase (called also BACE) in fact cuts βAPP molecule between residues Met 671 and Asp672 residues; two fragments are generated: a βCTF, linked to the membrane and a N-Ter peptide named sAPPβ. The trans-membrane fragment becomes substrate for the γ secretase enzyme that produces different small peptides (Aβ 40/42/43) resulting by Ile712, Tnr 714 e Val 715 cleavage.

βA 40-42 peptide, synthesized mainly in the endhoplasmatic reticulum and in Golgi system are majar form of Aβ produced during βAPP metabolism. In the pathogenesis of AD, accumulation of Aß in the brain, particularly Aß42, is considered to be an important step (Small DH and McLean CA, 1999). Aß40 is the major form of secreted Aß. However, Aß42, the minor form, aggregates more readily and is thought to seed amyloid fibril polymerization during the early stages of plaque formation (Jarrett TJ et al., 1993). Amyloid aggregates form insoluble filaments that are about 7-9 nm in diameter. The fibrillar forms of Aß, ß-pleated amyloid fibrils, consist of antiparallel-pleated sheets, thought to be especially neurotoxic. Understanding how and where Aß aggregation begins may elucidate the mechanism of AD pathogenesis. Recent reports suggest that Aß is generated and accumulates intracellularly (Turner RS et al., 1996; Skovronsky DM et al., 1998; Gouras JK et al., 2000). It has also been reported that

intraneuronal accumulation of Aß peptides may precede the detection of extracellular amyloid plaques and NFTs (Gouras JK et al., 2000), and that this may be associated with neurodegeneration (Chui DH et al., 1999). Masliah et al. showed by electron microscopy that neuronal processes near plaques can display fine intracellular amyloid fibrils adjacent to rough ER and coated vesicles (Masliah E et al., 1996). Recent evidence suggests that neurotoxic effects of Aß may be independent of plaque formation *in vivo* (Hsia AY et al., 1999; Chui DH et al., 1999) and independent of ß-pleated Aß formation *in vitro* (Lambert JC et al., 1998; Hartley DM et al., 1999; Walsh DM et al., 1999).

Inflammatory hypothesis

The hypothesis that inflammation may participate in Alzheimer's disease pathogenesis was first articulated about 20 years ago, and despite two decades of work, many of the central questions regarding the inflammatory response in the Alzheimer's disease brain remain unanswered (

One of the hypothesis is that the presence of amyloid plaques and neurofibrillary tangles may stimulate a chronic inflammatory reaction to clear this debris.

Inflammatory response is a very complex process, slightly regulated that involves the synthesis and the release of numerous factors such as cytokines, inflammatory mediators, histamine, prostaglandine and also some hormones (McGeer EG and McGeer PL, 1998).

In AD brain, in fact, inflammatory response appears to be alterated: high levels of cytokines as Tumor Necrosis Factor (TNF), Interleukin-1 (IL-1), IL-6, IL-8 IL-10 and some interferon seems to be elevated (Baumann H and Gauldie J, 1994). A recent report showed that alpha 1 antichymotrypsin (ACT) levels were higher in AD patients than in CIND (cognitive impairment but not dementia) or in controls (Porcellini E, et al 2008).

In addition pro-inflammatory cytokines enhance Aβ40 and Aβ42 peptides production and inhibit amyloid precursor protein (APP) production, on the whole and especially the soluble fraction of APP with neuronal protective effect.

Astrocytes and microglia have a pivotal role in the inflammatory activation. Astocytes represent about the 40% of the total population of the CNS and are involved in important brain functions such as the regulation of neuronal growth and are able to repair to neuronal damages.

In AD brain astrocytes have been found associated to β amyloid plaques (Norenberg MD., 1994; Masliah E et al., 2000).

Astrocites produce a large number of citokines as IL-1 (Griffin WS, 1989), IL-6 (Bauer J, 1992), TNF (Sawada M, 1989) and alpha 1 antichymotrypsin (ACT) that might have a pivotal role in AD pathogenesis since they could modify the normal metabolism of APP pathway (Goldgaber D, 1989; Altstiel LD, 1991).

In fact, many Authors have demonstrated that in Alzheimer's disease, several of inflammatory molecules and cytokines are increased. A recent report showed that ACT levels were higher in AD patients than in CIND

(cognitive impairment but not dementia) or controls (Porcellini E et al., 2008).

Also microglia is located inside the neuropathological lesions associated to AD. These cells are phenotipically similar to blood monocytes and tissue macrophages and replace their functions in the brain (Ransohoff RM and Perry VH, 2009). Microglial cell expose on their surface complement receptors, MHC I and MHC II molecules and release cytokines and molecules involving in acute phase inflammation.

Microglia, such as astrocytes, have a double role in the cellular response against neuronal damage: one pathogenetic function of promoting inflammation promoter and a protective role (Gonzales-Scarano F et al., 1999).

Anyhow, it is not clear whether inflammation in AD is an early event or a secondary process inducted by a pre existing damage.

The importance of inflammation in AD is further strengthened by epidemiological data showing that the routine use of the non-steroid anti-inflammatory drugs (NSAIDs) was associated with a decreased incidence of AD (Breitner JC et al., 1994.; In't Veld BA et al., 2001).

Moreover, molecular genetic studies have indicated also that single nucleotide polymorphisms (SNPs) located in inflammatory genes could be linked to Alzheiemer disease (Licastro et al., 2007). These SNPs may act both as risk and/or protective factors for the disease.

Vascular Hypothesis

The vascular hypothesis of Alzheimer disease, first proposed by De La torre in 1993, provides substantial evidence that suggests vascular risk factors (VRF) play a critical role in the development of cognitive decline and AD during aging (de la Torre JC and Mussivand T, 1993).

There are many notable observational epidemiological studies that have helped to clarify the role of vascular risk factors for AD; these include the Honolulu Asia Aging Study, the Goteborg Study ,and the Frammingam Study. All these investigations have underlined the possible role of hypertension, diabetes, smoking, lipids homocysteine, physical inactivity, fat intake, systemic marker of atherosclerosis and other vascular factors that may be associated with increasing or decreasing risk of cognitive impairment and AD. Moreover, several studies suggest an important role for blood vessels alterations in the pathogenesis of AD dementia (Skoog I et al., 1996; Hofman A et al., 1997; Snowdon DA et al., 1997)

Numerous structural and functional abnormalities of the cerebro-microvasculature in AD have been also identified, (de la Torre JC and Mussivand T, 1993; Kalaria RN, 1996) including decreased microvascular density and vascular distortions such as vessel kinking, twisting, tortuosity, and looping. In addition, several active functions of the blood-brain barrier, including glucose transport, are diminished in AD. Furthermore, AD brain vessels are oxidatively damaged, express inflammatory mediators, and over-produce nitric oxide. (Dorheim MA et al., 1994). Elevated vascular

production of nitric oxide, derivatives with potential neurotoxin action, could contribute to neuronal injury and death in AD.

We have just discussed that results of epidemiological studies suggest that chronic use of certain drugs (NSAIDs) significantly decreases the risk of Alzheimer's disease (Breitner JC et al., 1994.; In't Veld BA et al., 2001). Also if brain inflammation has became a major focus for Alzheimer's disease research, brain inflammation cannot, however, explains the risk reduction conferred by drugs that may lack substantial anti-inflammatory activity. Some putative Alzheimer's disease preventive drugs, such lovastatin, pravastatin, H2 antagonist, aspirin, also inhibit angiogenesis. These observations have led to consider again the role of the brain vascular endothelial cells in the pathogenesis of the disease.

Endothelial cells in fact could respond to both hypoxia and inflammation by regulating angiogenesis response. Brain angiogenesis is a tightly controlled process that requires chemotactic, proteolytic and mitogenic activities of the endothelial cells (Plate KH, 1999). For instance an increasing expression of vascular endothelial growth factor (VEGF), transforming growth factor b (TGFb) and tumor necrosis factor a (TNFa) may control angiogenesis in the brain (Tarkowsky E et al., 2002)

Ultrastructural studies have shown that β-amyloid plaques are closely associated with brain microvessels, and that Alzheimer's disease brain capillaries contain preamyloid deposits (Miyakawa T, 1997) Furthermore the β-amyloid plaque generates reactive oxygen species that damage brain endothelium (Liu F et al., 2000).

Progressive deposition of amyloid precursor protein leads to accumulation of the β-amyloid plaque, which generates more reactive oxygen species and further induces endothelial damage. By this way endothelial-dependent events may contribute to β-amyloid accumulation in the brain of patients with Alzheimer's disease and neuronal death (Vagnucci A, 2003)

Viral Hypothesis

Alzheimer's disease is the leading cause of dementia in developed countries. Its etiology is recognized as multifactorial, with the possible inclusion of infectious agents. In the 1960s and 1970s, researchers observed elevated levels of antibodies to herpes simplex virus type 1 (HSV-1) in patients with psychiatric disorders (Cleobury JF et al., 1971; Lycke E et al., 1974).

On the basis of these results, Sequiera et al, studied HSV-1 nucleic-acid sequenze in the brain of demented and psychiatric patients (Sequiera LW et al., 1979) and found that the HSV-1 genome was present in brain samples of elderly patients with dementia.

HSV-1 was found in both AD and normal aged brains (Jamieson GA et al., 1991; Jamieson GA et al., 1992; Wozniak MA et al., 2005).

The association of virus, in particular herpes virus, with AD, could be involved for several reason:

1) in acute HSV-1 encephalitis, infection targets particular regions, including hippocampus and the frontal and temporal cortices, which are also prominently affected in AD (Denaro FJ et al., 2003)

2) viral DNA has been found in the same regions as those most affected in AD (Honjo K et al., 2009)

3) HSV-1 DNA was detected in only a very small proportion of brains from younger people, indicating that the virus can enter the brain when an individual becomes older, perhaps because of the age related decline of the immune system (Wozniak MA et al. 2005)

A recent pubblication on Nature Genetics (Lambert et al. 2009) about a Genome Wide Association (GWA) showed indeed, that a genetic cluster on chromosome 19 was strongly associated with Alzheimer's disease. All these genes were located near APOE gene, the main gene associated to sporadic AD. The first set of genes was located in close vicinity of the APOE locus on the chromosome 19 (table 1) and consisted of the poliovirus receptor-related 2 or nectin-2 (NC-2), apolipoprotein E (APOE), the translocase of outer mitochondrial membrane 40 homolog (TOMM-40), the glycoprotein carcinoembryonic antigen related cell adhesion molecule-l6 (CEACAM-l 6) and B-cell/lymphoma-3 (Bcl-3) genes.

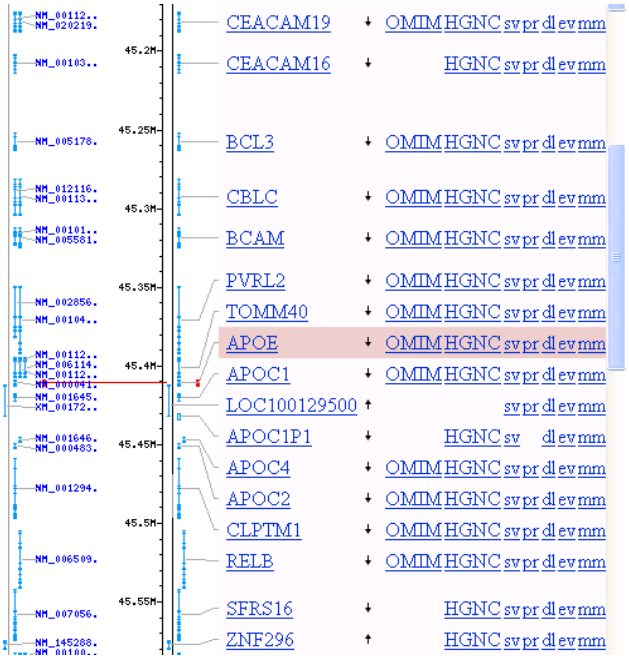

Table 1: genetic locus on chromosome 19 near APOE gene

These gene are involved in several viral pathway, it has been suggested that they result in a genetic signature that might affect individual brain susceptibility to infection by herpes virus family during aging, leading to neuronal loss, inflammation and amyloid deposition (Porcellini E et al., 2010).

It is likely that in genetically predisposed individuals, i.e with the above genetic signature, and with a defective immune response latent viruses might in the brain reactivated with an increased frequency and contribute to neuro-degenerative processes leading to cognitive impairment and AD.

Genetic of Alzheimer's disease

The vast majority of Alzheimer's disease cases are sporadic.

This mean that there is not a dominant genetic transmission, however some genes may act as risk factors promoting late onset AD. On the other hand, about 1% of AD cases are indeed familial forms with an autosomal-dominant inheritance; in this cases the disease has an onset before the age of 65 years (Early onset AD).

Familial form of early onset AD

Three genetic locus seem to be involved in the mendelian autosomal dominant form of Alzheimer:

1) Precurson β amyloid protein (APP) on chromosome 21
2) Presenil 1 (PS1) on chromosome 14
3) Presenilin 2 (PS2) on chromosome 1

The mutant gene APP is located on chromosome 21. Down Syndrome (DS) is the consequent of the presence of an extra copy of the chromosome 21. DS subjects often suffer of Alzheimer and show brain alterations with neuropathological features similar to those of AD, but with an early onset.

As we just mentioned before, APP is the precursor of Aβ peptide, the main component of senile plaques.

It has been reported that mutations on β and γ secretase, the two enzyme involved in the cleavage of APP leading to Aβ formation, could be modify

the normal processing of APP resulting in neuronal death (Goldgaber D et al.,1987; Goate A, et al., 1991).

PS1 gene is located on chromosome 14 and was identified by Rogaev in 1995 (Rogaev EI et al., 1995).

PS1 function is not clear and seems to be involved in the regulation of protein traffic and transduction signal during the development.

60 mutations in PS1 have been identified: many of these mutation are miss-sense mutation localized in transmembrane domain. Mutations in exon 5 and 8 have been correlated to the age of onset of the disease (Cruts M, 1996;). Mutations in presenilin 2 (PS2) on chromosome 1 were first described in 1995 and only 18 potentially pathogenic mutations have been reported (Levy-Lahad E et al., 1995; Rogaev EX et al., 1995).

Recent studies have shown that the lack of PS1 and PS2 prevents the γ-secretase cleavage; this leads to an accumulation of C-terminal fragments and increased production of beta-fragment amyloid that is the basis of the formation of amyloid plaques. In addition, PS1 and PS2 appear to be also targets for the cleavage of some proteins by caspases, activated during apoptosis and this could suggests a role for PS1 and PS2 in neuronal death.

However, most AD cases are late onset (>65 years of age), account for the majority of clinical AD and do not show a clear mendelian pattern of segregation. Other genetic factors may also play an important role in determining late onset AD risk.

Sporadic form of late onset AD

Complex disease, such as sporadic AD, are presumed to be the results of the interactions of many genes and environmental factors. Genetically, AD is an heterogeneous and complex disease , displaying no single or simple mode of inheritance. In 1994, two biochemists W. Strittmatter e G. Salvesen hypotized that in AD, other protein might deposit in brain plaques

One of this protein was found to be Apolipoprotein E (ApoE) the main cholesterol transporter in the brain (Strittmatter WJ et al., 1994). The ApoE is a glycoprotein of about 299 amino acids and whose gene is located on chromosome 19. It is present in three different isophorm called E2, E3 and E4 derived form three different allelic variations: APOE ε2, ε3 e ε4. The combination of these three alleles leads to the identification of six different genotypes being the ε3/ ε3 the most frequent. These isoforms derived from the substitution Arg-Cys in aminoacidic residues 112 and 158.

The apolipoprotein E ε4 allele is the only known genetic variant that has been constantly associated with increased late-onset AD risk. This association has been confirmed in a large number of study and in different ethnic group (Ganguli M et al., 2000). In other studies a protective effect of the APOE ε2 allele in AD patients has been demonstrated (Morrow JA 1999).

The presence of the allele ε4 predispose to an increased risk and is not by itself sufficient to establish the clinical diagnosis since many people with AD do not possess this allele (Laws M et al., 2003; Rubinsztein DC et al., 1999). By contrast, susceptibility for late-onset AD shows less obvious or

no apparent familiar aggregation, and is likely to be governed by an array of common risk alleles across a number of different gene. These genes affect various pathways, many of which are likely to be involved in inflammation, cholesterol metabolism, β-amyloid metabolism, angiogenesis, oxidative stress, and other less defined genes (Combarros O et al., 2002).

In recent years, many studies have tried to clarify whether polymorphisms present in genes regulating inflammation or cholesterol pathway were correlated to a differential risk of developing AD or to a different rate of cognitive decline. For example, Interleukin-1 (IL-1) is a cytokine involved in inflammation. IL-1 beta when released in the blood at high levels induces fever, sleep, anorexia and ipotension. This cytokine is expressed by activated microglia in AD (Sheng JG et al., 1996). On the promoter region of IL-1 beta gene is present a polymorphism at position -511 and the TT genotype increased the risk of AD (Licastro F et al., 2000; Chiappelli M et al., 2006).

IL-10 is another inflammatory gene found associated with Alzheimer's disease. IL-10 gene, located on chromosome 1, is synthesized in central nervous system and its function is to limit the inflammatory response. Many single nucleotide polymorphism (SNP) have been identified in this gene and the most informative one is at position -1082, in the promoter region (Tagore A et al., 1999).

Moreover, studies from our laboratory also confirmed that the presence of AA genotype in the promoter region of IL-10 gene increased the risk of developing AD and the rate of cognitive decline (Lio D et al., 2003).

Alpha-1 antichymotrypsin gene is localized in the chromosome 14 and codes for a phase acute protein. ACT is also present as secondary component in senile plaques and amyloid deposits (Furby A et al., 1991).

ACT is secreted in the brain by reactive astrocytes surrounding amyloid plaques. High levels of ACT were present in cerebro spinal fluid and blood from AD patients (Licastro F et al., 1995; Morgan K et al., 2001).

A SNP in the promoter region at the position -51 resulted strongly associated with AD and with an accelerated rate of cognitive deterioration (Licastro F et al., 2005).

The hydroxyl-methyl-glutaryl Coenzime A reductase (HMGCR) is a gene coding for the limiting step enzyme of cholesterol synthesis. HMGCR is also the target of statins, a group of drugs that act decreasing the cholesterol levels (Chong PH et al., 2002) and some epidemiological studies reports a negative association between the statin use and AD incidence (Kuodinov AR et al., 1998). A polymorphism in the promoter region at position -911 (tranversion C/A) is associated with AD and with a fast cognitive decline (Porcellini E et al., 2007).

Recently, it has been suggested that AD could be an angiogenesis-dependent disorder (Sun Y et al. 2003). Vascular Endothelial Growth Factor (VEGF), a molecule able to stimulate neo-angiogenesis, is localized on chromosome 6. VEGF has also a neuroprotective function stimulating the neuronal survival and the growth, regeneration and differentiation of axons. Moreover, VEGF levels were increased in the neurocortex of AD brains. A SNP in the promoter region at position -2578 (substitution C/A) in Italian population is

associated with an increased risk of developing AD, with an accelerated cognitive decline and an increased rate of progression from MCI to AD (Chiappelli M et al., 2006).

Single SNPs are not very informative to predict the individual risk to develop AD. In fact, all these SNPs explain a little percentage of all cases of Alzheimer's disease, whereas the vast majority (especially for late-onset forms of the disease) have other, more complex genetic determinants (Campion D et al., 1999).

More than 550 other genes have been proposed as candidates for Alzheimer's disease susceptibility, but thus far none has been confirmed to have a role in Alzheimer's disease pathogenesis (Gatz M. et al., 2006).

In the last decades scientists, to understand the pathogenetic mechanisms leading to neurodegeneration and dementia, focused their study on one-two or few genes and on few SNPs. This approach, is very limiting because it attempts to explain a complex and multifactorial disease as Alzheimer with approximate methods.

Recent genome-wide association approaches have delivered several additional AD susceptibility loci that are common in the general population.

Genome-wide association (GWA) studies are best understood as an extension of candidate gene association studies, scaled up to cover hundreds of thousands of markers across the genome in samples usually from several thousand of cases and controls.

The GWA approach allows the detection of much smaller effect sizes than the previous linkage-based genome-wide studies.

However, this sensitivity makes them vulnerable to false positive findings caused by subtle differences between cases and controls that may arise as a result of issues, such as genotyping errors, population stratification, and sample mix-ups as well as the more obvious issue of multiple testing.

In 2009 a great number of GWA studies have been proposed to find strong association with AD (Beecham GW et al., 2009; Carrasquillo MM et al., 2009; Lambert JC et al., 2009; Harold et al., 2009)

The two large GWAS from the UK (Harold D et al., 2009) and France (Lambert JC et al., 2009) were published back-to-back highlighting three novel AD genes, i.e., *CLU* (clusterin; apolipoprotein J), *CR1* (complement component (3b/4b) receptor 1), and *PICALM* (phosphatidylinositol binding clathrin assembly protein). These loci have since received overwhelming support from independent follow-up studies (Carrasquillo MM et al., 2010; Jun G et al., 2010) and currently rank at the very top of the AlzGene meta-analyses, directly following *APOE*. In addition, there are several other SNPs in each of these loci showing highly significant association (p values $< 1 \times 10^{-5}$) with AD risk, leaving essentially no doubt that variants in these or nearby genes represent genuine AD susceptibility loci.

It is important to note, the risk effects exerted by the new GWA loci are small, i.e., they confer a mere ~0.10-fold to 0.15-fold increase or decrease in AD risk in carriers versus non carriers of the associated alleles, compared to a nearly 4-fold increase in AD risk related to the presence of the *APOE* ε 4 allele.

Gene-Gene interaction: epistasis

In typical case-control association studies of complex diseases, candidate genes are examined individually, either evaluating one marker at a time or forming haplotypes over multiple neighbouring loci in and around one gene. These methods make the implicit assumption that susceptibility loci can be identified through their independent marginal contribution to the trait variability (Gambaro G et al., 2000).

Critics have pointed out that findings from many genetic association studies were inconsistent, with many failures of replication (Ioannidis JPA et al., 2001).

It has been suggest that this lack of replication can be a "signature of epistasis" or a gene-gene interaction (Moore JH et al. 2005.; Wade MJ, 2001).

Epistasis was first described by Bateson (1909) as the effect of one gene masking (or literally *standing upon*) the effect of another. The Bateson view of epistasis has also been described as *biological epistasis* (Moore JH and Williams SM, 2005), where variation in the physical interaction of biomolecules affects a phenotype. From a statistical perspective, epistasis was also observed as multiallelic segregation patterns by Fisher (1918) who mathematically described the phenomenon as deviation from additivity in a linear model of genotypes. Statistical epistasis and biological epistasis eventually converge as scientific understanding progresses.

But the study of epistasis has suffered severly from the lack of appropriate statistical methods. Logistic regression analysis and methods based on it, such as synergy factor analysis, are best used only for the examination of binary interactions. Various methods have been proposed for the study of higher order interactions, but several suffer from problems of interpretation. Therefore, methods for the formal analysis of complex gene-gene interactions and gene-protein interaction remain an open question.

For Alzheimer's disease two different new epistasis approaches have been proposed in these last years: the use of Grade of Membership (GoM) method and the Artificial Neuronal Network (ANN).

These two new statistical models have the potential of analyzing the relationship between factors and disease and the degree of interaction of all factors together and with the disease.

Grade of Membership (GoM)

The increasing amount of clinical, genetic, and phenotypic data of multifactorial diseases such AD, requires specific tools able to gather and recompose this information. These tools are not easily available today as the traditional statistical reductionistic approach tends to 'see' things individually, to simplify, and to look at one single element at a time.

Grade of Membership analysis identifes typologies or set group in rich datasets represented by profiles of response frequencies for the variables (Manton et al., 1991; Manton et al., 1992)

This approach has identified sufficient genetic risk sets for Alzheimer's disease (Corder EH et al.2006), vulnerable and robust sets of gene variants in mitochondrial complex I in Parkinson's disease (Corder EH et al.,2006), and multilocus genotypes specific to breast cancer and fibroadenoma (Corder EH et al. 2006).

Using GoM, the user specifies a number of latent groups, extreme pure type risk sets or profiles, to be identified.

The GoM model likelihood can be described after first identifying some indices. One is the number of subjects *I* (*i*=1, 2, . . ., *I*). The second index is the number of variables *J* (*j*=1, 2, . . ., *J*). The third index is *Lj*: the set of response levels for the *J*th variable. This leads to the definition of the basic GoM model where the probability that the *i*th subject has the *Lj*th level of the *J*th variable is defined by a binary variable (i.e. *yijl* = 0, 1).

The model with these definitions is $prob(yijl = 1.0) = \sum_k gik\lambda kjl$ where the *gik* are convexly constrained scores for subjects and the λkjl are probabilities that, for the *K*th latent group, the *Lj*th level is found for the *J*th variable.

A recent paper showed the attempt to find independent risk groups including several genetic variant for cognitive decline and Alzheimer's disease using this fuzzy latent statistic (Licastro F et al., 2007).

Licastro et al. identified four group representing the status and the genetic background: Set I represents low intrinsic risk: there is a low density of pro-inflammatory gene variants at the investigated loci. Sets II, III, and IV represent sufficient risk sets for AD.

According to this model, each risk set is defined by probabilities for each outcome AD status, rate of cognitive decline age group and the various genotypes (IL10, IL6, HMGCR, APOE, ACT, INFg, TNF) found for the loci.

At the same time, individuals are related to the groups via membership scores ranging from zero denoting no resemblance to the risk set to one, i.e. the individual matches the risk set exactly. The scores for highrisk sets were then input into logistic models to estimate the odds of AD and produce 95% CI. To evaluate each variable's information content, statistic '*H*' (Shannon, Bell Laboratories) was estimated for each variable. *H* is close to zero if each group has similar frequencies. Higher values denote increasing information content and differences in displayed frequencies from group to group. Here, the clinical status variables had the highest information content: *H* was 1.33 for AD status/ age and 1.11 for rate of cognitive decline. *IL-10* was the most informative genetic variable (*H*= 1.06), more informative than *APOE g*enotype (*H*= 0.44).

Artificial Neuronal Network (ANN)

Classical statistics predictive models like discriminant analysis, logistic regression, etc., are able to utilize a number of factors simultaneously higher than a human mind. This number generally ranges between 8-15 variables. However, it is not unusual to have at hand, especially when faced with treatment planning for a chronic degenerative disorder, hundreds of different variables, consisting of clinical history data, objective findings,

symptomatology, multi-item scales of different meanings, laboratory examinations and imaging procedures.

With the increased availability and use of functional genomics and digital imaging we now tentatively have at our disposition thousands of data per subject. More features imply more information and potentially higher accuracy. Unfortunately an important paradox is that more features we have, the more difficult information extraction is.

A part from quantitative features, non linearity, complexity, fuzzy interaction are new emerging qualitative features of chronic degenerative diseases which account for most morbidity and mortality in western world. New statistical approaches, based on new mathematical and logic assumptions broadly belonging to artificial adaptive system family allow to tame these intractable data sets.

Actually the coupling of computer science and these new theoretical bases coming from complex systems mathematics allows the creation of "intelligent" agents able to adapt themselves dynamically to problem of high complexity: the Artificial Adaptive Systems, which include Artificial Neural Networks(ANNs) (Grossi E and Buscema M, 2007; Grossi E and Buscema M, 2006)

ANNs are adaptive models for the analysis of data which are inspired by the functioning processes of the human brain (McCulloch WS et al., 1943).

They are systems which are able to modify their internal structure in relation to a function objective. They are particularly suited for solving problems of the non linear type. ANNs are able to reconstruct the approximate rules that

put a certain set of data which describes the problem being considered - with a set of data which provides the solution. The base elements of the ANN are the nodes, also called processing elements (PE), and the connections.

Each node has its own input, from which it receives communications from other nodes and/or from the environment and its own output, from which it communicates with other nodes or with the environment. Finally, each node has a function f through which it transforms its own global input into output.

Each connection is characterized by the strength with which pairs of nodes are excited or inhibited. Positive values indicate excitatory connections, the negative ones inhibitory connections.

The connections between the nodes can modify themselves over time. This dynamic starts a learning process in the entire ANN. The way through which the nodes modify themselves is called "Law of Learning".

The learning process is, therefore, one of the key mechanisms that characterize the ANN, which are considered adaptive processing systems.

The learning process is one way to adapt the connections of an ANN to the data structure that makes up the environment and, therefore, a way to "understand" the data base itself and its internal relations (Rumelhart DE et al., 1986; Personnaz L et al., 1986).

In summary, the aim of the "analyzer" is not to analyze the language of each individual variable, but to evaluate the meta-language which considers the holistic contribution of all the recorded variables (Grossi E, 2010).

Artificial Adaptive Systems and in particular Neural Networks are already emerging as new tools in medical statistics ranging from heart diseases,

gastroenterology and neurology with special regard to Alzheimer disease, stroke and Amyotrophic Lateral Sclerosis (Penco S et al., 2008; Rossini PM et al., 2008; Licastro F et al., 2010; Grossi E, 2006).

In conclusion, data mining by ANN could show a non linear relationship between genetic and environmental variables and show a connectivity map among a high number of variables. This approach may be today very useful to understand the complex mechanisms of multifactorial disease as AD.

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Chapter II

Elevated plasma levels of α-1-antichymotrypsin in age-related cognitive decline and Alzheimer's disease: a potential therapeutic target

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Elevated Plasma Levels of α-1-Anti-Chymotrypsin in Age-Related Cognitive Decline and Alzheimer's Disease: A Potential Therapeutic Target

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Abstract: α-1-antichymotrypsin (ACT), is an acute phase protein and a protease inhibitor produced by the liver and brain. ACT is involved in the pathogenesis of Alzheimer's disease (AD), since elevated ACT concentration was found in cerebrospinal fluid (CSF) and brain from AD. ACT has also been shown to influence amyloid deposition *in vitro* and in animal models of AD.

In this investigation 830 healthy controls, 69 subjects with cognitive impairment and not dementia (CIND), 53 patients with severe clinical AD and 142 patients with mild AD were investigated. Plasma levels of ACT were measured with a new competitive immune enzyme linked immune-assay (ELISA). ACT levels were higher in AD patients than in CIND or controls. An age dependent increase of plasma ACT was present in both healthy elderly and CIND. Patients with mild clinical AD were followed up for two years and stratified according to the rate of clinical deterioration.

CT plasma levels were elevated in AD patients that showed an accelerated rate of cognitive deterioration during the follow up; this increment being prominent in AD with the Apolipoprotein E (APOE) ε4 allele. Therefore, increased peripheral ACT levels in APOE 4 positive patients appear to predict an accelerated clinical progression. Plasma ACT might be used as a surrogate marker to monitor the conversion of pre-dementia stages to AD and the progression of the disease. The development of compounds able to interfere with the ACT biological activity (protease inhibition and/or promotion of amyloid deposition) might have therapeutic relevance for the disease.

Key Words: AD, cognitive decline, CIND, ACT plasma levels, APOE allele.

INTRODUCTION

The major cause of cognitive deterioration in Western societies is Alzheimer's disease (AD). AD is a chronic, complex and clinically heterogeneous neurodegenerative disease, characterized by a progressive impairment of cognitive functions and memory loss [1].

The major pathological hallmarks of AD are the presence of neurofibrillary tangles and β-amyloid plaques associated with hyperactive microglia, activated astrocytes and degenerating neurons [2]. Inflammatory processes are thought to be important contributors to the pathogenesis of AD [3]. The association of pro-inflammatory gene variants with increased risk of AD [4] have reinforced the notion that impaired immune functions indeed play a pathogenetic role in the neurodegeneration associated with the disease.

α-1-antichymotrypsin (ACT), also known as serine protease inhibitor 3 or SERPINA3, is an acute phase protein mainly produced by the liver, that is also widely distributed in the central nervous system. Several lines of evidence suggest that ACT is involved in the pathogenesis of AD. In fact, ACT is produced by astrocytes in the brain and is a secondary component of amyloid deposits in AD brains [5]. In affected brain regions, ACT and Apolipoprotein E (APOE) colocalize with Aβ deposits and reactive astroglia overexpress these molecules [6]. It has been suggested that ACT binds Aβ peptide and affects the rate of amyloid fibril formation *vitro* [7,8]. Findings from a transgenic mouse model of AD have also shown that ACT over-expression promotes Aβpeptide deposition in the brain of these animals [9]. Moreover, both APOE and ACT molecules affected amyloid deposition and cognitive performances in an animal model for AD [10]. More recently, ACT has been shown to influence TAU protein phosphorylation and apoptosis in neuronal cells [11].

Whether peripheral levels of ACT may be of practical use as AD biomarker or indicator of the disease clinical progression, however, remains an open question. In fact, after the initial reports of increased blood ACT concentrations in AD patients [12,13], several studies measured ACT concentrations in blood samples drawn from subjects with AD, with other forms of dementias, and control subjects. Findings from these studies have produced conflicting results; some investigations reporting increased serum ACT levels [14,15], others showing normal ACT blood levels in AD [16,17]. Several reasons such as, different techniques for ACT detection, different criteria for the selection of controls and AD patients and small numbers of cases and controls included in

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the studies, may account for contradictory results regarding the association of abnormal ACT plasma levels with AD.

Increased peripheral blood ACT was also shown to correlate with decreased cognitive function in patients with AD [18]. More recent reports confirmed that the ACT levels were elevated in both cerebrospinal fluids and blood from patients with AD and that ACT plasma levels correlated with cognitive decrement in AD patients [19-21]. It is of interest that thereafter, a population study showed that increased ACT serum levels correlated with decreased cognitive performances in non-demented elderly [22].

Finally, gene polymorphism in the ACT gene was associated with increased ACT plasma levels in AD patients [15].

In the present study we have reported data regarding ACT plasma levels detection by a sensitive ELISA method from a large cohort of cognitively healthy elderly subjects with cognitive impairment and not dementia (CIND) and two populations of patients with mild or severe clinical AD. ACT plasma levels were also correlated with cognitive performances in a cohort of AD patients followed up for 2 years. Our findings showed that ACT plasma levels progressively increased with cognitive deterioration in CIND and AD patients.

MATERIALS AND METHODS

Patients and Controls

Different cohorts of healthy, cognitively impaired or demented patients were included in this study. The first population belongs to the "Conselice study of brain aging" [23] from Northern Italy and included 830 cognitively healthy control subjects (controls). From this population study 69 subjects with cognitive impairment and non dementia (CIND) and 53 patients with clinical severe dementia (mini mental state evaluation, MMSE=12±6) of AD type were also investigated. Another group of 142 patients from Northern Italy (Milan) with the clinical diagnosis of probable moderate or mild AD (MMSE=18±5) was also included. This latter group of AD patients was followed up for two years and their cognitive performances recorded. Patients and controls were Caucasians and informed consent from each control and a relative of each AD patient was obtained.

Diagnosis of probable AD was performed according to standard clinical procedures and followed the NINCDS/ADRDA [24] and DSM-IV-R criteria [25]. Cognitive performances were measured according to MMSE. Cognitive decline during the longitudinal follow up in AD patients was also assessed by the MMSE scores, according to the method suggested elsewhere [26], and AD patients were divided into three groups with different degree of deterioration rate: (FAST=F, patients with a decrement of more than five points of MMSE per year; INTERMEDIATE=I, patients loosing 2–4.9 points/year; SLOW=S, AD loosing less than 2 points/year).

Subjects from the "Conselice" study scoring below 24 at the MMSE underwent further examination with mental deterioration battery and those with cognitive impairment at neuropsychological testing but not meeting the DSM-IV criteria for dementia were labelled as CIND [23]. A group of CIND were followed up for 4 years and their cognitive evolution monitored.

DNA Extraction and Polymorphism Detection

DNA extraction from peripheral blood leukocytes, APOE and ACT -51 promoter polymorphism genotype were assessed, as previously described [27].

CRP Detection

Plasma levels of C-reactive protein (CRP) was measured on venous blood using the N-high sensitivity CRP assay with latex-enhanced immunonephelometricy assay on a BN II analyser (Dade Behering, Milan, Italy).

ACT Serum Levels Detection

Plasma ACT detection was made by a competitive ELISA assay as previously described [14,15] with slight modifications. Briefly, 96 well plates were coated with 100 µl of purified ACT, (Sigma, Milan) at the concentration of 1µg/ml in buffer (15mM Na_2CO_3, 34mM $NaHCO_3$, 3mM NaN_3 pH=9.6) and incubated overnight at 4°C. Plates were washed 3 times with a 300 µl of Dulbecco washer solution (DBSS) (0.88 mM $CaCl_22H_2O$, 0.138M NaCl, 2.7mM KCl, 7.86mM $Na_2HPO_42H_2O$, 1.47mM KH_2PO_4 pH7.4) + 0.5% of bovine serum albumin (BSA), blocked with 100µl of DBSS + 1% of BSA and then incubated at 37°C for 30 minutes. Two fold serial dilutions (50 µl) of ACT (standard curve,: 0-60 µg/ml: 0 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml and 60 µg/ml) or plasma sample (1:200 dilution) were incubated with rabbit anti-human ACT antibody (Dako, Milan). Polyclonal rabbit antibody anti human ACT (50µl; 1:1000 dilution, DAKO, Milan) was added.

Plates were incubated for 2 hours at 37°C, thereafter washed 3 times with 300µl of DBSS+0.5%BSA and 100µl of secondary anti-rabbit IgG peroxidase conjugate (SIGMA, Milan 1:4000 dilution) were added. Plates were incubated for 2 hours at 37°C, washed and peroxidase substrate (100µl; ROCHE, Milan) finally added for 15 minutes at 37°C. Absorbance was read by an automatic ELISA reader at 405nm (BIORAD, Milan).

Statistical Analysis

Statistical analysis between the mean value of different variables from AD, controls and CIND were performed by one way ANOVA test followed by appropriate post-hoc comparison and Bonferroni correction (SPSS 11.01). Genotype and allele distribution was evaluated by using the Fisher test. Linear regression analysis to assess correlation coefficients was also performed. Continuous variable values are shown as mean ± 1 standard deviation.

RESULTS

Number of subjects, age, gender and MMSE scores from the healthy elderly, patients with CIND, and from two groups of patients with clinical probable mild or severe AD is reported in Table **1**.

Plasma levels of blood ACT in the different groups of elderly with or without cognitive impairment are shown in

Table 1. Clinical Features of Elderly Populations with or without Cognitive Alteration, Mild or severe AD. Cognitive Performance were Assessed by MMSE score. n.a. Not Available

	N	Age	Gender	MMSE	Education	BMI	Smoke (%) Yes ex no
Healthy elderly control (ctr)	830	73±6	380M/450F	28±1	4.7±2.4	28.7 ± 4.5	10 32 58
Cognitive impairment non demented (CIND)	69	78±8	32M/37F	21±2	3.1±1.6	28.8 ±5.6	6 26 68
Mild /moderate clinical AD	142	77±8	48M/94F	18±5	n.a	n.a	n.a
Senile severe dementia	53	85±6	11M/42F	12±6	2.5±1.5	25.5 ±4.9	2 13 85

Fig. (1). Only one ACT plasma level determination was performed in all groups at the beginning of the study and ACT levels were not measured during or at the end of the follow up.

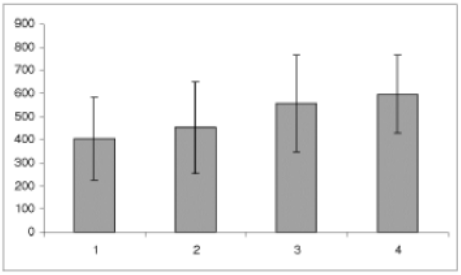

Fig. (1). Plasma levels of ACT in healthy controls (ctr = 1; 406±178 ug/ml), CIND (CIND=2; 452±196 ug/ml), and in patients with mild (mild AD=3; 557±209 ug/ml) or severe AD (severe AD=4; 597±168 ug/ml); data are shown as mean ± SD. Statistics: ANOVA test, F=41.823 and p=0.0001.* p<0,001.

Circulating levels of ACT were higher in patients with mild AD (557±209 μg/ml; p=0.0001) or severe clinical AD (597±168 μg/ml; p=0.0001) than in CIND (452±196 μg/ml) and in controls (406±178 μg/ml); patients with severe clinical AD showing the highest plasma ACT levels.

Subjects with CIND showed a slight increment of plasma ACT, however, difference with control group was not statistically significant (p= 0.18).

A light age dependent progressive increase of blood ACT levels was present in healthy elderly, as assessed by regression analysis between these two variables (R = 0.11; p = 0.001); the oldest elderly showing the highest ACT levels (451±182). ACT blood levels was also influenced by age in subjects with CIND (R=0.34; p=0.004). However, this age dependent effect was not evident in patients with mild or severe AD (R=0.12, p=0.35).

Genetic polymorphism in the promoter region of ACT at position -51 gene was also investigated. No difference was found in allele frequencies and genotype distribution among controls, CIND, mild and severe AD subjects (χ^2=1.562; p=0.955).

A possible correlation of ACT plasma levels with the presence of APOE ε 4 allele, the most common genetic risk factor for AD, was also tested in all groups (two AD populations were pooled to increase statistical power). The APOE ε 4 allele did dot affected the ACT concentrations in controls (ε 4^+=396±176; ε 4^-=407±178, p=0,49), or CIND (ε 4^+=464±162; ε 4^-=454±202, p=0,88) and AD (ε 4^+=549±176; ε 4^-=585±219, p=0,24).

CRP levels were also measured in controls, CIND and patients with severe AD. AD patients showed higher (p = 0.0001) CRP levels than CIND and controls, as shown in Table **2**. Differences in CRP levels from CIND and controls were also statistically significant (p=0.016).

Table 2. Plasma Levels of CRP in Healthy Controls, CIND and in Patients with Severe AD: Data are Shown as Mean ± SD. Anova test F=12,225 p=0,0001

	CRP mg/dl	SD
Healthy Control	0,471	0,667
CIND	0,617	1,104
Severe AD	1,008	1,624

Moreover, patients with mild AD (n=142) were also followed up for 2 years. During the follow up MMSE scores were recorded and patients stratified in three groups according to the rate of cognitive decline: fast (F), intermediate (I) and slow (S). ACT plasma levels at the beginning of the follow up were higher in AD patients showing later on a F cognitive decline than those from patients showing an I or S deterioration rate of cognitive decline. However, differences were not statistically significant (Table **3**; p=0.280).

The three groups of AD patients with cognitive follow up records were further stratified according to the presence of the APOE ε 4 allele (Table **4**). Among patients carrying the APOE ε 4 allele, those with the fast cognitive decline showed the highest ACT plasma levels (p=0.046).

Plasma ACT was measured in a small group of CIND that was also cognitively monitored for 4 years. ACT plasma levels at the beginning of the 4 year follow up were higher in CIND subjects developing AD (n=24, ACT=456±154 μg/ml) than in those not developing AD (n=9, ACT=351±141

μg/ml). Once again, ACT plasma levels in CIND developing AD were higher than those of control subjects (456±154 vs 351±154 μg/ml).

Table 3. ACT Plasma Levels in Patients with Mild AD Stratified According to Three Different Rates of Cognitive Decline: S=slow, I=intermediate and F=fast. p=0.280

Rate of Cognitive Decline in AD Patients	ACT Plasma Levels (ug/ml)
S (59)	527±161
I (51)	566±251
F (32)	598±213

Table 4. ACT Plasma Levels in Patients Stratified According to the Presence of the APOE ε4 and the Rate of Cognitive Decline: S=slow, I=intermediate and F=fast. Anova test F=2.020 p=0.138. Anova test (S+I vs F) F=4.173 p=0.046

Rate of Cognitive Decline in APOE ε 4 Positive AD	ACT Plasma Levels (ug/ml)
S (31)	540±163
I (22)	540±184
F (6)	696±232
S+I (53)	540±170

DISCUSSION

Epidemiological investigations have shown that the routine use of non steroid anti-inflammatory drugs (NSAIDs) was associated with a decreased incidence of AD in a co-twin study [28]. Findings from other more recent USA or Canada population longitudinal studies also confirmed that the use of anti-inflammatory medications decreased the incidence of dementia [29,30]. A Meta-analysis [31] and a systematic review of published results [32] confirmed that NSAIDs offered some protection against the development of AD. Thereafter, it was suggested that APOE genotype could influence the effect of NSAIDs on AD incidence, since the effect of these drugs in decreasing dementia incidence was more pronounced among APOE ε4 carriers [33]. It is of interest that NSAIDs have been shown to influence beta amyloid fibril formation *in vitro* [34] or beta amyloid processing in an AD animal model [35].

It is likely that not all NSAIDs have a comparable effect and different individuals may have a differential response to these compounds. Therefore, it is important to search for biological markers which will help in identifying subjects with increased risk of the disease and, among these, those who would benefit the most by early NSAIDs intervention.

In our previous investigations, we have shown that ACT plasma levels were higher in AD than in controls or patients with vascular dementia [14]. ACT levels were elevated in AD even in the presence of normal levels of other acute phase proteins [18].

It is interesting to note that high plasma levels of ACT were associated with an increased risk of dementia in subjects from the Rotterdam study [36]. In fact, recently high serum ACT levels were reported to be associated with an increased risk of decline on MMSE scores in elderly non-demented participants in the Longitudinal Aging Study from Amsterdam [22]. In a pilot population study from Italy, high serum ACT was associated with incident dementia and AD, when increased C Reactive Protein and Interleukin-6 serum levels were also present [37].

Present findings reinforce and extend the association of ACT plasma levels with age-associated cognitive decline, as observed here in CIND subjects. Plasma ACT levels increased as a function of age in both non demented elderly and CIND. However this increment was higher in the oldest CIND (> 80 years) than in age comparable controls (504±185 vs 451±182, p=0.059).

Our data suggest that increased ACT levels may be associated with progressive cognitive deterioration in subjects with CIND and those with the highest levels of the SERPINA 3 might thereafter develop late onset AD. In fact, preliminary data showed that ACT plasma level in CIND subjects developing AD were higher than those from subjects remaining CIND. However, longitudinal studies on larger cohorts of subjects with CIND or mild cognitive impairment (MCI) are needed to confirm this observation.

CRP plasma levels were also higher in AD patients and CIND than controls; however, a positive linear correlation between ACT and CRP was only present in controls but not in CIND or AD (data not shown). These finding suggest that CRP elevation may be another independent sign of abnormal immune responses associated with age related cognitive alterations.

Our findings confirm in human patients previous observations [10] showing that cognitive impairment in a animal model for AD (PDAPP mice) depended on murine APOE and human ACT genetic backgrounds. These two factors also affected amyloid burden in hippocampus from these mice [38]. A link of ACT peripheral levels and cognitive status was in fact observed in AD patients, since the highest ACT plasma levels were detectable in patients with clinical AD and the fastest cognitive decline during a 2 year follow up. It is of interest that this association was prominent in AD patients carrying the APOE ε 4 allele. These data suggest that the interaction of the ACT phenotype with the APOE ε 4 allele plays a deleterious role in the clinical progression of AD. Our findings reinforce the notion that the APOE ε 4 allele carriers are prone to abnormal inflammatory responses in the brain which in turn may adversely influence cognitive performances.

It has been suggested that APOE genotype affected ACT plasma levels [39]. Thereafter, our data showed that ACT plasma levels were affected by ACT genetic background, since a polymorphism in the ACT promoter region was associated with elevated circulating levels of the SERPINA 3 in both APOE ε 4 positive and negative patients [15,30].

Our recent findings also showed that the APOE ε4 allele was over-represented in subjects with MCI who converted to AD, but did not independently influenced the rate of cognitive deterioration in patients with clinical AD [40].

In the present study no difference in ACT allele or genotype frequencies was observed in CIND or AD when compared with controls. These findings are in accordance with our previous observations that ACT allele and genotype were associated in early onset AD, but not in late onset AD [27].

A limitation of our study is that data are group statistics and the predictive values of ACT detection for individual risk of progression to CIND or MCI and from these conditions to AD are not available. However, these data and others recently reported [36] suggest that ACT peripheral levels, in combination with ACT and APOE genetic backgrounds, might be a reliable surrogate marker to evaluate the differential progression of pre-clinical AD conditions to dementia in population longitudinal studies. Our findings suggested that NSAID might have a positive effect only in those subjects with elevated blood levels of ACT and the APOE ε 4 allele. In other words the potential preventive effect of these compounds might be restricted to a group of at risk subjects with defined biological features.

It has been suggested that some inflammatory molecules could influence the expression of ACT. For example, it has been showed that interleukin 1 (IL-1) and oncostatin (OSM), two pro-inflammatory cytokines localized also in affected areas of AD brain, could directly increase *in vitro* ACT synthesis from human astrocytes, while IL-6 modulated ACT release *via* OSM [41, 42]. IL-1 blood levels in AD, control or CIND were not measured, since these subjects were infections free and did not show clinical inflammatory diseases. However, plasma levels of IL-6 were indeed found elevated in AD patients from the Conselice study [37], and high serum ACT was associated with incident dementia and AD, when increased C Reactive Protein and Interleukin-6 serum levels were also present [37]. These data suggested that different immunological factor might be altered during the developing of cognitive deterioration leading to dementia.

Drugs specifically decreasing the peripheral blood ACT levels might affect the rate of cognitive decline and retard the clinical progression of the disease. For instance, compounds with the ability of regulating the biological activities of SERPINA 3 (protease inhibitory activity and/or aggregation of beta amyloid peptide) might have therapeutic potential and when used in animal models of the diseases might decrease brain amyloid deposition and improve cognitive performances.

Conversely, ACT blood levels might be sensitive to the action of tarenflurbil, a NSAID derived compound with minimal anti-inflammatory activity, able to decrease A beta amyloid peptide levels and to ameliorate cognitive deterioration in patients with mild AD [43,44]. In this clinical situation the modulation of ACT levels by tarenflurbil might be used as a marker to further assess the drug biological and clinical activity.

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ABBREVIATIONS

ACT	=	Alpha1-antichymotrypsin
AD	=	Alzheimer's disease
APOE	=	Apolipoprotein E
CIND	=	Cognitive Impairment and Not Dementia
CRP	=	C-reactive Protein
CSF	=	Cerebrospinal Fluid
MCI	=	Mild Cognitive Impairment
MMSE	=	Mini Mental state Evaluation
NSAIDs	=	Non Steroid Anti-Inflammatory Drugs

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Chapter III

Multivariable network associated with cognitive decline and dementia

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Multivariable network associated with cognitive decline and dementia

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Abstract

Data mining of a large data base from the population longitudinal study named "The Conselice Study" has been the focus of the present investigation. Initially, 65 years old or older participants were interviewed, underwent medical and cognitive examination, and were followed up for 5 years: 937 subjects completed the follow-up. Relationships of 35 genetic and/or phenotypic factors with incident cognitive decline and dementia were investigated. The new mathematical approach, called the Auto Contractive Map (AutoCM), was able to show the differential importance of each variables. This new variable processing created a semantic connectivity map that: (a) preserved non-linear associations; (b) showed connection schemes; (c) captured the complex dynamics of adaptive interactions. This method, based on an artificial adaptive system, was able to define the association strength of each variable with all the others. Few variables resulted to be aggregation points and were considered as major biological hubs. Three hubs were identified in the hydroxyl-methyl-gutaryl-CoA reductase (HMGCR) enzyme, plasma cholesterol levels and age. Gene variants and cognate phenotypic variables showed differential degrees of relevance to brain aging and dementia.

This data analysis method was compared with another mathematical model called mutual information relevance network and results are presented and discussed.

Keywords: Brain aging; Dementia; Data base analysis; Connectivity map; Predictive factors

1. Introduction

Alzheimer' disease (AD) is a chronic progressive disease and the most frequent cause of mental disability and loss of independence among the elderly (Aronson et al., 1991). The disease is characterized by neuro-pathological hallmarks such as, synapsis loss, extracellular amyloid deposition, intracellular fibrillary tangle deposits and neuronal degeneration (Terry, 1994; Trojanowski et al., 1997). A prominent neuropathological feature of the AD brain is also represented by astrogliosis and microglia activation (Griffin et al., 1989; McGeer et al., 1993; Rogers et al., 1988). Abnormal activation of glia cells is now considered an early phenomenon associated with the development of the disease (Griffin et al., 1998) and has been suggested to be implicated in the pathogenesis of AD (Mrak et al., 1995). Genetic studies on inflammatory gene polymorphism associated with the disease have reinforced the notion that abnormal immune responses in the brain play a pivotal role in the disease (Licastro, 2002; Licastro and Chiappelli, 2003).

Some inflammatory genetic markers and the levels of their cognate proteins in the blood have been related to the conversion of pre-dementia states, such as subjects with mild cognitive impairment (MCI) or cognitive impairment and no dementia (CIND) to AD (Chiappelli et al., 2006a,b). A gene

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polymorphism in the promoter region of an acute phase protein called alpha-1 antichymotrypsin (ACT) or SERPINA 3, has been found to be associated with an increased risk of early onset AD and levels of the ACT protein were elevated in sera from AD and CIND patients (Licastro et al., in press). These findings have raised the question whether genetic or phenotypic markers might be used for the screening of persons at high risk of developing cognitive decline and dementia before clinical manifestation of the diseases. The answer to this question might open the possibility of starting preventive protocols for high-risk healthy subjects with the goal of decreasing AD incidence.

AD is a complex multi-factorial disease and it is unlikely that a single biomarker may carry enough information for screening the potential risk of cognitive decline and dementia. Therefore, the use of several biomarkers, either genetic or phenotypic, may be necessary for a comprehensive screening protocol.

To approach this complex situation, informative biomarkers should be generated during longitudinal studies that can validate the clinical endpoints, e.g. cognitive decline, dementia or healthy cognitive performance.

The statistical evaluation of multiple variables in a sufficiently large population is another complex issue and new statistical models able to connect several factors with the disease, to evaluate the degree of linkage among variables and their association with the disease or its absence are needed.

The Conselice Study of brain aging is a population-based prospective study focused on an homogeneous elderly population from Northern Italy (Forti et al., 2001; Ravaglia et al., 2001). The principal aim of this investigation was to explore environmental, epidemiological and intrinsic risk factors for dementia in the elderly (Ravaglia et al., 2001).

From this study a biological and clinical data base during the 5-year follow-up has been generated and biological markers have been found individually associated or not with AD risk, incident cognitive decline and incident AD (Ravaglia et al., 2005; Ravaglia et al., 2006a, Sep. 28; Ravaglia et al., 2006b; Ravaglia et al., 2007). However, results were not conclusive or completely satisfactory, because of the limited power of classical statistical analysis and the difficulty in solving multiple concomitant variable analysis.

Here, we applied a novel data mining process to concomitantly explore the possible association of 35 different variables with CIND and AD and the possible presence of patterns or systematic relationship among variables, as recently described in other topics of medicine (Buscema and Grossi, in press).

This method of data mining is an analytical process designed to search a data base for consistent patterns and/or systematic relationships between variables. The method has the aim to detect patterns from new subsets of data. The ultimate goal of data mining is to discover hidden trends and associations among variables.

The more common algorithms of linear projections of variables are the principal component analysis (PCA) and the independent component analysis (ICA); the former requires a Gaussian distribution of data, while the latter does not require any specific distribution. These classical statistical techniques have limited power when the relationships between variables are non-linear. Moreover, PCA and ICA are not able to preserve the geometrical structure of the original space. Application of these methods may lose important information and establishing precise association among variables having only the contiguity as a known element is difficult. Another limitation of currently used statistical methods is that mapping is generally based on a specific kind of "distance" among variables (e.g. Euclidean, City block, correlation, etc.) and gives origin to a "static" projection of possible associations. In other words, the intrinsic dynamics due to active interactions of variables in living systems of the real world (which could be captured by means of artificial adaptive systems) is completely lost.

A connection scheme able to hypothesize links among variables, i.e. minimum spanning tree (MST) algorithm, as described by Kruskal (1956), could increase the information obtained by the map. The Kruskal MST algorithm of graph theory finds a minimum spanning tree for a connected weighted graph. MST method finds a subset of the edges that form a tree that includes every vertex, where the total weight of all the edges in the tree is minimized. This function has been recently applied in the medical field, especially in biology and medical imaging. However, the MST algorithm is still rare in medical clinics (Frimmel et al., 2004; Lee et al., 2006).

Here, we describe a new paradigm of variables mapping able to create a semantic connectivity map in which: (a) non-linear associations are preserved; (b) there are explicit connections schemes; (c) the complex dynamics of adaptive interactions is captured.

Data recorded during the 5-year follow-up from The Conselice Study participants were elaborated in relation to three different clinical endpoints: no cognitive decline, CIND and dementia. Three major biological hubs connecting variables with the three different cognitive conditions were identified in hydroxyl-methyl-gutaryl-CoA reductase enzyme (HMGCR), plasma cholesterol levels and age.

Biological hubs of variables are detected by the analysis. Related dependent variables converge to these hubs, that in turn may be considered as relevant biological variables in the connectivity map.

Several gene variants of different inflammatory genes and their cognate phenotypic factors showed a variable degree of relevance to brain aging and development of dementia. This is the first attempt to describe an integrated approach illustrating 35 variables in association with the risk of developing cognitive impairment and dementia in the elderly. The identification of biological hubs suggests possible patterns of pharmacological and non-pharmacological intervention with preventive potential against cognitive impairment.

2. Materials and methods

2.1. Data base generation

Data were collected from 1200 elderly, 65 years old or older, living in Conselice county in northern Italy. Female and male participants were interviewed and underwent medical examination and cognitive evaluation in 1999. A blood sample from each subject was taken and each participant was given a computerized radiogram scan of the brain. After 5 years subjects underwent medical and cognitive re-evaluation and 937 elderly completed the follow-up. A detailed description of the clinical protocol and the assessed variables has been already described elsewhere (Ravaglia et al., 2001; Ravaglia et al., 2007).

Diagnosis of dementia was performed according to DSM-IV criteria and clinical AD was defined using the NINCDS-ADRDA criteria (McKhann et al., 1984). Vascular dementia (VD) was diagnosed using NINDS-AIREN criteria (Roman et al., 1993).

Diagnosis of CIND was performed according methods already described (Ravaglia et al., 2004).

2.2. Statistical analysis

The Conselice data base has the aim of increasing our understanding of the pathogenetic pathway leading to cognitive decline and dementia. This goal has been achieved through a new mathematical approach able to point out the relative relevance of each variable in representing a major biological hub. This new paradigm of variable processing aims to create a semantic connectivity map in which: (a) non-linear associations are preserved; (b) connections schemes are explicit; (c) the complex dynamics of adaptive interactions is captured. This method is based on an artificial adaptive system able to define the association strength of each variable with all the others in any dataset, named the Auto Contractive Map (AutoCM). The architecture and mathematics of AutoCM were invented, tested and implemented in C language, as described elsewhere (Buscema and Grossi, in press).

An appendix describing the mathematics and equations supporting the methodology is provided (see Appendix A).

This approach highlights affinities among variables as related to their dynamical interaction rather than to their simple contingent spatial position. This approach describes a context typical of living systems where a continuous time dependent complex change in the variable value is present. After the training phase, the matrix of the AutoCM represents the warped landscape of the dataset. We apply a simple filter (minimum spanning tree by Kruskal) to the matrix of AutoCM system to show the map of main connections between and among variables and the principal hubs of the system. These hubs can also be defined as variables with the maximum amount of connections in the map. The AutoCM learning equations, the specific mathematics linked to the "contractive factor" and the association to minimum spanning tree (MST) algorithm, are described in detail in Appendix A.

The stability of the MST statistical method was verified with a validation protocol here described. From the original dataset, 10 different and independent random samples, each one including the 90% of data points of the original dataset were generated. Thereafter, 10 different and independent AutoCM on the 10 new datasets were trained and an independent MST for each AutoCM matrix was built. A cell by cell comparison regarding the zero–one squared matrix of each MST was performed (end point: 0 = no link; 1 = link). For each possible connection the summation of the agreement coefficient among the 10 MST was made: in each $a_{i,j}$ cell the 10 MST may agree from 0.5 (no agreement) to 1 (full agreement).

Finally, data analysis was also performed according to another mathematical model, i.e. the mutual information score, following the method elsewhere described (Butte and Kohane, 2000).

3. Results

A summary of data from The Conselice Study at the beginning and after the 5-year follow up is reported in Table 1.

A list of variables investigated and their functional definition used in this study is reported in Tables 2 and 3.

Two time points are considered; the first one represents the baseline time point (time 0) where clinical, biological and genetic data have been collected in 1999. The second one represents the follow-up time point (time 1) where clinical data from participants have been collected in 2004; this latter point also represents the cognitive function outcome. After the training phase of the statistical process, AutoCM has been applied using all records from all subjects in the data base. The connectivity map related to 35 variables from The Conselice Study data base is shown in Fig. 1. The map illustrates the most relevant associations present in the data base. Three major biological hubs or points of variable aggregation were identified: (1) a SNP in the HMGCR (non-mutated allele); (2) plasma cholesterol levels; (3) age.

Different genotypic, phenotypic, clinical, pharmacological or habit variables converged to the three hubs. Females with no history of smoking or alcohol consumption converged to the first HMGCR hub. Males with past history of smoking, present alcohol consumption and carriers of the mutated alleles in the ACT, APOE and IL-6 genes also converged to this first MHGCR hub. These two gender related pathways led to the second major hub; blood cholesterol levels. Other different genotypic, phenotypic and clinical states converged to the cholesterol hub, each variable showing a differential degree of relation with cholesterol. For instance, ACT, HDL and triglycerides serum levels, as well as BMI, were highly connected with blood cholesterol. Incident CIND cases (CIND 2004) also showed a significant correlation with cholesterol levels. The degree of correlation between

Table 1
Description of the population investigated at the beginning (1999/2000) and at the end of the follow up (2003/2004)

Eligible individuals	Non-participants[a] at the beginning	Final participants	Prevalent dementia	Cognitively non-classificable	Dementia free cohort
1999/2000					
$N = 1353$	$n = 337$	$n = 1016$	$n = 60$	$n = 19$	$n = 937$
Reassessed population	**Non-reassessed[b] population**	**Final population**	**Incident dementia**	**Cognitively non-classificable**	**Dementia free cohort**
Follow up 2003/2004					
$N = 937$	$n = 133$	$n = 804$	$n = 109$	$n = 4$	$n = 695$

[a] Refusals $n = 271$; deceased $n = 59$; not found $n = 7$.
[b] Refusals $n = 74$; deceased $n = 28$; not found $n = 31$.

variables is described by the number between each variable showed in the connectivity map: the higher the score, the higher the link between the two variables.

Age represented the third hub and cholesterol blood levels were highly correlated with this chronological variable. Most clinical states such as, incident AD (AD 2004), incident VD (VD 2004), patients with cancer or BPCO converged in to this third hub; incident AD showing the highest degree of association (4.25).

Pharmacological variables, e.g. the use of statins and non-steroid anti-inflammatory drugs (NSAID), also converged to this third hub. Statins, a major prescription for decreasing blood cholesterol, converged to the age hub through cardiovascular diseases (CVD). On the other hand, NSAID directly converged on age.

The stability of the MST statistical method was verified by a validation protocol, as described in the material and methods. For each possible connection the summation of the agreement coefficient among the 10 MST was made: in each $a_{i,j}$ cell the 10 MST may agree from 0.5 (no agreement) to 1 (full agreement). Results from this validation analysis are reported in Table 4. All variables showed a stability index very close to full agreement (full agreement = 1) and the mean stability index of the variables from the validation protocol resulted 98.58% with a variance of 0.003.

Table 2
Genetic and phenotypic variables used in the statistical analysis

Genetic variable (gene polymorphism)
ACT = alpha-1 antichymotrypsin SNP* at −51 in the promoter region, allele mutation = T
APOE = apolipoprotein E gene variation 2, 3 and 4 alleles, allele mutation = 4
HMG = hydroxy-methyl-glutayl CoA reductase, SNP at −694, allele mutation = A
IL-1 beta = interleukin-1 beta, SNP at −511 in the promoter region, allele mutation = T
IL-6 = interleukin-6, SNP at −674 in the promoter region, allele mutation = C
Phenotypic blood variable
ACT = serum level (μg/ml) assessed by competitive non-commercial ELISA
Cholesterol = serum levels (mg/dl) assessed by commercial clinical lab assay
CRP = serum levels (mg/l) assessed by commercial nephelometric assay
HDL = serum levels (mg/dl) assessed by commercial clinical lab methodology
IL-6 = serum level (pg/ml) assessed by commercial ELISA
TNF = tumor necrosis factor-alpha (pg/ml) assessed by commercial ELISA
Triglycerides = serum levels assessed by commercial clinical lab assay
Other clinical variable
BMI = body max index

Table 3
Definitions of functional and non-parametric variables used in the statistical analysis

Clinical states
AD 2004 = patients with Alzheimer's disease at the end of the follow up
Age = chronological age
Alcohol yes, alcohol no = alcohol consumption habit
COPD = patients with chronic obstructive lung disease
CIND 2004 = patient with clinical diagnosis of cognitive impairment but no dementia
CVD = cardiovascular diseases
Diabetes = clinical diagnosis of non-autoimmune diabetes
Female, male = gender definition
Healthy 2004 = cognitively non-impaired at the end of the follow up
Smokers, non-smokers, ex-smokers = cigarette smoking habit
Tumor = patients with diagnosis of cancer
VAD 2004 = patients with vascular dementia at the end of the follow up
Therapy
NSAID = non-steroid anti-inflammatory drugs
Statins = drugs with anti cholesterol action

Data were also analyzed by a different algorithm, i.e. the mutual information score, and the map of relationship among variables is shown in Fig. 2. This second mathematical approach used mutual information distance to map the variables after a MST filtering. This second map was partially but significantly different; triglycerides, BMI, age and ACT blood levels were major aggregation points. Furthermore, the clinical status of incident AD, VAD and CIND converged to the point representing the controls (healthy 2004).

4. Discussion

AD is a complex and multi-factor disease. Therefore, it is unlikely that a single biomarker may be determinant in the diagnosis or monitoring the progression of the disease.

This is the main reason why we chose to concomitantly evaluate several biological and clinical variables. These variables were selected according our previous experience showing them associated or linked to pathogenetic mechanisms

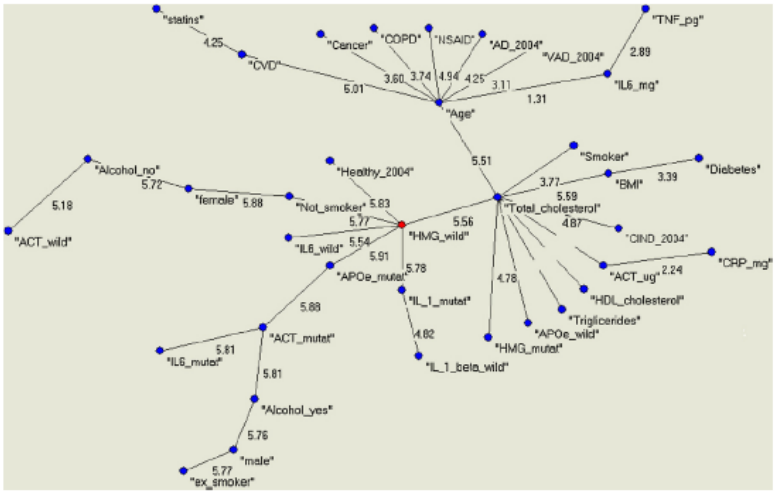

Fig. 1. Connectivity map of 35 variables from The Conselice Study database.

involved in AD (Chiappelli et al., 2006a,b; Ravaglia et al., 2006a,b).

The statistical analysis applied to elaborate biological and clinical data was a new entry in the field of biology and medicine. In fact, the statistical power of most common algorithms used in medicine have been influenced by the following limitations: (1) the analyses usually did not preserve the geometrical structure between variables when non-linear relationships among variables were not evident; (2) methods to establish precise associations between variables without predefined contiguity have been poorly explored.

Here, we used a new algorithm aimed to map variables and search for connectivity. In this analysis non-linear associations were preserved, explicit connection schemes were investigated and the complexity of dynamic interactions were preserved. The mathematical approach of this analysis has been described in detail elsewhere (Buscema and Grossi, in press and appendix to this paper). Some applications of this analysis have already been focused upon AD investigations with interesting findings (Grossi et al., 2007).

Results described here generated a connectivity map among variables and illustrated a rational path of biological variables leading to cognitive decline and incident dementia.

Major hubs among the 35 variables in the map were found; HMGCR genotype, cholesterol serum levels and age being the three major connectivity variables.

HMGCR genotype has been recently described as a genetic risk factor for AD (Porcellini et al., 2007). HMGCR is the rate limiting enzyme in cholesterol synthesis and controls cholesterol availability by affecting the synthesis of mevalonate and isoprenoid compounds which are necessary for the attachment of several proteins to biological membranes (Zhang and Casey, 1996). The presence of the mutated allele also affected the rate of cognitive decline in AD patients (Porcellini et al., 2007). Our data from The Conselice Study confirmed that this enzyme was a relevant factor for the developing of dementia. Statins inhibited HMGCR and this

Table 4
The stability of the MST statistical method was verified with a validation protocol

Variable	Stability index	Variable	Stability index
Male	0.9917	VAD_2004	1
Female	1	IL-6 pg	1
Age	0.975	CRP mg	0.9944
Smoker	0.9722	TNF pg	1
Ex smoker	1	ACT ug	0.9917
Non-smoker	0.9722	Total cholesterol	0.9556
Alcohol no	0.9889	HDL cholesterol	0.9972
Alcohol yes	1	triglycerides	1
BMI	0.9667	IL-1beta wild	1
CVD	0.9806	IL-1 beta mutat	0.9944
Diabetes	1	ACT wild	0.9722
Cancer	0.9889	ACT mutat	0.975
COPD	1	APOE wild	1
NSAID	0.9861	APOE mutat	0.9472
statins	0.9833	IL-6wild	0.9889
Healthy 2004	0.9833	IL-6 mutat	0.9722
CIND 2004	0.9833	HMGCR wild	0.9333
AD 2004	1	HMGCR mutat	0.9944

The stability index of each variable is shown (0–05 = no agreement, 0.6–1 = agreement). Mean stability of all variables from the 10 different MST was 98.58% and its variance = 0.003.

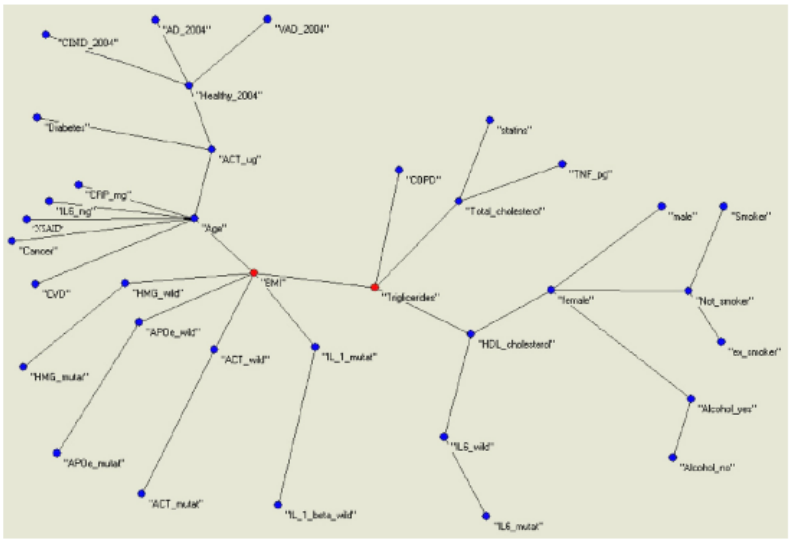

Fig. 2. Mutual information map from the Conselice data base.

enzyme might be a pharmacological target for AD prevention. In fact, data regarding the possible preventive effect of statins in AD were on record (Jick et al., 2001), although another investigation did not confirm these observations (Rea et al., 2005). However, recent findings showed that statin therapy was associated with reduced AD neuropathology (Li et al., 2007). Variations in HMGCR gene might be responsible for different effectiveness of statins in the prevention of dementia and this topic deserves further experimental and clinical attention.

Other interesting points of aggregation upon HMGCR were also found. For instance, the interaction of the mutated ACT allele with the APOE ε4 allele in men appeared of interest. This APOE-ACT gene interaction has been already described in an animal model for neuro-degeneration and atherosclerosis such as APOE deficient mice (Licastro et al., 1999). Furthermore, an influence of APOE ε4 allele upon brain ACT concentration has been found in AD patients (Licastro et al., 1999).

Epidemiological data presented here reinforced the notion of a functional linkage of APOE and ACT and are in accordance with recent data showing an influence of these two genetic factors upon the rate of cognitive decline in a short term follow up of AD cases (Licastro et al., 2005, 2007).

There was a strong linkage (5.56) of the HMGCR genotype hub with the second hub, i.e. serum cholesterol. Several other apparently independent variables converged upon this second major hub and among these the following variables were found: serum levels of triglycerides and HDL, ACT serum levels, carrier status of the mutated allele of HMGCR enzyme, APOE ε2 allele and BMI.

The link of lipids variables to the cholesterol hub was not surprising; present findings were in accordance with observations suggesting a pathogenetic link of cholesterol to AD (Koudinov et al., 1998).

A new unexpected link between blood ACT levels and serum cholesterol also emerged and deserves further experimental attention. The CRP levels also converged through ACT levels to the cholesterol hub, suggesting that these inflammatory components could play a relevant role in AD by affecting lipid metabolism and/or turnover. Both ACT and CRP might be pharmacological targets of potential relevance to dementia.

The mutated allele of HMGCR was significantly linked to the cholesterol hub. We already described that the transfection of HMGCR mutated allele in human cell cultures did not influence transcription of gene reporter under physiological conditions (Porcellini et al., 2007). However, we could not exclude that this SNP might show functional relevance under cell stressing conditions or be in linkage with other SNP in the same gene or other genes directly affecting cholesterol synthesis and/or turnover. This data suggested that SNP might play a relevant role in the developing of abnormal cholesterol metabolism in the aging brain.

The status of APOE ε4 non-carrier, i.e. the presence of APOE ε 2,3 or 3,3 alleles (APOE wild) was also linked to

cholesterol hub. APOE ε2 allele has been shown to affect cholesterol and lipid serum levels (Eto et al., 1986) and to be linked to cardiovascular diseases (Lahoz et al., 2001). On the other hand it is well known that many subjects with the APOE ε 3,3 have developed AD (Bickeboller et al., 1997).

The incident clinical CIND status, after the five year follow up (CIND 2004), also converged to the cholesterol hub, suggesting that alterations of cognitive performances in these elderly were partially dependent upon an abnormal regulation of cholesterol synthesis or turnover.

Cholesterol hub was linked with the third major one, i.e. chronological age. Age has been considered the major risk factor for dementia and AD (Blennow et al., 2006). However, old age has also been associated with a variety of other diseases, named age-related diseases. Therefore, convergence of cancer, obstructive lung alterations (BPCO) and cardiovascular diseases (CVD) to this last major hub was not surprising.

The weak (1.31) association of phenotypic immune variables such as TNF and IL-6 serum levels with the age hub indicated an age-related serum level alterations of these cytokines. These findings confirmed previous observations reporting increased IL-6 blood levels in the elderly (Cohen et al., 2003). These age-related immune alterations may independently contribute to neurodegenerative processes in the central nervous system and influence the clinical appearance of cognitive alterations and dementia. In fact, a special role in neuro-protection and neuro-degeneration for IL-6 has been suggested (Gruol and Nelson, 1997).

Finally, incident AD and VD (AD 2004 and VAD 2004, respectively) strongly converged to the age hub. In particular, AD was highly associated (4.25) with age, showing the second highest correlation coefficient after CVD (5.01).

A different map was generated by applying a different statistical model, i.e. mutual information analysis (Butte and Kohane, 2000). Results from this algorithm were substantially different, since triglycerides, BMI and age were major hubs of the map. Moreover, incident AD, VD and CIND converged to incident healthy status (healthy 2004) or diverged from this point and all four conditions were connected with the ACT blood levels. In our opinion the connectivity map shown in Fig. 1 displayed a higher resolution power in connecting different variables and increased focus in systematically showing pattern aggregation than that represented in Fig. 2. The reduced power of the connectivity map generated by the mutual information analysis could be ascribed to the fact that this mapping method was based on specific kind of "distance" among variables. In fact, the mutual information method evaluated a couple of variables at each time and joint information was not calculated when the number of variables increased. This model appeared to generate a static projection of the possible associations and the active interactions among variables might be underestimated. On the other hand, AutoCM was able to simultaneously compute multiple or "many to many" associations among variables, since it was a non-linear auto-associative method. Finally, a validation analysis of the AutoCM methods has been performed (see Table 4) and it showed a high statistical stability of the method. The AutoCM statistical analysis was able to point out affinities among variables, as related to their dynamical interaction rather than to their simple contingent spatial position. This was obtained through a dynamic processing with a particular neural network which reproduced the value of a given variable using the information of all other variables. In the AutoCM analysis each variable influenced all other variables and was influenced by all other ones (n-order of effects). AutoCM could be considered a dynamic system, since the system adjusted its weight gradually, computing all records several times. During the learning phase variables could dynamically negotiate the value of their connections. The implications of this method for better understanding AD are substantial, since the method avoids limitation of data analysis linked to the reductionist approach of probability based statistics which might lead to missed information regarding the associations among variables. In addition the explicit connection schemes allow clear cut hypotheses generation at variance with clustering methods in which associations are often vague.

In conclusion, the connectivity map presented here on incident dementia extended previous observations from case/control investigations and confirmed that some immune factors could indeed play a role in the pathogenesis of age-associated dementia. Our findings also showed a new link between immunity, cholesterol metabolism and age in relation with cognitive deterioration.

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Appendix A. Auto Contractive Map analysis

A.1. Learning equations

The Auto Contractive Map (CM) presents a three layers architecture: an Input layer, where the signal is captured from the environment, an Hidden layer, where the signal is modulated inside the CM, and an Output layer by which the CM influences the environment according to the stimuli previously received (Fig. 1A).

Each layer is composed by N units. Then the whole CM is composed by $3N$ units. The connections between the Input layer and the Hidden layer are Mono-dedicated, whereas the ones between the Hidden layer and the Output layer are at maximum gradient. Therefore, in relation to the units the number of the connections Nc, is given by: $Nc = N(N+1)$.

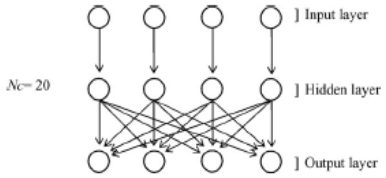

Fig. 1A. The figure gives an example of a Auto CM with $N=4$.

All the connections of CM may be initialized both by equal values and by values at random. The best practice is to initialize all the connections with the same positive value, close to zero.

The learning algorithm of CM may be summarized in four orderly steps:

1. Signal Transfer from the Input into the Hidden layer;
2. Adaptation of the connections value between the Input layer and the Hidden layer;*
3. Signal Transfer from the Hidden layer into the Output layer;*
4. Adaptation of the connections value between the Hidden layer and the Output layer.

(*) Steps 2 and 3 may take place in parallel.

We define as $m^{[s]}$ the units of the Input layer (sensors), scaled between 0 and 1, as $m^{[h]}$ the ones of the Hidden layer and as $m^{[t]}$ the ones of the Output layer (system target). We define v the vector of monodedicated connections, w the matrix of the connections between Hidden layer and Output layer, and n the discrete time of the weights evolution.

The signal forward transfer equations and the learning ones are four:

a. Signal transfer from the Input to the Hidden:

$$m^{[h]}_{i_{(n)}} = m^{[s]}_{i_{(n)}} \left(1 - \frac{v_{i_{(n)}}}{C}\right) \tag{1}$$

where C = positive real number, named contractive factor.

b. Adaptation of the connections $v_{i_{(n)}}$ through the $\Delta v_{i_{(n)}}$ trapping the energy difference generated by the Eq. (1):

$$\Delta v_{i_{(n)}} = (m^{[s]}_{i_{(n)}} - m^{[h]}_{i_{(n)}}) \left(1 - \frac{v_{i_{(n)}}}{C}\right); \tag{2}$$

$$v_{i_{(n+1)}} = v_{i_{(n)}} + \Delta v_{i_{(n)}}; \tag{3}$$

c. Signal transfer from the Hidden to the Output:

$$\mathrm{Net}_{i_{(n)}} = \sum_{j}^{N} m^{[h]}_{j_{(n)}} \left(1 - \frac{w_{i,j_{(n)}}}{C}\right); \tag{4}$$

$$m^{[t]}_{i_{(n)}} = m^{[h]}_{i_{(n)}} \left(1 - \frac{\mathrm{Net}_{i_{(n)}}}{C}\right). \tag{5}$$

d. Adaptation of the connections $w_{i,j_{(n)}}$ through the $\Delta w_{i,j_{(n)}}$ trapping the energy differences generated by the Eq. (5):

$$\Delta w_{i,j_{(n)}} = (m^{[h]}_{i_{(n)}} - m^{[t]}_{i_{(n)}}) \left(1 - \frac{w_{i,j_{(n)}}}{C}\right) m^{[h]}_{j_{(n)}}; \tag{6}$$

$$w_{i,j_{(n+1)}} = w_{i,j_{(n)}} + \Delta w_{i,j_{(n)}} \tag{7}$$

The value $m^{[h]}_{j_{(n)}}$ of (6) is used for proportioning the change of the connection $w_{i,j_{(n)}}$ to the quantity of energy liberated by the node $m^{[h]}_{j_{(n)}}$ in favor of node $m^{[t]}_{i_{(n)}}$.

In CM the learning process, conceived as adjustment of the connections in relation to the minimization of Energy, corresponds to the continuous acceleration and deceleration of velocities of the signals inside the ANN connection matrix.

The first step to make the precedent sentence evident is to show the CM convergence equation:

$$\lim_{n\to\infty} v_{i_{(n)}} = C. \tag{8}$$

In fact, when $v_{i_{(n)}} = C$, then $\Delta v_{i_{(n)}} = 0$ Eq. (2), and $m^{[h]}_{i_{(n)}} = 0$ Eq. (1) and, consequently, $\Delta w_{i,j_{(n)}} = 0$ Eq. (6)

During this mathematic analysis, we will introduce four new variables, that we consider the key points of the AutoCM learning process:

1. ε is the contractive factor of the first layer of AutoCM weights:

$$\varepsilon = 1 - \frac{v_{i_{(n)}}}{C}.$$

2. η is the contractive factor of the second layer of AutoCM weights:

$$\eta = 1 - \frac{w_{i,j_{(n)}}}{C}$$

3. φ is the contractive factor between the Hidden nodes and the Input nodes:

$$\varphi = m^{[s]}_{i_{(n)}} - m^{[h]}_{i_{(n)}};$$

4. λ is the contractive factor between the Output nodes and the Hidden nodes:

$$\lambda = m^{[h]}_{i_{(n)}} - m^{[t]}_{i_{(n)}}.$$

The second step is to demonstrate how $\Delta v_{i_{(n)}}$ increases and decreases during the CM learning phase.

Let us suppose that:

$\frac{v_{i_{(n)}}}{C} = 1 - \varepsilon$, where ε is a small positive real number close to zero.

At this point, we can re-write the Eq. (2) in this way:

$$\Delta v_{i_{(n)}} = \left(m^{[s]}_{i_{(n)}} - m^{[s]}_{i_{(n)}}\left(1 - \frac{v_{i_{(n)}}}{C}\right)\right)\left(1 - \frac{v_{i_{(n)}}}{C}\right) = m^{[s]}_{i_{(n)}}\frac{v_{i_{(n)}}}{C}\left(1 - \frac{v_{i_{(n)}}}{C}\right); \quad (2a)$$

But, because $(v_{i_{(n)}}/C) = 1 - \varepsilon$, then:

$$\Delta v_{i_{(n)}} = m^{[s]}_{i_{(n)}}(1-\varepsilon)(1-(1-\varepsilon)) = m^{[s]}_{i_{(n)}}(1-\varepsilon)\varepsilon. \quad (2b)$$

The Eq. (2b) shows the parabolic dynamics of Δv_i.

Considering (2b) we can write:

$$\Delta v_{i_{(n)}} < \varepsilon. \quad (2c)$$

The Eq. (2c) means that the increment of $\Delta v_{i_{(n)}}$ will be always smaller than the quantity that $v_{i_{(n)}}$ needs to reach up C.

At this point we can re-write the Eq. (3) in this form:

$$v_{i_{(n+1)}} = C(1-\varepsilon)_{i_{(n)}} + m^{[s]}_{i_{(n)}}(1-\varepsilon)\varepsilon. \quad (3a)$$

Consequently:

$$\lim_{\varepsilon \to 0} v_{i_{(n)}} = C \quad (3b)$$

Further, the contractive factor of the Eqs. (1) and (5) makes evident this relation:

$$m^{[t]}_{i_{(n)}} < m^{[h]}_{i_{(n)}} < m^{[s]}_{i_{(n)}}; \quad (1\text{-}5)$$

In fact:

$$m^{[h]}_{i_{(n)}} = m^{[s]}_{i_{(n)}}\varepsilon; \quad (1a)$$

and:

$$m^{[t]}_{i_{(n)}} = m^{[h]}_{i_{(n)}}\varepsilon(1 - Net_{i_{(n)}}). \quad (5a)$$

Now it is possible maker clear the relationship between $\Delta v_{i_{(n)}}$ and $\Delta w_{i,j_{(n)}}$.

From the Eq. (1-5) we can suppose:

$m^{[h]}_{i_{(n)}} = m^{[s]}_{i_{(n)}} - \varphi$; where φ is a small positive real number close to 0; (1b)

And

$m^{[t]}_{i_{(n)}} = m^{[h]}_{i_{(n)}} - \lambda$; where λ is a small positive real number close to 0; (5b)

$$m^{[t]}_{i_{(n)}} = m^{[s]}_{i_{(n)}} - (\varphi + \lambda). \quad (5c)$$

At this point we write again the Eq. (2) in this way:

$$\Delta v_{i_{(n)}} = (m^{[s]}_{i_{(n)}} - (m^{[s]}_{i_{(n)}} - \varphi))\left(1 - \frac{v_{i_{(n)}}}{C}\right) = \varphi\left(1 - \frac{v_{i_{(n)}}}{C}\right) = \varphi\varepsilon. \quad (2d)$$

In a similar way we can re-write the Eq. (6):

$$\Delta w_{i,j_{(n)}} = ((m^{[s]}_{i_{(n)}} - \varphi) - (m^{[s]}_{i_{(n)}} - (\varphi + \lambda))(m^{[s]}_{i_{(n)}} - \varphi) \times (1 - w_{i,j_{(n)}}) = \lambda(m^{[s]}_{i_{(n)}} - \varphi)(1 - w_{i,j_{(n)}}). \quad (6a)$$

Now we can re-write $(w_{i,j_{(n)}}/C) = 1 - \eta$ (where η has to be a positive real number smaller than 1).So:

$$\Delta v_{i_{(n)}} = \varphi\varepsilon; \quad (2e)$$

and

$$\Delta w_{i,j_{(n)}} = \lambda(m^{[s]}_{i_{(n)}} - \varphi)\eta; \quad (6b)$$

So, considering the Eq. (5a) in this form:

$$m^{[s]}_{i_{(n)}}\varepsilon \mathrm{Net}_{i_{(n)}} = m^{[s]}_{i_{(n)}}\varepsilon - m^{[t]}_{i_{(n)}};$$

$$\mathrm{Net}_{i_{(n)}} = \frac{m^{[s]}_{i_{(n)}}\varepsilon - m^{[t]}_{i_{(n)}}}{m^{[s]}_{i_{(n)}}\varepsilon} = \frac{m^{[s]}_{i_{(n)}}\varepsilon - (m^{[s]}_{i_{(n)}}\varepsilon - \lambda)}{m^{[s]}_{i_{(n)}}\varepsilon} = \frac{\lambda}{m^{[s]}_{i_{(n)}}\varepsilon}. \quad (5b)$$

It is now possible to size the λ contractive factor between Hidden and Output units:

$$\lambda = m^{[s]}_{i_{(n)}}\varepsilon \mathrm{Net}_{i_{(n)}}. \quad (5c)$$

From (5c) we can write:

$$\lambda = m^{[s]}_{i_{(n)}}\left(1 - \frac{v_{i_{(n)}}}{C}\right)\mathrm{Net}_{i_{(n)}} \quad (5d)$$

and so:

$$\lim_{v_{i_{(n)}} \to C} \lambda = 0 \quad (5e)$$

Now we can substitute (5c) in (6b):

$$\Delta w_{i,j_{(n)}} = m^{[s]}_{i_{(n)}}\varepsilon \mathrm{Net}_{i_{(n)}}(m^{[s]}_{i_{(n)}} - \varphi)\eta; \quad (6c)$$

But because $m^{[s]}_{i_{(n)}} - \varphi = m^{[s]}_{i_{(n)}}\varepsilon$, then

$$\Delta w_{i,j_{(n)}} = (m^{[s]}_{i_{(n)}}\varepsilon)^2 \mathrm{Net}_{i_{(n)}}\eta; \quad (6d)$$

and

$$\lim_{\varepsilon \to 0} \Delta w_{i,j_{(n)}} = 0 \quad (6e)$$

Now we have to consider the Eq. (7):

$$w_{i,j_{(n+1)}} = C(1-\eta)_{i,j_{(n)}} + (m^{[s]}_{i_{(n)}}\varepsilon)^2 \mathrm{Net}_{i_{(n)}}\eta; \quad (7a)$$

From (7a) we can conclude:

$$\lim_{\varepsilon \to 0} w_{i,j_{(n)}} = C - C\eta_{i,j_{(n)}} \quad (7b)$$

So this means that at the beginning of the training Input and Hidden units will be very similar (Eq. (1)), and, consequently, $\Delta v_{i_{(n)}}$ will be very small (Eq. (2e)), while for the same reason λ, at the beginning will be very big (Eq. (5c)) and $\Delta w_{i,j_{(n)}}$ bigger than $\Delta v_{i_{(n)}}$ (Eq. (5c)).

During the training, all the same, while $v_{i_{(n)}}$ slowly increases, $m^{[h]}_{i_{(n)}}$ decreases, so φ increases and, consequently, $\Delta w_{i,j_{(n)}}$ monotonically continues to decreases (λ becomes always smaller, see equation (5c)) and $\Delta v_{i_{(n)}}$ increases faster. When λ becomes close to zero this means that $m^{[h]}_{i_{(n)}}$ is only a bit bigger that $m^{[t]}_{i_{(n)}}$ (see Eq. (5b)). At this point, $\Delta v_{i_{(n)}}$ is on the global maximum of the equation $(1-\varepsilon)\cdot\varepsilon$ (see (2b)), so after this critical point $\Delta v_{i_{(n)}}$ will becomes a symmetrical decreasing toward zero.

Auto Contractive Maps do not behave as a regular ANN:

a. They learn also starting from all connections set up with the same values. So they do not suffer the problem of the symmetric connections.
b. During training, they develop for each connection only positive values. Therefore, Auto CM do not present inhibitory relations among nodes, but only different strengths of excitatory connections.
c. Auto CM can learn also in hard conditions, that is, when the connections of the main diagonal of the second connections matrix are removed. When the learning process is organized in this way, Auto CM seems to find a specific relationships between each variable and any other. Consequently, from an experimental point of view, it seems that the ranking of its connections matrix is equal to the ranking of the joint probability between each variable and the others.
d. After learning process, any input vector, belonging to the training set, will generate a null output vector. So, the energy minimization of the training vectors is represented by a function trough which the trained connections absorb completely the input training vectors. Auto CM seems to learn to transform itself in a dark body.
e. At the end of the training phase ($\Delta w_{i,j} = 0$), all the components of the weights vector **v** reach up the same value:

$$\lim_{n\to\infty} v_{i_{(n)}} = C. \tag{8}$$

f. The matrix **w**, then, represents the CM knowledge about all the dataset.

It is possible to transform the **w** matrix also in probabilistic joint association among the variables m:

$$p_{i,j} = \frac{w_{i,j}}{\sum_{j=1}^{N} w_{i,j}}; \tag{9}$$

$$P(m_j^{[s]}) = \sum_{i}^{N} p_{i,j} = 1 \tag{10}$$

The new matrix **p** can be read as the probability of transition from any state-variable to anyone else:

$$P(m_i^{[t]} | m_j^{[s]}) = p_{i,j}. \tag{11}$$

g. At the same time the matrix **w** may be transformed into a non-Euclidean distance metric (semi-metric), when we train the CM with the main diagonal of the **w** matrix fixed at value N.

Now, if we consider N as a limit value for all the weights of the **w** matrix, we can write:

$$d_{i,j} = N - w_{i,j} \tag{12}$$

The new matrix **d** is also a squared symmetric matrix where the main diagonal represents the zero distance between each variable from itself.

A.2. The contractive factor

There is another way to interpret the squared weights matrix of the AutoCM system. We have to assume each variable of the dataset as a vector composed of the all its values. At this point, the dynamic value of each connection between two variables represents the local velocity of their mutual attraction caused by their mutual vectors similarity: more is the vectors similarity, more is their attraction speed. When two variables are attracted by each other, they contract proportionally the original Euclidean space between them. The limit case is when two variables are identical: the space contraction should be infinitive and the two variables should collapse in the same point.

We can extract from each weight of a trained AutoCM this specific contractive factor:

$$F_{i,j} = \left(1 - \frac{w_{i,j}}{C}\right)^{-1}; \quad 1 \le F_{i,j} \le \infty. \tag{9a}$$

This equation is interesting for three reason:

1. it is the inverse of the equation used as contractive factor during the AutoCm training;
2. considering the Eq. (3b), each mono-connection v_i at the end of the training will reach the value C. In this case the contractive factor will be infinitive because the two variables connected by the weight are really the same variable.
3. considering, instead, the Eq. (7b), each weight $w_{i,j}$, at the end the training will be always smaller than C. This means that the contractive factor for each weight of the matrix that we are considering will be always non-infinitive. That is correct. In fact in the case of the weight $w_{i,i}$, the variable is connected with itself, but the same variable has also received the influences of the other variables (remind that the matrix **w** is a squared matrix where each variable is linked to the other). Consequently, this variable has not be exactly the same.

At this point, we are able to calculate the contractive distance between each variable and the other, modifying the original Euclidean distance with a specific contractive factor.

The Euclidean distance among the variables in the dataset is given by the following equation:

$$d_{i,j}^{[\text{Eucliden}]} = \sqrt{\sum_{k}^{R}(x_{i,k} - x_{j,k})^2}; \tag{10a}$$

where R = the number of the records of the assigned dataset; $x_{i,k}$ and $x_{j,k}$ = the i-th value and the j-th value of two variables in the k-th record.

And, consequently, the AutoCM distance matrix among the same variables is:

$$d_{i,j}^{[\text{AutoCM}]} = \frac{d_{i,j}^{[\text{Euclidean}]}}{F_{i,j}}. \tag{11a}$$

A.3. Auto CM and minimum spanning tree

Eq. (12) transforms the squared weights matrix of Auto CM into a squared matrix of distances among nodes. Each distance between a pair of node becomes, consequently, the weighted edge between these pair of nodes.

At this point, the matrix **d** may be analyzed trough the graph theory.

A graph is a mathematical abstraction that is useful for solving many kinds of problems. Fundamentally, a graph consists of a set of vertices, and a set of edges, where an edge is something that connects two vertices in the graph. More precisely, a graph is a pair (V,E), where V is a finite set and E is a binary relation on V, to whom it is possible to attribute a scalar value (in this case the weights is the distance $d_{i,j}$).

V is called a vertex set whose elements are called vertices. E is a collection of edges, where an edge is a pair (u, v) with u, v in V. In a directed graph, edges are ordered pairs, connecting a source vertex to a target vertex. In an undirected graph edges are unordered pairs and connect the two vertices in both directions, hence in an undirected graph (u, v) and (v, u) are two ways of writing the same edge.

It does not say what a vertex or edge represents. They could be cities with connecting roads, or web-pages with hyperlinks. These details are left out of the definition of a graph for an important reason; they are not a necessary part of the graph abstraction.

An adjacency-matrix representation of a graph is a two-dimensional $V \times V$ array, where rows represent the list of vertices and the columns represent the edges among the vertices. Each element in the array is stored with a Boolean value saying whether the edge (u, v) is in the graph.

A distance matrix among V vertices represents an undirected graph, where each vertex is linked with all the other, but itself (Table A1).

At this point is useful to introduce the concept of minimum spanning tree (MST).

Table A1
Adjacency matrix of a distance matrix

	A	B	C	D	...	E
A	0	1	1	1	1	1
B	1	0	1	1	1	1
C	1	1	0	1	1	1
D	1	1	1	0	1	1
..	1	1	1	1	0	1
E	1	1	1	1	1	0

The minimum spanning tree problem is defined as follows: find an acyclic subset T of E that connects all of the vertices in the graph and whose total weight is minimized, where the total weight is given by

$$d(T) = \sum_{i=0}^{N-1}\sum_{j=i+1}^{N} d_{i,j}, \forall d_{i,j}. \tag{13}$$

T is called spanning tree, and MST is the T with the minimum sum of its edges weighted.

$$\text{Mst} = \text{Min}\{d(T_k)\} \tag{14}$$

Given a undirected Graph G, representing a **d** matrix of distances, with V vertices, completely linked each other, the total number of their edges (E) is:

$$E = \frac{V(V-1)}{2}; \tag{15}$$

And the number of its possible tree is:

$$T = V^{V-2}. \tag{16}$$

Kruskal in the 1956 found out an algorithm able to determinate the MST of any undirected graph in a quadratic number of steps, in the worse case. Obviously, the Kruskal algorithm generates one of the possible MST. In fact in a weighted graph more than one MST are possible.

From conceptual point of view the MST represents the *energy minimization* state of a structure. In fact, if we consider the atomic elements of a structure as vertices of a graph and the strength among them as the weight of each edge, linking a pair of vertex, the MST represents the minimum of energy needed because all the elements of the structure continue to stay together.

In a closed system, all the components tend to minimize the overall energy. So the MST, in specific situations, can represent the most probable state where a system tends to.

To define the MST of a undirected graph, each edge of the graph has to be weighted. The Eq. (12) shows a way to weight each edge whose nodes are the variables of a dataset and whose weights of a trained AutoCM provides the metrics.

Obviously, it is possible to use any kind of AutoAssociative ANN or any kind of Linear Auto-Associator to generate a weight matrix among the variables of a assigned dataset. But it is hard to train a two layer AutoAssociative Back Propagation with the weights main diagonal fixed (to avoid variables auto-correlation). In the most of the cases, the Root Mean

Square Error stops to decrease after few epochs. Especially when the orthogonally of the records increase. And that is usual when it is necessary to weight the distance among the records of the assigned dataset. In this case, in fact, it is necessary to train the transposed matrix of the assigned dataset.

By the way, if a Linear Auto-Associator is used, all the non-linear association among variables will be lost.

So, actually, AutoCM seems to be the best choice to compute a complete and a non-linear matrix of weights among variables or among records of any assigned dataset.

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Chapter IV

Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease

nature genetics

Genome-wide association study identifies variants at *CLU* and *CR1* associated with Alzheimer's disease

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The gene encoding apolipoprotein E (*APOE*) on chromosome 19 is the only confirmed susceptibility locus for late-onset Alzheimer's disease. To identify other risk loci, we conducted a large genome-wide association study of 2,032 individuals from France with Alzheimer's disease (cases) and 5,328 controls. Markers outside *APOE* with suggestive evidence of association ($P < 10^{-5}$) were examined in collections from Belgium, Finland, Italy and Spain totaling 3,978 Alzheimer's disease cases and 3,297 controls. Two loci gave replicated evidence of association: one within *CLU* (also called *APOJ*), encoding clusterin or apolipoprotein J, on chromosome 8 (rs11136000, OR = 0.86, 95% CI 0.81–0.90, $P = 7.5 \times 10^{-9}$ for combined data) and the other within *CR1*, encoding the complement component (3b/4b) receptor 1, on chromosome 1 (rs6656401, OR = 1.21, 95% CI 1.14–1.29, $P = 3.7 \times 10^{-9}$ for combined data). Previous biological studies support roles of *CLU* and *CR1* in the clearance of β amyloid (Aβ) peptide, the principal constituent of amyloid plaques, which are one of the major brain lesions of individuals with Alzheimer's disease.

Alzheimer's disease is a neurological disorder primarily affecting the elderly that manifests through memory disorders, cognitive decline and loss of autonomy. Two principal types of neuropathologic lesions are observed: (i) neurofibrillary degeneration resulting from the intraneuronal accumulation of hyperphosphorylated Tau proteins and (ii) amyloid deposits resulting from the extracellular accumulation of amyloid plaques, which are primarily composed of Aβ peptides. Currently, the processes leading to the formation of these lesions and their combined association with Alzheimer's disease are not adequately understood[1].

Genetic studies have provided significant insights into the molecular basis of Alzheimer's disease. Rare hereditary early-onset forms of the disease have been linked to mutations in three different genes: *APP*, encoding amyloid precursor protein, on chromosome 21; *PS1*, encoding presenilin 1, on chromosome 14; and *PS2*, encoding presenilin 2, on chromosome 1 (ref. 2). These mutations, however, explain less than 1% of all cases of Alzheimer's disease, whereas the vast majority (especially for late-onset forms of the disease) have other, more complex genetic determinants[3].

Genetic studies have led to the consistent identification of the ε4 allele of *APOE* as a susceptibility locus for late-onset Alzheimer's disease[4]. Twin studies suggest that genes may have a role in more than 60% of Alzheimer's disease susceptibility[5] and that *APOE* may account for as much as 50% of this genetic susceptibility[6]. More than 550 other genes have been proposed as candidates for Alzheimer's disease susceptibility, but thus far none have been confirmed to have a role in Alzheimer's disease pathogenesis[7].

As with other multifactorial diseases, this knowledge gap has motivated more comprehensive investigations using genome-wide association studies (GWAS). The first GWAS of case-control Alzheimer's disease data collections have examined a relatively small number of cases (<1,000)[8–12]. Similar to studies done on other multifactorial disorders, these GWAS have shown that, except in the case of *APOE*, larger samples will be necessary to locate common genetic factors of Alzheimer's disease. Here, we report results from a large two-stage GWAS of late-onset Alzheimer's disease.

In the first stage of this study, we undertook a GWA analysis of 537,029 SNPs in 2,032 French Alzheimer's disease cases and 5,328 French controls. Patients with probable Alzheimer's disease were ascertained by neurologists. Individuals without symptoms of

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Table 1 Association of SNPs at the *CLU* locus with Alzheimer's disease in the Stage 1 and Stage 2 samples

	rs2279590						
	N		MAF			Association test	
	Cases	Controls	Cases	Controls	HWE	OR (95% CI)	*P* value
Stage 1	**2,025**	**5,328**	**0.36**	**0.41**	$\mathbf{3.1 \times 10^{-1}}$	**0.83 (0.77–0.90)**	$\mathbf{1.0 \times 10^{-6}}$
Stage 2	**3,803**	**3,097**	**0.38**	**0.41**	$\mathbf{4.9 \times 10^{-1}}$	**0.88 (0.81–0.95)**	$\mathbf{8.2 \times 10^{-4}}$
Belgium	1,071	505	0.38	0.41	3.0×10^{-1}	0.82 (0.69–0.98)	3.1×10^{-2}
Finland	587	645	0.40	0.42	4.3×10^{-1}	0.92 (0.78–1.09)	3.5×10^{-1}
Italy	1,410	1,206	0.38	0.41	3.4×10^{-1}	0.87 (0.77–0.97)	1.3×10^{-2}
Spain	738	806	0.38	0.40	5.6×10^{-1}	0.91 (0.79–1.06)	2.4×10^{-1}
Stage 1 & 2	**5,828**	**8,425**	**0.37**	**0.41**	$\mathbf{2.9 \times 10^{-1}}$	**0.86 (0.82–0.91)**	$\mathbf{8.9 \times 10^{-9}}$
APOE ε4 carriers	3,060	1,714	0.36	0.40	7.5×10^{-1}	0.81 (0.74–0.89)	1.9×10^{-5}
APOE ε4 non carriers	2,727	6,697	0.39	0.41	1.8×10^{-1}	0.92 (0.85–0.98)	1.3×10^{-2}
	rs11136000						
Stage 1	**2,016**	**5,266**	**0.35**	**0.39**	$\mathbf{6.0 \times 10^{-1}}$	**0.83 (0.77–0.90)**	$\mathbf{1.5 \times 10^{-6}}$
Stage 2	**3,775**	**3,154**	**0.35**	**0.38**	$\mathbf{2.6 \times 10^{-1}}$	**0.88 (0.81–0.95)**	$\mathbf{8.8 \times 10^{-4}}$
Belgium	987	467	0.35	0.37	5.2×10^{-2}	0.80 (0.66–0.97)	2.2×10^{-2}
Finland	596	640	0.38	0.41	2.5×10^{-1}	0.92 (0.78–1.08)	3.1×10^{-1}
Italy	1,454	1,241	0.35	0.38	6.0×10^{-1}	0.88 (0.80–0.99)	2.8×10^{-2}
Spain	738	806	0.35	0.37	6.1×10^{-1}	0.91 (0.79–1.06)	2.4×10^{-1}
Stage 1 + 2	**5,791**	**8,420**	**0.35**	**0.38**	$\mathbf{2.7 \times 10^{-1}}$	**0.86 (0.81–0.90)**	$\mathbf{7.5 \times 10^{-9}}$
APOE ε4 carriers	3,053	1,707	0.36	0.41	9.0×10^{-1}	0.81 (0.74–0.88)	2.7×10^{-5}
APOE ε4 non-carriers	2,693	6,699	0.39	0.41	1.9×10^{-1}	0.91 (0.85–0.97)	7.0×10^{-3}
	rs9331888						
Stage 1	**2,025**	**5,328**	**0.31**	**0.28**	$\mathbf{8.9 \times 10^{-1}}$	**1.19 (1.11–1.30)**	$\mathbf{1.8 \times 10^{-5}}$
Stage 2	**3,862**	**3,180**	**0.31**	**0.29**	$\mathbf{7.9 \times 10^{-1}}$	**1.12 (1.04–1.21)**	$\mathbf{2.9 \times 10^{-3}}$
Belgium	1,072	501	0.29	0.28	9.3×10^{-1}	1.16 (0.96–1.40)	1.2×10^{-1}
Finland	586	638	0.38	0.37	1.3×10^{-1}	1.09 (0.92–1.29)	3.2×10^{-1}
Italy	1,474	1,241	0.30	0.26	9.9×10^{-1}	1.22 (1.07–1.39)	4.5×10^{-3}
Spain	730	800	0.29	0.28	5.4×10^{-1}	1.06 (0.90–1.24)	4.8×10^{-1}
Stage 1 + 2	**5,887**	**8,508**	**0.30**	**0.28**	$\mathbf{3.3 \times 10^{-1}}$	**1.16 (1.10–1.23)**	$\mathbf{9.4 \times 10^{-8}}$
APOE ε4 carriers	3,098	1,723	0.32	0.29	8.6×10^{-1}	1.21 (1.10–1.33)	7.8×10^{-5}
APOE ε4 non-carriers	2,748	6,770	0.30	0.28	3.1×10^{-1}	1.09 (1.02–1.18)	1.8×10^{-2}

P values and ORs with the associated 95% CI have been calculated under an additive model using logistic regression models adjusted for age, gender and centers when necessary. MAF, minor allele frequency; HWE, *P* value for the test of Hardy-Weinberg equilibrium in controls.

dementia from French Three-City (3C) prospective population-based cohort were obtained as controls (Supplementary Note). Samples were genotyped with Illumina Human 610-Quad BeadChip and subjected to standard quality control procedures. The resulting GWA data were then analyzed with logistic regression taking into account sex and age and using principal components to adjust for possible population stratification. The genomic control parameter was 1.20 before this adjustment but 1.04 afterward. Comparison of the observed and expected χ^2 distributions (Supplementary Fig. 1) did not indicate substantial inflation of the test statistics after adjustment. We carried out additional tests to establish the robustness of the statistical results as described in the online methods section.

Several *APOE*-linked SNPs gave strong evidence of disease association (Supplementary Table 1). Outside of *APOE*, one marker (rs11136000) within *CLU* on chromosome 8p21-p12 showed significance ($P = 9.0 \times 10^{-8}$) in the association test. This slightly surpassed the criteria for genome-wide significance as evaluated with a conservative Bonferroni correction ($P < 9.3 \times 10^{-8}$). We observed markers in several chromosome regions with suggestive evidence of association ($P < 10^{-5}$) as shown in a Manhattan plot (Supplementary Fig. 2). The results of the entire GWAS are available online. Finally, we imputed genotypes using the HapMap CEU samples to increase the number of SNPs examined in these regions. The genotyped and imputed markers from the regions that gave $P < 10^{-5}$ are shown in Supplementary Table 2.

In the second stage, we sought replication of association by genotyping a selection of markers at the loci identified in Supplementary Table 2 in additional collections totaling 3,978 probable Alzheimer's disease cases and 3,297 controls obtained from Belgium, Finland, Italy and Spain (Supplementary Table 3 and Supplementary Note). Genotyping of the second-stage samples was performed using either Taqman or Sequenom assays. Data were analyzed using logistic regression under an additive genetic model taking into account sex and age at diagnosis (cases) or at confirmation of the absence of dementia (controls). Five SNPs at two loci showed association, with *P* values ranging from 1.6×10^{-2} to 8.2×10^{-4}. The first of these loci encompasses *CLU* on 8p21-p12, and the second spans the gene encoding complement component (3b/4b) receptor 1 (*CR1*) on 1q32.

At *CLU*, three markers (rs2279590, rs11136000, rs9331888) showed statistically significant association with Alzheimer's disease in the replication collections, even with a conservative Bonferroni correction for all 11 SNPs tested in stage 2 ($P < 4.5 \times 10^{-3}$, Table 1). The odds ratios were statistically homogeneous across the stage 1 and 2 study collections (Supplementary Table 4). We found strong evidence for association in the combined GWA and replication datasets taking into account the sample origin in the logistic regression, with two markers exceeding the criterion for genome-wide significance ($P < 9.3 \times 10^{-8}$). For the marker showing the strongest evidence of association (rs11136000), the odds ratio for the minor

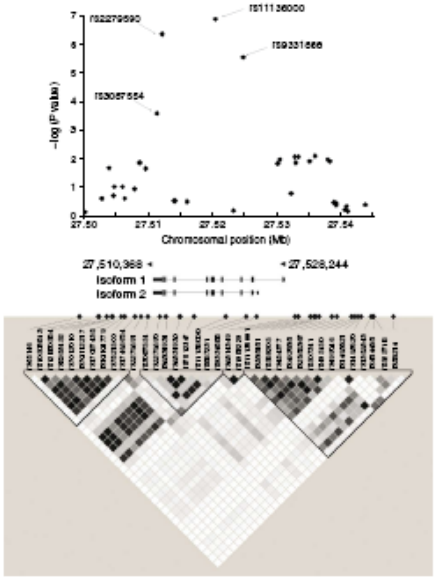

Figure 1 Schematic overview of *CLU* and LD patterns at the *CLU* locus. *P* values for association of SNPs (resulted from imputation and with minor allele frequency (MAF) >5%) encompassing the *CLU* locus with Alzheimer's disease risk under an additive model with adjustment for age and gender are plotted against physical distance. The LD plot is shown for SNPs at the *CLU* locus in controls (stage 1 data in Haploview 4.0, solid spine haplotype block definition, r^2 color scheme).

allele was 0.86 (95% CI 0.81–0.90, $P = 7.5 \times 10^{-9}$). Similar results were obtained using a Mantel-Haenszel statistic for the combined analysis (Supplementary Table 5).

We detected a statistical interaction between the *APOE* ε4 status and the *CLU* SNPs (ranging from 3.0×10^{-2} to 5.2×10^{-2} from the SNP tested). For rs11136000, although the association was significant in both ε4 carriers and non-carriers, it was more significant in carriers (Table 1).

The three *CLU* locus markers replicated in stage 2 are within a linkage disequilibrium (LD) block that encompasses only the *CLU* gene (Fig. 1). They define three common haplotypes (frequency >2%) that together account for 98.2% of the observations in stage 1 controls. Compared to the most frequent haplotype, TTC, the other two most frequent haplotypes were all associated with a statistically significant increased disease risk, with similar odd ratios in the stage 1 and stage 2 collections (Table 2). The odds ratio was highest for the CCG haplotype compared to the TTC haplotype (OR = 1.22, $P = 5.6 \times 10^{-10}$ for the combined samples).

A second locus of potential interest lies within an LD block that encompasses *CR1* on 1q32 (Fig. 2). We tested two SNPs at this locus in the second stage, and one (rs6656401) showed evidence of association with Alzheimer's disease in the replication collections ($P = 8.2 \times 10^{-4}$, Table 3). There was no significant heterogeneity by origin (Supplementary Table 4), and the odds ratio and *P* values in the combined data were 1.21 (95% CI 1.14–1.29) and 3.7×10^{-9}. We found similar results when using the Mantel-Haenszel method (Supplementary Table 5). At this locus, we also detected a statistical interaction with *APOE* ε4 status and risk of disease ($P = 9.6 \times 10^{-3}$) with significant association in both carriers and non-carriers, but with a more significant association in the former. Although the association of the second marker tested at this locus (rs3818361) only showed a suggestive significance overall in the stage 2 collections (OR = 1.11, 95% CI 1.02–1.22, $P = 1.6 \times 10^{-2}$), there was evidence of association of this SNP in the *APOE* ε4 carriers in stage 1, stage 2 and combined samples (respectively, OR = 1.38, 95% CI 1.19–1.60, $P = 2.3 \times 10^{-5}$; OR = 1.29, 95% CI 1.08–1.56, $P = 5.4 \times 10^{-3}$; OR = 1.34, 95% CI 1.20–1.50, $P = 2.9 \times 10^{-7}$). The genotyped markers define two principal haplotypes that account for 97.8% of the observations at the *CR1* locus, and a third haplotype has an estimated frequency of 1.2% in the combined control population (Table 4). The odds ratio was highest for the AA haplotype compared to the GG haplotype (OR = 1.22, $P = 3.1 \times 10^{-10}$ for the combined samples).

The association results for *CLU* are supported by a recent meta-analysis of linkage studies of Alzheimer's disease, in which a region of 15.1 Mb on chromosome 8p that encompasses *CLU* had the strongest evidence genome wide for linkage[13]. The biological functions of the *CLU* markers associated with Alzheimer's disease, and of other markers from the region that may be in LD with them, is presently unknown. However, *CLU* is the only described gene located within the association region, and several lines of evidence suggest that it is a strong candidate for involvement in the disease.

APOE and CLU are the most abundantly expressed apolipoproteins in the central nervous system[14,15], with strong analogies in terms of possible impact on the Alzheimer's disease physiopathological process. Like APOE, CLU is present in amyloid plaques[16,17] and can bind Aβ[18,19]. Consistent with this, several experiments in transgenic PDAPP mice lacking *CLU* have shown that this protein may strongly influence Aβ fibrillogenesis and increase Aβ neurotoxicity *in vivo*[20]. It has also been proposed that CLU may participate in Aβ clearance from the brain across the blood-brain barrier, mainly of the highly pathogenic Aβ42 peptide[21,22], which is a function similar to that of APOE[23–25].

In addition, we observed an association between Alzheimer's disease risk and markers spanning *CR1*, the gene that encodes for the main receptor of the complement C3b protein[26]. The biological function of these CR1 markers is unknown. The LD block containing these markers

Table 2 Association results for haplotypes at the *CLU* locus

	Stage 1				Stage 2				Stage 1 + 2			
Haplotypes	Cases	Controls	OR (95% CI)	*P* value	Cases	Controls	OR (95% CI)	*P* value	Cases	Controls	OR (95% CI)	*P* value
TTC	0.344	0.388	Ref.	–	0.343	0.372	Ref.	–	0.344	0.382	Ref.	–
CCC	0.334	0.329	1.14 (1.04–1.24)	3.0×10^{-3}	0.338	0.327	1.10 (1.01–1.21)	3.3×10^{-2}	0.336	0.328	1.12 (1.06–1.20)	8.0×10^{-5}
CCG	0.302	0.265	1.28 (1.17–1.41)	1.5×10^{-7}	0.277	0.258	1.19 (1.09–1.31)	1.5×10^{-4}	0.286	0.263	1.22 (1.14–1.29)	5.6×10^{-10}

The results have been calculated using the THESIAS program with adjustment for age, gender and center (see Online Methods). The *P* values for the global association were 1.8×10^{-6}, 2.9×10^{-2} and 2.7×10^{-9}, respectively, for the data from stage 1, stage 2 and stage 1 and 2 combined. The markers as ordered from left to right (5′ to 3′) are rs2279590, rs11136000 and rs9331888. Minor alleles are underlined.

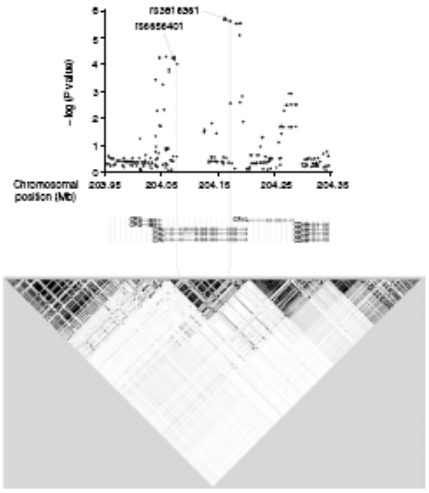

Figure 2 Schematic overview of *CR1* and LD patterns at the *CR1* locus. *P* values for association of SNPs (resulted from imputation and with MAF >5%) encompassing the *CR1* locus under an additive model with adjustment for age and gender are plotted against physical distance. The LD plot shown is for SNPs at the *CR1* locus in controls (stage 1 data in Haploview 4.0, solid spine haplotype block definition, r^2 color scheme).

only includes *CR1*. Several observations suggest that pathways involving C3b and CR1 are involved in the Alzheimer's disease process, particularly in Aβ clearance. Briefly, complement activation leads to the formation of C3-cleaving enzymes, known as C3 convertases, on the surface of the pathogen or protein undergoing complement attack. Cleavage of C3 results in the formation of C3a and C3b fragments. Whereas C3a is involved in the chemotaxis of phagocytes, C3b binds covalently to acceptor molecules and can mediate phagocytosis through CR1[27,28]. Such a mechanism has been proposed to participate in Aβ clearance for several reasons: (i) aggregated Aβ are able to activate and become bound by C3b[29–31]; (ii) in a transgenic mouse model, an increase in C3 expression coincides with a smaller degree of Aβ deposition and neuropathology[32,33]; (iii) conversely, expression of a C3 convertase inhibitor such as the rodent complement receptor 1-related gene/protein y (Crry) results in increased Aβ deposition and neurodegeneration[33]; (iv) using human erythrocytes (the cell type that most abundantly expresses CR1), it has been observed that this cell type is able to sequester $A\beta_{42}$ (refs. 31,32) and to favor its clearance via the C3b-mediated adherence to erythrocyte CR1 (ref. 32); (v) finally, this mechanism may be deficient in individuals with Alzheimer's disease[31]. Altogether, these data support a protective role for CR1 via the generation and binding of C3b, which may contribute to Aβ clearance[33].

We calculated the attributable fractions of risk to be 25.5% for *APOE*, 8.9% for *CLU* and 3.8% for *CR1*. As these calculations are based on the combined stage 1 and 2 samples, the estimates are biased upward. Nevertheless, if the estimate that 60–80% of the Alzheimer's disease risk is due to genetic factors[5] is correct, additional genetic susceptibility loci remain to be identified, which is also true for many other diseases in which loci have been successfully mapped by GWA[34,35]. In a recent GWAS, the *PCDH11X* gene was found to be a genetic determinant of Alzheimer's disease[12]. We examined the effect of 17 SNPs in the *PCDH11X* locus in the stage 1 samples. Although none reached our criteria for suggestive level of significance, we did observe $0.01 < P < 0.05$ for several SNPs (Supplementary Table 6). Thus, this locus may also contribute to the Alzheimer's disease risk in our dataset, albeit not as significantly as the other loci that we have identified.

Table 3 Association of SNPs at the *CR1* locus with Alzheimer's disease in the Stage 1 and Stage 2 samples

	N		MAF			Association test	
	Cases	Controls	Cases	Controls	HWE	OR (95% CI)	*P* value
rs6656401							
Stage 1	**2,025**	**5,324**	**0.22**	**0.18**	$\mathbf{9.9 \times 10^{-1}}$	**1.27 (1.16–1.39)**	$\mathbf{1.8 \times 10^{-7}}$
Stage 2	**3,880**	**3,198**	**0.20**	**0.18**	5.3×10^{-2}	**1.16 (1.06–1.27)**	$\mathbf{8.2 \times 10^{-4}}$
Belgium	1,066	500	0.22	0.18	2.2×10^{-1}	1.24 (0.99–1.24)	5.6×10^{-2}
Finland	608	654	0.21	0.17	2.9×10^{-1}	1.38 (1.12–1.70)	2.5×10^{-3}
Italy	1,472	1,243	0.20	0.20	2.2×10^{-1}	1.03 (0.88–1.17)	8.4×10^{-1}
Spain	734	801	0.20	0.16	4.2×10^{-2}	1.23 (1.02–1.47)	2.6×10^{-2}
Stage 1 + 2	**5,905**	**8,526**	**0.21**	**0.19**	1.1×10^{-1}	**1.21 (1.14–1.29)**	$\mathbf{3.5 \times 10^{-8}}$
APOE ε4 carriers	2,497	1,632	0.22	0.18	7.5×10^{-1}	1.38 (1.22–1.55)	1.8×10^{-7}
APOE ε4 non-carriers	2,761	6,780	0.21	0.19	4.3×10^{-2}	1.13 (1.04–1.23)	3.7×10^{-3}
rs3818361							
Stage 1	**2,018**	**5,324**	**0.22**	**0.18**	$\mathbf{8.5 \times 10^{-1}}$	**1.28 (1.17–1.40)**	$\mathbf{8.5 \times 10^{-8}}$
Stage 2	**3,717**	**3,094**	**0.22**	**0.20**	$\mathbf{1.1 \times 10^{-1}}$	**1.11 (1.02–1.22)**	$\mathbf{1.6 \times 10^{-2}}$
Belgium	972	436	0.24	0.24	9.9×10^{-1}	1.05 (0.84–1.48)	6.8×10^{-1}
Finland	590	634	0.22	0.19	1.5×10^{-1}	1.26 (1.03–1.57)	2.6×10^{-2}
Italy	1,423	1,232	0.20	0.20	9.2×10^{-1}	1.03 (0.89–1.18)	7.2×10^{-1}
Spain	732	792	0.21	0.18	1.0×10^{-2}	1.21 (1.01–1.44)	3.8×10^{-2}
Stage 1 + 2	**5,735**	**8,418**	**0.22**	**0.19**	$\mathbf{2.2 \times 10^{-1}}$	**1.19 (1.11–1.26)**	$\mathbf{8.9 \times 10^{-8}}$
APOE ε4 carriers	3,032	1,696	0.22	0.18	8.9×10^{-1}	1.34 (1.20–1.50)	2.9×10^{-7}
APOE ε4 non-carriers	2,661	6,707	0.21	0.19	1.8×10^{-1}	1.12 (1.03–1.22)	6.4×10^{-3}

P values and ORs with their associated 95% CIs have been calculated under an additive model using logistic regression models adjusted for age, gender and centers when necessary. MAF, minor allele frequency; HWE, *P* value for the test of Hardy-Weinberg equilibrium in controls.

Table 4 Association results for haplotypes at the *CR1* locus

	Stage 1				Stage 2				Stage 1 + 2			
Haplotypes	Cases	Controls	OR (95% CI)	*P* value	Cases	Controls	OR (95% CI)	*P* value	Cases	Controls	OR (95% CI)	*P* value
GG	0.772	0.813	Ref.	–	0.778	0.786	Ref.		0.776	0.803	Ref.	
GA	0.011	0.009	1.25 (0.87–1.80)	2.2×10^{-1}	0.016	0.017	1.01 (0.77–1.33)	6.4×10^{-1}	0.014	0.012	1.13 (0.89–1.43)	3.9×10^{-1}
AA	0.207	0.170	1.28 (1.17–1.40)	1.4×10^{-7}	0.198	0.182	1.17 (1.07–1.27)	6.8×10^{-4}	0.202	0.175	1.22 (1.15–1.30)	3.1×10^{-10}

The results have been calculated using the THESIAS program with adjustment for age, gender and center (see Online Methods). The *P* values for the global association were 7.5×10^{-7}, 3.0×10^{-2} and 1.5×10^{-10}, respectively, for the data from stage 1, stage 2 and stage 1 and 2 combined. The markers as ordered from left to right (5′ to 3′) are rs6656401 and rs3818361. Minor alleles are underlined.

In an independent study published in this issue of *Nature Genetics*[36], Williams and colleagues report an independent GWAS of late-onset Alzheimer's disease and also report association with markers at *CLU*. In comparing these two studies, the Belgian samples used in the replication phase of each were found to overlap. To test for the independence of these results, we tested our association results with the entire Belgian collection removed from the replication phase. We find that the association to *CLU* remains significant, with OR = 0.89, 95% CI 0.83–0.97 and $P = 5.3 \times 10^{-3}$ for association at rs1136000 in the stage 2 samples when the Belgian collection is excluded. Although this is marginally less significant than the conservative *P* value that we applied at replication phase, the combined stage 1 and 2 samples gave OR = 0.86, 95% CI 0.81–0.91 and $P = 4.8 \times 10^{-8}$ with the Belgian collection excluded, meeting our criterion for genome-wide significance. Thus these two studies provide strong independent evidence of an association of Alzheimer's disease risk with *CLU* markers. In addition, evidence of association with the *CR1* markers at rs3818361 in the GWA data from Williams and her colleagues[36] exceeded our criteria for replication in their study ($P = 9.2 \times 10^{-6}$), providing additional confirmation of the relationship between *CR1* markers and late-onset Alzheimer's disease (OR = 1.17, 95% CI 1.09–1.25). We also examined *PICALM* markers on chromosome 11 for which association has been identified and replicated in the second study. In our stage 1 data, we also detected evidence of association for *PICALM* markers with *P* values between 10^{-2} and 10^{-3} (rs541458 OR = 0.88, 95% CI 0.81–0.96, $P = 2.8 \times 10^{-3}$ and results available online in GWAS results).

In summary, in addition to the previously known *APOE* locus, we have identified loci at *CLU* and *CR1* that are potentially associated with the risk of late-onset Alzheimer's disease. Biological evidence suggests that the genes at these loci, along with *APOE*, are involved in Aβ clearance. These data may indicate that whereas familial early-onset forms of Alzheimer's disease are mainly linked to genes implicated in Aβ overproduction, genetic variants at *APOE* and these newly defined loci may influence susceptibility to late-onset forms of the disease as a result of roles in Aβ clearance.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Project conception, design, management. J.-C.L., M.L., P.A. **Phenotype collection, data management.** *France:* D.C., B.T., L.L., C.B., F.Pasquier, N.E, P.B.-G., O.H., C.L., C.D., C.J., T.L., D.H., K.R., H.B., J.-F.D., C.T., A.A. *Belgium:* K.S., K.B., S.E., P.D.D., C.V.B. *Finland:* M.H., S.Helisalmi, H.S. *Italy:* E.E, F.Bosco, M.M., F.Panza, B.N., P.Bossù, P.P., G.A., D.S., D.G., F.L. *Spain:* O.C., M.J.B., I.M., A.F., M.M.d.P., V.A. **Genome-wide, validation genotyping.** S.Heath., D.Z., I.G. **Data analysis:** J.-C.L., S.Heath., G.E., M.L., P.A. **Writing group:** J.-C.L., S.Heath., M.L., P.A.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturegenetics/.

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ONLINE METHODS

Sample populations. The case-control cohorts are described in the Supplementary Note.

Genotyping. DNA samples were transferred to the French Centre National de Génotypage for genotyping. First stage samples that passed DNA quality control were genotyped with Illumina Human610-Quad BeadChips. Genotype data were retained in the study for samples that had been successfully genotyped for >98% of the SNP markers. SNPs with call rates of <98%, with MAF <1% or showing departure from Hardy-Weinberg equilibrium in the control population ($P < 10^{-6}$) were excluded. We removed 134 Alzheimer's disease case and 980 control samples because the individuals who had provided them were found to be first- or second-degree relatives of other study participants or were assessed as being of non-European descent based on genetic analysis using methods described[37]. This led us to retain 537,029 autosomal SNPs genotyped in 2,032 cases and 5,328 controls. Stage 2 genotyping was performed using Taqman (Applied Biosystems) or Sequenom assays. The primer and probe sequences for the genotyping assays are available upon request. To avoid any genotyping bias, cases and controls were randomly mixed when genotyping, and laboratory personnel were blinded to case or control status. The genotyping success rate was at least 95%, and no departure from Hardy-Weinberg equilibrium was observed for the markers included in the second stage.

Statistical analysis. We evaluated the case and control differences using logistic regression, which optionally incorporated principal components that were significantly associated with disease status to account for possible population stratification as described[37,38]. We hypothesized that the relatively high genomic control parameter found in the absence of the principal-components adjustment was due to differences in the representation of various French regions in the case and control series. Therefore, we further explored the robustness of our conclusions by incorporating 6,734 anonymized samples from France and other European countries as additional controls (unpublished data and ref. 37). With the inclusion of the additional samples, the genomic control parameter was 1.04 without principle-components adjustment and 1.03 after the adjustment. Inclusion of the additional controls did not substantially modify the association statistics for markers in regions showing suggestive evidence of association ($P < 10^{-5}$) after correction for population structure in the primary analysis (Supplementary Tables 1 and 2). Markers in these regions were analyzed at stage 2. We included imputed markers in these regions and selected markers for genotyping at stage 2 if the marker showed at least moderate association with disease status ($P < 10^{-4}$) (either with or without correction for population structure), the marker was not in strong LD with another marker entering stage 2, and a genotyping assay for the marker could be successfully designed.

We did statistical analyses under an additive genetic model using logistic regression taking age, sex and disease status into account and using SAS software version 9.1 (SAS Institute). Population controls that were not genotyped specifically for this study were excluded, as were any samples with missing age or gender data. This gave a maximum of 2,025 Alzheimer's disease cases and 5,328 controls in stage 1 and 3,359 cases and 2,633 controls in stage 2. Information on age and gender of the cases and controls included in these analyses are shown in Supplementary Table 3. Homogeneity of the odds ratios in different collections was tested using the Breslow-Day test[39]. We also used the Mantel-Haenszel method as implemented in Review Manager 5.0 to evaluate odds ratios across collections. Interactions between *CLU* or *CR1* SNPs and *APOE* ε4 polymorphism were tested in logistic regression models adjusted for age, gender and center where the samples were taken. The solid spine haplotype block definition in Haploview 4.0 was used to generate LD blocks of the genomic regions encompassing the *CLU* or *CR1* genes from imputed SNPs (MAF >5%)[40]. Associations of the *CLU* and *CR1* haplotypes were estimated using either logistic regression or a proportional hazards models using THESIAS 3.0, which implements a maximum likelihood model and uses an s.e.m. algorithm[41]. The population attributable risk (PAR) fraction was estimated using the following formula: $PAR = F(OR-1)/(F(OR-1)+1)$, where F is the frequency of the deleterious allele in the sample and OR is the odds ratio of Alzheimer's disease risk associated with the deleterious allele.

The criterion for genome-wide significance was defined in stage 1 with a conservative Bonferroni correction ($P < 9.3 \times 10^{-8}$; 0.05/537,029 SNPs tested). In stage 2, the level for significance was defined at $P < 4.5 \times 10^{-3}$ (0.05/11 SNPs tested).

URLs. Haploview: http://www.broad.mit.edu/mpg/haploview/index.php; Revman: http://www.cc-ims.net/revman/; THESIAS: http://ecgene.net/genecanvas/uploads/THESIAS3.1/; HapMap: http://www.hapmap.org; website for GWAS results: http://www.cng.fr/alzheimer/.

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Chapter V

Multi factorial interactions in the pathogenesis pathway of Alzheimer's disease: a new risk charts for prevention of dementia

Licastro et al. Immunity & Ageing 2010, 7(Suppl 1):S4
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Multi factorial interactions in the pathogenesis pathway of Alzheimer's disease: a new risk charts for prevention of dementia

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Abstract

Background: The population longitudinal study named "The Conselice Study" has been the focus of the present investigation. 65 years old or older participants of this population study on brain aging were followed up for 5 years: 937 subjects completed the follow-up. Relationships of 46 genetic, phenotypic, clinical and nutritional factors on incident cognitive decline and incident dementia cases were investigated.

Results: A new statistical approach, called the Auto Contractive Map (AutoCM) was applied to find relationship between variables and a possible hierarchy in the relevance of each variable with incident dementia. This method, based on an artificial adaptive system, was able to define the association strength of each variable with all the others. Moreover, few variables resulted to be aggregation points in the variable connectivity map related to cognitive decline and dementia. Gene variants and cognate phenotypic variables showed differential degrees of relevance to brain aging and dementia. A risk map for age associated cognitive decline and dementia has been constructed and will be presented and discussed.

Conclusion: This map of variables may be use to identify subjects with increased risk of developing cognitive decline end/or dementia and provide pivotal information for early intervention protocols for prevention of dementia.

Background

Inflammatory responses during ageing

A dramatic increase in mean life span and life expectancy, coupled with a significant reduction in early mortality, has lead to a substantial increment in the number of elderly population in contemporary societies. This demographic picture parallels the merging of a new epidemic characterized by chronic age related diseases. Most age related diseases have complex aetiology and pathogenesis. Clinical diagnosis and therapy of these diseases imply multidisciplinary medical approaches and their cost is progressively increasing.

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The immune system is often implicated, with a variable degree of importance, in almost all age related diseases or associated with their clinical complications. Both innate and clonotypic immune system are usually involved in the pathogenesis of these chronic diseases. However, inflammatory responses appear to be the prevalent trigger mechanism driving tissue damages associated with different age-related [1].

Chronic inflammation is involved in the pathogenesis of all age-related diseases: Alzheimer's disease, atherosclerosis, diabetes, autoimmune diseases, sarcopenia and cancer have an important inflammatory component. Furthermore, increased levels of circulating inflammatory mediators may result from a constant, low-grade activation of cytokine producing cells or a dysregulated cytokine response following stimulation [2].

Licastro et al. Immunity & Ageing 2010, 7(Suppl 1):S4
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However, recent researches link an individual's exposure to precedent infections which have become latent infections and are able to induce chronic inflammation. A continuous chronic activation of immune responses then may lead and to increased risk of heart attack, stroke, and cancer. For example, the risk of heart attack and stroke is correlated with serum levels of inflammatory proteins such as CRP. Within individuals, CRP levels are also correlated to the number of seropositivities to common pathogens, indicating a history of infections (FINCH).

Low-grade increment of circulating TNF-α, IL-6, soluble IL-2 receptor (sIL-2R), and C reactive protein (CRP) and decreased levels of albumin and cholesterol, which also are indicators of inflammatory state, are strong predictors of all-cause mortality risk in longitudinal studies of several elderly cohorts. The effects of inflammatory mediators are independent of pre-existing morbidity and of other traditional risk factors for death (smoking, blood pressure, physical exercise, total cholesterol, co-morbidity, body mass index, and intake of anti-inflammatory drugs) in survival analyses suggesting that cytokines trigger/exaggerate pathological processes or act as very sensitive markers of subclinical disorders in elderly populations [2-8].

Therefore, innate immunity appears to play a pivotal role in several age related diseases and therapeutic control of chronic inflammation is becoming an emerging topics of modern gerontology and clinical geriatrics.

Brain degenerative diseases: Alzheimer's disease

Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disease that in Western societies accounts for the majority of clinical senile dementia and by 2050 the number of patients with AD is expected to rise from 4.6 to 16 millions cases in the USA [9]; worldwide statistical projections predict more that 45 million of AD patients within the above year. Neuropathological hallmarks of AD are extracellular amyloid deposits (neuritic plaques) and intracellular deposition of degenerate filaments (neurofibrillary tangles) [10]. Major clinical manifestations of the disease are memory loss and cognitive impairment [11].

Inflammation clearly occurs in pathologically vulnerable regions of the AD brain, and it does so with the full complexity of local peripheral inflammatory responses. In the periphery, degenerating tissue and the deposition of highly insoluble abnormal materials are classical stimulants of inflammation. Likewise, in the AD brain damaged neurons and neurites and highly insoluble Aβ42 peptide deposits and neurofibrillary tangles provide obvious stimuli for inflammation. Senile plaques in AD brains are associated with reactive astrocytes and activated microglial cells and cytokines and acute phase proteins are overexpressed in microglia and astrocytes surrounding neuropathological lesions in AD brains. Inflammatory factors, such as cytokines, chemokines, complement components and acute phase proteins co-localize as secondary components in neuritic or senile plaques or are over-produced in AD brains, and activated microglia surround senile plaques and areas of neurodegeneration [12,13]. There is accumulating evidence that Aβ peptide may promote or exacerbate inflammation by inducing glial cells to release immune mediators. Moreover, microglial and astroglial cells surrounding mature plaques in AD brains have been found to express activation markers. Enriched populations of human microglial cells isolated from mixed cell cultures prepared from embryonic human telencephalon tissues are able to express constitutively mRNA transcripts for cytokines and chemokines and treatment with pro-inflammatory stimuli as lipopolysaccharide or Aβ peptide led to increased expression of mRNA levels of these inflammatory molecules [14].

The role of inflammation is further emphasized by a number of clinical studies demonstrating that the long-term use of non-steroidal anti-inflammatory drugs may protect against AD. There are now a lot of published observational studies demonstrating that people who are known to be taking anti-inflammatory drugs considerably reduce their odds of developing AD and population studies have confirmed this negative association [15].

However, alternative hypothesises have been proposed. In particular, this effect has been suggested largely due to these drugs ability to inhibit angiogenesis. In fact, the brain endothelium secretes the precursor substrate for the beta-amyloid plaque and a neurotoxic peptide that selectively kills cortical neurons. So, antiangiogenic drugs targeting the abnormal brain endothelial cell might be able to prevent and treat this disease [16].

The long-term prospective association between dementia and the well known inflammation marker high-sensitivity C-reactive protein was evaluated in a cohort of Japanese American men who were seen in the second examination of the Honolulu Heart Program (1968-1970) and subsequently were re-examined 25 years later for dementia in the Honolulu-Asia Aging Study (1991-1996). In a random subsample of 1,050 Honolulu-Asia Aging Study cases and noncases, high-sensitivity C-reactive protein concentrations were measured from serum taken at the second examination; dementia was assessed in a clinical examination that included neuroimaging and neuropsychological testing and was evaluated using international criteria. Compared with men in the lowest quartile (<0.34mg/L) of high-sensitivity C-reactive protein, men in the upper three quartiles had a 3-fold significantly increased risk for all dementias combined, Alzheimer's disease, and

Licastro et al. Immunity & Ageing 2010, 7(Suppl 1):S4
http://www.immunityageing.com/content/7/S1/S4

vascular dementia. These data support the view that inflammatory markers may reflect not only peripheral disease, but also cerebral disease mechanisms related to dementia, and that these processes are measurable long before clinical symptoms appear [17].

On the other hand, several other investigations have shown increased blood levels of some cytokines, such as IL-1β and IL-6, and acute phase proteins (α-1-antichymotrypsin, ACT) in patients with clinical AD [18-21]. Therefore, altered immune responses in the brain and the peripheral blood appeared to be associated with the disease. Finally, plasma levels of ACT also correlated with the degree of cognitive impairment in AD patients form a case-control study [96] suggesting that peripheral markers of inflammation or impaired immune responses could be used for monitoring the progression of the disease.

Moreover, elevated levels of IL-6 in both brain homogenates and peripheral blood from AD patients have been reported [22]. These findings suggested that an important, but still largely unknown, interplay between brain and peripheral immune responses existed in the diseases.

In conclusion, the brain lesions associated with AD, which are referred to as neurofibrillary tangles and senile plaques, are characterized by the presence of a broad spectrum of inflammatory mediators, produced by resident brain cells, including neurons. Although secondary to the fundamental pathology caused by the presence of tangles and plaques, there is strong evidence that inflammation exacerbates the neuronal loss. Accordingly, several reports have appeared indicating that the risk of AD is substantially influenced by several polymorphisms in the promoter region, and other untranslated regions, of genes encoding inflammatory mediators. Alleles that favour increased expression of the inflammatory mediators or alleles that favour decreased expression of anti-inflammatory mediators are more frequent in patients with AD than in controls. The polymorphisms are fairly common in the general population, so there is a strong likelihood that any given individual will inherit one or more of the high-risk alleles [21].

Results

A summary of data derivation from the "Conselice" investigation at the beginning of the study and after the five year follow up is reported in Table 1.

A list of variable investigated and their functional definition used in this study is reported in Tables 2 and 3.

The connectivity map related to 42 variables from the Conselice study data base focused upon the AD, VD and CIND prevalent cases during the follow up interval is shown in Figure 1. The map depicts the most relevant associations present in the data base. The figures on the connections lines are proportional to the strength of connections. Chronological age was the closest variable to prevalent AD. However, several major biological hubs were identified: 1) low blood cholesterol, 2) high BMI index, 3) low blood HDL, 4) low blood folate.

Different genotypic, phenotypic, clinical, pharmacological or habit variables converged to these diverse hubs or cluster of connectivity. Low blood cholesterol levels was the first hub directly linked with age. Elevated IL-6 blood levels and ACT geneotype appeared to influence low cholesterol levels. The second hub was represented by high BMI index; several other variables were connected on high BMI. Increased blood cholesterol, APOE 4 allele, increased blood hcy, increased ACT and VitB12, and the mutate allele of HMGCR gene. Low blood HDL was the third hub and several variable were linked to this hub such as, male gender, increased blood CRP levels, the mutated allele of IL-1 beta gene. The fourth hub was low blood folate linked to APOE 3 and 2 alleles and the mutated ACT allele.

Third and fourth hubs in the connectivity map were shared by prevalent CIND and VD cases. Low age was directly connected with the CIND clinical state. Whereas, increased blood ACT levels were directly linked with prevalent VD.

Cognitive healthy status at the end of the follow up was on the other extremity of the connectivity map; far away from CIND, VD and on the opposite site of AD.

Table 1 Description of population investigated at the beginning (1999/2000) and at the end of the follow up (2003/2004)

1999/2000					
Eligible	Non participants[1]	Final population	Prevalent AD	Cognitively NC [2]	AD free
N= 1353	n = 337	n = 1016	n = 60	n = 19	n = 937
Followup2003/2004					
Reassessed population	Non reassessed [3]	Final population	Incident ADdementia	Cognitively NC non classified	AD free cohort
N = 937	n = 133	n = 804	n = 109	n = 4	n = 695

[1] Refusals n = 271; Deceased n = 59; Not found n = 7.
[2] NC = non classified.
[3] Refusals n =74; Deceased n = 28; Not Found n = 31.

Licastro et al. Immunity & Ageing 2010, 7(Suppl 1):S4
http://www.immunityageing.com/content/7/S1/S4

Table 2 Genetic variables used in the connectivity map

Genetic variable (gene polymorphism)		
gene	SNP	Allele mutated
ACT	rs 1884082	T
APOE	variation ε 2,3,4	ε 4
HMGCR	rs3761740	A
IL-1 beta	rs16944	T
IL-6	rs1800795	C

Discussion and conclusions

AD is a complex and multi-factorial disease, therefore, it is unlikely that a single biomarker may be determinant in the diagnosis or monitoring the progression of the disease.

The statistical analysis applied to elaborate biological and clinical data was a new enter in the field of biology and medicine. In fact, most common algorithms used in medicine are limited by the following limitations: 1) the analyses usually do not preserve the geometrical structure between variables when non linear relationships among variables are not evident. 2) another factor of uncertainty is how to establish precise associations between variables without predefined contiguity.

Here we used a new paradigm aimed to map variables and search for connectivity. In this analysis non linear association were preserved, explicit connection schemes were investigated and the complexity of dynamic interactions were preserved. The mathematics and philosophy of this analysis has been described in detail elsewhere [23]. Some application of similar kind of this analysis has already been focused to AD investigations with interesting findings [24].

Table 3 Phenotypic and clinical variables used in the connectivity map

Variable	level
high ACT	> 400 ug/ml
high cholesterol	> 200 mg/dl
high CRP	> 0.3 mg/ml
high HDL	> 65 mg/dl
low HDL	< 40 mg/dl
high IL-6	> 5 pg/ml
high TNFa	> 20 pg/ml
high triglicerids	> 175 mg/dl
high Hcy	> 17 umol/l
high folate	> 5.3 mg/dl
high TSH	>0,4 mU/L
high vitB12	> 341 pg/ml
high BMI	> 28
Hypertension	> 140 mm hg
type II diabetes	positive

Findings described here generated a connectivity map among variables and illustrated a rational path of biological variables leading to prevalent dementia.

Data presented here suggest that age, low cholesterol, high BMI, low HDL and low folate are major variable associated with the risk of AD, VD and CIND. CIND, as expected, were associated with a lower age at onset.

Our findings showed four major connecting nodes from the Conselice data base; these hubs linked apparently different factors to cognitive impairment and dementia via cholesterol, cholesterol gene dependent pathway, BMI and age. A new association among different immunological factors and lipid metabolism with incident dementia has also emerged.

In conclusion the connectivity map presented here on prevalent dementia extents previous observations from case/control investigations and population investigations and confirm that some immune factors indeed play a role in the pathogenesis of age-associated dementia by modifying metabolic and lipid variables and also show a new link between immunity, cholesterol metabolism and age related cognitive deterioration.

Material and methods

Data base generation

Data were collected from the elderly (65 year old or older) living in Conselice county in Northern Italy. Participants were interviewed, medically examined and cognitively evaluated in 1999. A blood sample from each subject was taken and each participant was given a computerized scan radiogram of the brain. After five years subjects underwent medical and cognitive re-evaluation. 937 elderly completed the follow up. A detailed description of the clinical protocol and the assessed variable has been already described elsewhere [25,26].

Diagnosis of dementia was performed according criteria of DSM-IV (1994). Clinical AD was defined using the NINCDS-ADRDA criteria [27]. Vascular dementia (VD) was diagnosed using NINDS-AIREN criteria [28].

Diagnosis of CIND was performed according methods already described [29].

Statistical analysis

Conselice data base has the aim of increasing our understanding of the pathogenetic pathway leading to cognitive decline and dementia. This goal has been achieved through a new mathematical approach able to point out the relative relevance of each variable in representing major biological hub or aggregation point. This new paradigm of variables processing aims to create a semantic connectivity map in which: a) non linear associations are preserved, b) there are explicit connections schemes, c) the complex dynamics of adaptive interactions is captured. This method is based on an artificial adaptive

Licastro et al. Immunity & Ageing 2010, 7(Suppl 1):S4
http://www.immunityageing.com/content/7/S1/S4

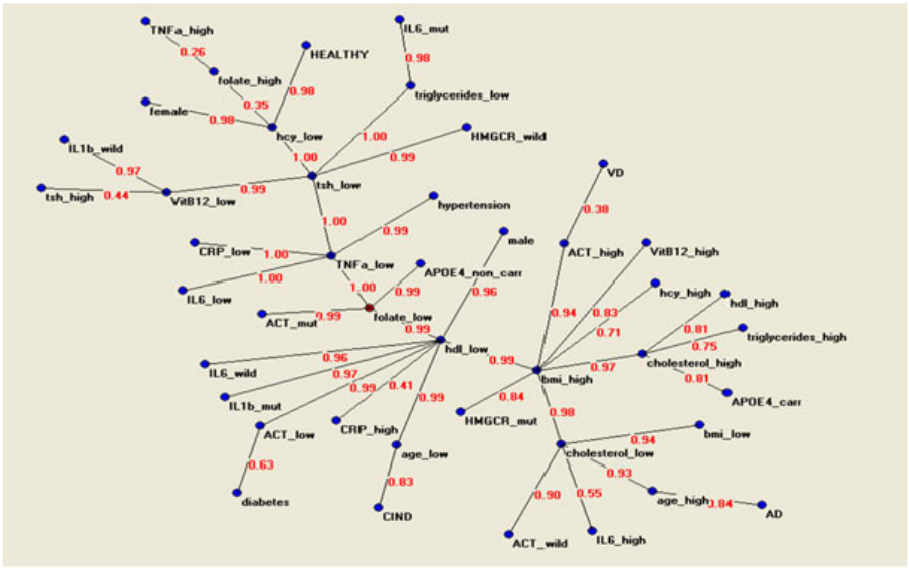

Figure 1 Connectivity map of 42 epidemiological, genetic and clinical variables showing different output such as prevalent AD, prevalent VD, prevalent CIND and control cases

system able to define the strength of the associations of each variable with all the others in any dataset, named the Auto Contractive Map (AutoCM). The architecture and the mathematic of AutoCM was invented, tested and implemented in C language, as described elsewhere [24]. The philosophy behind this approach is to pick up affinities among variables related to their dynamical interaction rather than to their simple contingent spatial position. This approach is suggested more suitable to describe a context typical of living systems in which there is a continuous complex change in the variables values among time. After the training phase, the weights matrix of the AutoCM represents the warped landscape of the dataset. We apply a simple filter (minimum spanning tree by Kruskal) to the weights matrix of AutoCM system to show the map of main connections between and among variables and the principal hubs of the system.

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Abbreviations
ACT: alpha-1 antichymotrypsin; APOE: Apolipoprotein E; HMGCR: hydroxsil-methyl-glutayl CoA reductase; IL-1 b: Interlukin-1 beta; IL-6: Interleukin-6; TNFa: tumor necrosis factor alpha; AD: patients with Alzheimer's disease; CIND: patient with clinical diagnosis of cognitive impairment but no dementia; VD: patients with vascular dementia; Hcy: homocystein; Tsh: thyroid stimulating hormone; BMI: Body max index; HDL: high density lipoprotein

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Authors' contribution
EG and MB created and developed the Auto Contractive Map; EP and IC performed laboratory analysis and genotyping; PF and GR collected samples and conceived of the Conselice Study of Brain Ageing; FL coordinated the application of statistical analysis of Conselice data base and contributed to design the clinical, epidemiological and genetic study.

Competing interests
The authors declare that they have no competing interests.

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Chapter VI

Altered glycosylation profile of purified plasma ACT from Alzheimer's disease

Ianni et al. Immunity & Ageing 2010, 7(Suppl 1):S6
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Altered glycosylation profile of purified plasma ACT from Alzheimer's disease

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Abstract

Background: Alzheimer's disease (AD) is one of the most frequent cause of neurodegenerative disorder in the elderly. Inflammation has been implicated in brain degenerative processes and peripheral markers of brain AD related impairment would be useful. Plasma levels of alpha-1-antichymotrypsin (ACT), an acute phase protein and a secondary component of amyloid plaques, are often increased in AD patients and high blood ACT levels correlate with progressive cognitive deterioration. During inflammatory responses changes in the micro-heterogeneity of ACT sugar chains have been described.

Methods: N-Glycanase digestion from Flavobacterium meningosepticum (PNGase F) was performed on both native and denatured purified ACT condition and resolved to Western blot with the purpose to revealed the ACT de-glycosylation pattern.
Further characterization of the ACT glycan profile was obtained by a glycoarray; each lectin group in the assay specifically recognizes one or two glycans/epitopes. Lectin-bound ACT produced a glyco-fingerprint and mayor differences between AD and controls samples were assessed by a specific algorithms.

Results: Western blot analysis of purified ACT after PNGase F treatment and analysis of sugar composition of ACT showed significantly difference in "glyco-fingerprints" patterns from controls (CTR) and AD; ACT from AD showing significantly reduced levels of sialic acid. A difference in terminal GlcNac residues appeared to be related with progressive cognitive deterioration.

Conclusions: Low content of terminal GlcNac and sialic acid in peripheral ACT in AD patients suggests that a different pattern of glycosylation might be a marker of brain inflammation. Moreover ACT glycosylation analysis could be used to predict AD clinical progression and used in clinical trials as surrogate marker of clinical efficacy.

Background

Alzheimer's disease (AD) is a neurodegenerative disorder clinically defined by progressive impairment of memory and cognitive functions. Brain pathology hallmarks of AD are extra-cellular amyloid plaques and intracellular neurofibrillary tangles, along with hyperactive microglia, activated astrocytes, degenerating neurons and synapsis loss [1].

Alpha-1-antichymotrypsin (ACT) is a secondary component of amyloid plaques [2]; it belongs to the superfamily of Serpins (serine protease inhibitors) and is also known as SERPINA3 [3]. ACT is synthesized in the liver and in other tissues, including lungs and brain. In the brain ACT is produced by activated astrocytes found near brain beta-amyloid (Aβ) deposits [4]. It has been suggested that ACT binds Aβ peptide and affects the rate of amyloid fibril formation *in vitro*[5,6]. Findings from mouse models of AD have also shown that ACT over-expression promotes Aβ peptide deposition in the brain of AD animal models [7] and affected their cognitive performances [8]. More recently, ACT has

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Ianni et al. Immunity & Ageing 2010, 7(Suppl 1):S6
http://www.immunityageing.com/content/7/S1/S6

been shown to influence TAU protein phosphorylation and apoptosis in neuronal cells [9].

Interleukin- α (IL–1α), IL-1ß, IL-6, tumor necrosis factor α (TNF-α) and other cytokines are up-regulated and are associated with AD lesions. The inflammatory cytokines IL-1, IL-6, and TNF-α are produced by both activated microglia and astrocytes. Moreover, IL-6 and oncostatin M have been reported to modulate ACT production in brain astrocytes [10]. These data have suggested the notion that ACT might be a critical factor affecting both neurodegenerative process induced by amyliod deposition and brain inflammatory processes. The association of gene variations in ACT and other cytokine genes with the increased risk of AD has further reinforced the above hypothesis [11].

Whether peripheral levels of ACT may be of practical use, as AD biomarker or an indicator of the disease clinical progression, remains an open question. In fact, after the initial reports of increased blood and CSF ACT concentrations in AD patients [12-14], several studies measured ACT concentrations in blood samples drawn from subjects with AD, with other forms of dementias, and control subjects. Findings from these studies have produced conflicting results; some investigations confirming increased serum ACT levels [12,15,16] others showing normal ACT blood levels in AD [17,18]. Recent findings indeed showed that peripheral blood ACT levels were increased in AD patients or subjects with cognitive alteration and no dementia and high ACT levels correlated with progressive cognitive deteriorations [19]. These data paralleled other findings showing that ACT blood levels correlated with cognitive performances in elderly without dementia [20]. Different techniques for ACT detection, different criteria for the selection of controls and AD patients or small numbers of cases and controls included in the studies may account for contradictory results regarding the association of abnormal ACT plasma levels with AD. Moreover, alterations in molecular forms of ACT present in tissues and/or blood might also account for increased variability of ACT detection in AD and controls. However, no investigation has focused upon ACT molecular rearrangement in AD.

ACT plays a role in the modulation of brain amyloid deposition and immune responses, both processes are thought to be important contributors to the pathogenesis of AD [21]. Inflammatory states are usually associated with changes in the glycosylation pattern of acute phase proteins [22,23]. ACT is a glycoprotein and carbohydrates accounts approximately for 25% of its molecular weight. The sugar chain composition of ACT was studied by affinity immune-electrophoresis with Concanavalin A [24], by high resolution ^{1}H-NMR spectroscopy [25] and, more recently, by mass spectrometry techniques [26]. ACT contains six N-glycosylation sites and shows four oligosaccharide side-chains with disialyl diantennary and trisialyl triantennary type glycan structures with traces of disialylated triantennary oligosaccharides. Studies from other biology fields showed that inflammatory responses causes changes in the microheterogeneity of ACT sugar chains. Such changes were observed in several disease states, such as prostate cancer, myocardial infarction, ovary cancer, septic inflammation, metastatic breast cancer, connective tissue disease and pulmonary sarcoidosis [24,25,27-29] .

In AD altered glycosilation pattern of presenilin-1, a molecule forming the catalytic core of the γ-secretase complex and able to generate amyloidogenic peptides [30] and an abnormal glycosylation of reelin, a glycoprotein essential for the correct cyto-architectonic organization of the developing CNS, were previously shown [31].

No data on plasma ACT glycosylation patterns in AD are on record. Here we have shown that glycosylation pattern of this molecule from the peripheral blood of AD patients and healthy controls is partially different.

Methods

Patients

The control plasma samples were from the "Conselice Study of brain aging" [32] and the demented patients were also from a different Northern Italy clinical longitudinal study, where AD patients were followed up for two years and their cognitive performances recorded. Patients and controls were Caucasians and informed consent from each control and AD relative was obtained.

Diagnosis of probable AD was performed according to standard clinical procedure and followed the NINCDS/ADRDA and DSM-IV-R criteria [33,34]. Cognitive performances were measured according to MMSE. Cognitive decline during the 2 year longitudinal follow up in AD patients was also assessed by the MMSE scores, according to the method suggested elsewhere [35].

Purification of ACT from plasma of CTR and AD

Plasma samples from CTR and AD patients with comparable ACT levels were chosen. ACT levels in plasma were measured by using a competitive ELISA assay, as described elsewhere [19]. Plasma samples from 20 CTR or 19 AD patients were pooled in 2 distinct experimental sets (CTR 1 and 2 and AD 1 and 2, respectively). All experiments were performed using purified ACT obtained from these plasma sample pools.

Purification of ACT was performed by affinity chromatography using Hitrap NHS-activated HP columns (1 ml) (GE Lifesciences, Milan). 10 mg of sheep anti-human ACT antibody (AbCam, Cambridge) was coupled to the column matrix according to the manufacturer's instructions.

Ianni et al. Immunity & Ageing 2010, 7(Suppl 1):S6
http://www.immunityageing.com/content/7/S1/S6

Pooled plasma samples (100 μl) containing about 70 μg ACT were diluted to 10 ml with PBS, filtered through a 0.45 μm filter and applied to the column. Each sample was left re-circulating for 2h at room temperature using a peristaltic pump at a flow rate of 0.2 ml/min. Thereafter, the column was washed with 10 ml of PBS and bound ACT was eluted with 0.2 M glycine, pH 2.8; the purified protein was immediately neutralized with 5N NaOH and dialyzed against H_2O and concentrated under reduced pressure.

Assessment of purified ACT concentration by sandwich ELISA assay

96 well maxisorp plates (Nunc, Milan) were coated with 100 μl of sheep anti-human ACT antibody (AbCam, Cambridge), diluted 1:1000 in 50 mM Na/CO_3 pH 8.5, incubated overnight at 4°C and washed. If not otherwise specified, washing of plates was always performed with 4 x 200 μl/well of PBS+0,05% Tween 20x (PBST) and incubation steps throughout the assay always lasted 2h, at 37°C, with shaking. After washing, plates were incubated with 100 μl/well of PBST+5% BSA and washed again.

Thereafter, 100 μl of commercially available ACT (Sigma, Milan) (dissolved in PBST + 1% BSA), in several dilutions ranging from 0 to 200 ng/ml to generate a standard curve, and test samples were added to the plate wells. After incubation and washing, plates were incubated with primary antibody (100 μl/well of rabbit anti-human ACT antibody (Dako, Milan), diluted 1:1000 in PBST+1% BSA) and secondary HRP-conjugated antibody (goat anti-rabbit-HRP (Santa Cruz, Heidelberg), diluted 1:1000 in PBST+1% BSA).

Following the usual PBST washes, an additional wash with 200 μl of PBS without Tween was performed and 100 μl of peroxidase substrate (ABTS) (Roche, Milan) diluted in ABTS buffer (Roche, Milan) was added to the wells.

Absorbance was recorded by an automatic ELISA reader at 405 nm (Biorad, Milan).

De-glycosylation by N-Glycanase digestion of purified ACT

N-Glycanase from Flavobacterium meningosepticum (PNGase F) was used (BioLabs, Milan). De-glycosylation was performed on both native and denatured purified ACT.

Reaction in native conditions was performed by incubating 1 μg of purified ACT with 500 U of PNGase F in 50 mM sodium phosphate pH 7.5, 1% NP-40 at 37°C for 1 and 3 h.

Denaturation of purified ACT was obtained by heating the protein at 100°C for 10 min in the presence of 0.5% SDS and 40 mM dithiothreitol (DTT). After denaturation, ACT was reacted with PNGase F, as described above.

De-glycosylated ACT samples were resolved on a 10% SDS-polyacrylamide gel, blotted on a PVDF membrane, visualized by immune reaction with a specific antibody (rabbit anti-human ACT (Dako, Milan) and revealed by a Cy5-labelled secondary antibody (GE Lifesciences, Milan).

Glycan composition analysis of purified ACT

The glycan profile of purified ACT samples was obtained by using the Qiagen Qproteome™ GlycoArray. Briefly, 5 μg of purified ACT were absorbed onto the surface of the GlycoArray slide, following the manufacturer's instructions. Lectin-bound ACT was revealed by immune reaction using the rabbit anti-human ACT antibody (QIAGEN, Dako, Milan) and the Cy5-labelled secondary antibody (GE Lifescience, Milan). The entire process was performed in parallel without samples on a separate control array. At the end of the procedure, array slides were scanned and analyzed using the Scann Array 4000 scanner (Packard Biochip Technologies, Milan). Array image data were analyzed using the Qproteome Glycoarray Analysis Software (QIAGEN), which calculates the "glyco-fingerprint" of the sample protein by subtracting the control array signals from the experimental sample array signals. Fingerprint deconvolution was performed by algorithms using rule-based technology calibrated to a wide range of standard proteins. Each lectin group in the assay specifically recognizes one or two glycans/epitopes, although a degree of interdependence between these groups is present. This algorithm according to manufacturer calculates relative abundance of glycan epitopes and provides array-binding information on the proportion of various features within a glycoform population.

Results

Clinical, cognitive and epidemiological variables along with number of subjects, ACT plasma levels, purified pooled ACT samples, age, gender, cognitive status assessed by MMSE scores at the time of clinical diagnosis and two years later from controls (CTR) and AD are summarized in Table 1. The AD 1 showed a higher cognitive deterioration during a 2 year follow up than the AD 2. Plasma samples from 2 different group of control (CTR 1 and 2) and AD patients (AD 1 and 2) were used for the purification of ACT and the biochemical analysis. Mean plasma ACT levels between 2 groups of controls and AD patients were comparable, as well as those of the collected ACT after the purification procedures.

Figure 1 shows Western Blot analysis of purified ACT from CTR 1 and 2 or AD 1 and 2 treated with PNGase F. De-glycosylation of native purified ACT form both

Ianni et al. Immunity & Ageing 2010, 7(Suppl 1):S6
http://www.immunityageing.com/content/7/S1/S6

Table 1 Epidemiological and clinical features from investigated subjects

	CTR 1	CTR 2	AD 1	AD 2
N° of samples	10	10	9	10
ACT mean (μg/ml)	763	789	695	876
ACT purify (μg/ml)	26	38	27	32
Age	73	75	80	77
Gender	9 F - 1 M	4 F – 6 M	7 F - 2 M	7 F - 3 M
Evolution	4 S- 1 I - 5 NA	2 S- 5 I- 3 NA	2 F – 6 I – 1 S	1 F – 5 I – 4 S
MMSE time 0	28	28	19	19
MMSE after 2 year follow up	27	26	13	18

Number of subjects, ACT plasma levels, purified ACT of subjects's pool, age, gender, group of cognitively state, MMSE scores at the beginning of the study and two years later are summarized in this table.

CTR and AD samples resolved into three protein bands and no qualitative differences were observed between CTR and AD. On the contrary, when PNGase F treatments was performed on denatured purified ACT, four bands were detected in both CTR 1 and 2, whereas ACT from AD samples resolved again into three bands. Semi quantitative evaluation of fluorescence band intensity from Figure 1 is reported in Table 2. Total fluorescence from ACT PAGE electrophoresis and western blot analysis in band 1 from CTR and AD was comparable (CTR 1 = 37977; AD 1 = 44469; CTR 2 = 35449; AD 2 = 23722). Results regarding total fluorescence and its percentage in band 1, 2 and 3 (native) and 1, 2, 3 and 4 (denatured) are reported in Table 2. Some difference was observed in native samples from CTR 2 and AD 2 after 3 hours of incubation with PNGase F enzyme. Under mild denaturing treatment band 4 was never detected in AD 1 and AD 2 and strong difference in fluorescence intensity in band 1, 2 and 3 after 3 hours of PNGase F digestion were found. Moreover, fluorescence intensity in band 1, 2 and 3 from AD 1 and AD 2 was quite different from those of CTR 1 and CTR 2, especially after 3 hour treatment. In fact, in these condition both total fluorescence intensity and its percentage, were higher in bands 1 and 2 from AD 1 and AD 2 samples than those detected in CTR 1 and/or CTR 2.

Further analysis of sugar composition in purified ACT from CTR 1 and 2 or AD 1 and 2 was performed by using the Qproteome™ GlycoArray kit. This glyco-array consisted of 24 lectins covering a large pattern of glycan specificity. Binding of a glycoprotein to the array results in a characteristic fingerprint pattern that is highly sensitive to the glycan structure and composition. Glycan structure semi-quantitatively detectable by the array include: N-glycans (bi-antennary, tri/tetra antennary, high mannose, sialic acid, terminal N-acetyl glucosamine (GlcNac), terminal N-acetyl galactosamine (GalNac) and bisecting GlcNac and presence or absence of O-glycans. The fingerprint is interpreted by proprietary knowledge-based algorithms to produce the glycoanalysis results, a list of epitopes and their relative abudance.

Fingerprint data, analyzed by the Qproteome™ GlycoArray software, produced a detailed profile of ACT glycosylation status and a glycan epitope prediction pattern by the specific algorithm (Table 3). The Qproteome™ GlycoArray method provides four levels quantification output for most epitopes: not Detected=up to 10%, low=11-30%, medium=31-70%, high=71-100% and a qualitative glycan profile for other epitopes (detected/not detected). Quantitative difference in purified ACT lectin reactivity between the experimental sets, i.e. Purified ACT from AD 1 and 2 showed significantly reduced levels of sialic acid when compared to those from CTR 1 and 2. Moreover, a difference in terminal GlcNac residues was found between AD 1 and AD 2 groups. It is interesting to note that AD 1 showed a faster cognitive deterioration than AD 2 in a 2 years follow up. In fact, as shown in Table 1, AD 1 patients loosed 5 points in the MMSE score and AD 2 patients only 1 point.

Discussion

Glycosylation is a versatile biochemical mechanism and one of the most abundant post-translational modification of protein; however, glycosylation of proteins is not a template driven process, is difficult to predict [36] and affects molecule stability, resistance to proteolysis, solubility and molecule functional activity. Therefore, this protein modification may play a role in affecting biological activity of molecules with a special role in the metabolic events related to neuro-degeneration and AD.

ACT is a glycoprotein and carbohydrate content reach 24% of molecular weight. This acute phase proteins is mainly synthesized by the liver, however, other tissues are able to produce and release this molecule. In fact, astrocytes synthesized and release ACT and increased levels of this protein have been found in the brain, CSF and blood from AD patients [15,19,37]. ACT levels in

Ianni et al. Immunity & Ageing 2010, 7(Suppl 1):S6
http://www.immunityageing.com/content/7/S1/S6

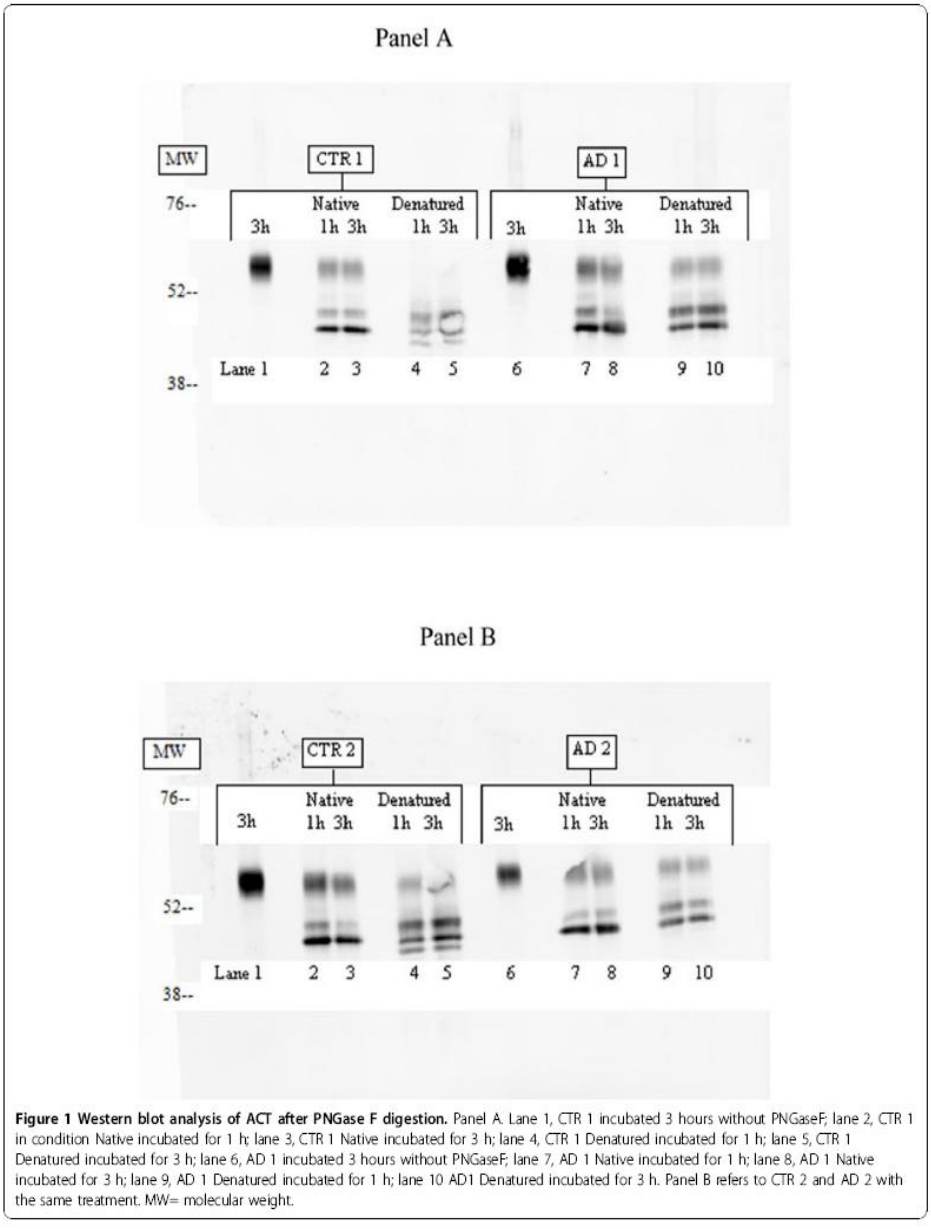

Figure 1 Western blot analysis of ACT after PNGase F digestion. Panel A. Lane 1, CTR 1 incubated 3 hours without PNGaseF; lane 2, CTR 1 in condition Native incubated for 1 h; lane 3, CTR 1 Native incubated for 3 h; lane 4, CTR 1 Denatured incubated for 1 h; lane 5, CTR 1 Denatured incubated for 3 h; lane 6, AD 1 incubated 3 hours without PNGaseF; lane 7, AD 1 Native incubated for 1 h; lane 8, AD 1 Native incubated for 3 h; lane 9, AD 1 Denatured incubated for 1 h; lane 10 AD1 Denatured incubated for 3 h. Panel B refers to CTR 2 and AD 2 with the same treatment. MW= molecular weight.

Ianni et al. Immunity & Ageing 2010, 7(Suppl 1):S6
http://www.immunityageing.com/content/7/S1/S6

Table 2 Fluorescence intensity analysis after PNGase F treatment

PANEL A	CTR 1				AD 1			
Band	Native 1 1 h	Native 3 3 h	Denatured 1 h	Denatured 3 h	Native 1 1 h	Native 3 3 h	Denatured 1 h	Denatured 3 h
1	12271 (41.2%)	11658 (38.6%)	8608 (57%)	978 (5.5%)	20528 (40.5%)	16066 (37.4%)	10821 (30.5%)	10480 (25.3%)
2	5297 (17.8%)	5272 (17.4%)	4313 (28%)	9264 (52.6%)	10135 (20%)	5651 (13.1%)	12959 (36.6%)	15418 (37.1%)
3	12229 (41%)	13257 (44%)	2240 (15%)	4689 (26.6%)	19972 (39.5%)	21315 (49.5%)	11668 (32.9%)	15594 (37.6%)
4				2702 (15.3%)				
PANEL B	CTR 2				AD 2			
Band	Native 1 1 h	Native 3 3 h	Denatured 1 h	Denatured 3 h	Native 1 1 h	Native 3 3 h	Denatured 1 h	Denatured 3 h
1	18537 (46.7%)	12721 (44.9%)	6925 (25.1%)	5234 (15.7%)	10371 (44.4%)	10937 (37.2%)	6649 (37.1%)	5579 (36.7%)
2	6293 (15.9%)	2825 (10%)	11136 (40.4%)	14381 (43.3%)	2916 (12.5%)	4048 (13.8%)	5527 (30.8%)	3913 (25.8%)
3	14817 (37.4%)	12804 (45.1%)	6094 (22.1%)	10005 (30.1%)	10083 (43.1%)	14385 (49%)	5759 (32.1%)	5706 (37.5%)
4			3428 (12.4%)	3632 (10.9%)				

Band fluorescence intensity after PNGase F treatment (see Western Blot bands in figure 1, panels A and B).

the blood markedly increases after tissue damages or infections [38]. We already postulated that a proportion of plasma ACT in AD might derive from the brain as a by-product of neurodegenerative processes and inflammation in the central nervous system [39]. As for other glycoproteins, micro-heterogeneity of ACT may be ascribed to differences in carbohydrate structure and indeed different patterns of ACT micro-heterogeneity has been shown in different diseases [40,41].

Table 3 Glycan epitope pattern of Cy5 labeled ACT

Glycan epitope	CTR 1	AD 1	CTR 2	AD 2
N-linked:				
Bi Antennary	Not Detected	Not Detected	Not Detected	Not Detected
Tri/Tetra Antennary	High	High	High	High
High Mannose	Not Detected	Not Detected	Not Detected	Not Detected
Sialic Acid	High	Medium	High	Medium
Terminal GlcNAc	Low	Low	Low	Not Detected
Terminal GalNAc	Not Detected	Not Detected	Not Detected	Not Detected
Bisecting GlcNAc	Not Detected	Not Detected	Not Detected	Not Detected
O-Glycans	Not Detected	Not Detected	Not Detected	Not Detected

Glycan profile produced for ACT using the Qproteome Glycoarray method. ND=not detected (up to 10%); Low=11-30%; Medium=31-70%; High=71-100%.

To obtain usable level of purified ACT, samples from AD or control were pooled; plasma samples showing comparable levels of this serpin, as assessed by competitive ELISA, i.e. moderately high ACT levels, were chosen. This step is relevant, since plasma levels of ACT and other serpins increase in different pathological conditions; however, in this investigation both patients controls were free from cancer, infections and inflammatory diseases.

Here we showed that after partial denaturation, purified ACT from AD plasma samples were less sensitive to enzymatic digestion by N-glycanase than ACT from plasma samples of healthy donors. This first observation suggested a different glycosylation pattern in ACT form AD patients, since denaturation was shown to increase deglycosylation by glycanase [25]. Different deglycosylation patterns of denatured ACT between AD and CTR may be ascribed to differentially presence of fucose residues linked α(1-3) to ASN bound N-acetylglucosamine that resistant to PNGase F action.

Purified ACT was then analysed by a lectin array specifically developed for investigating protein glycan content and composition [42]. This analysis resulted in a partially different pattern of glycan profiles between ACT from AD and controls; sialic acid content being different between AD and CTR.

This alteration may have several explanations. For instance, a proportion of circulating ACT in AD plasma may derive from other tissues than liver, possibly the

brain and this molecules might show a different glycosylation signature.

On the other hand, we can not exclude another interpretation suggesting that altered ACT glycan profile from AD samples may reflect a generalized impairment of glycosylation processes involving other glycoproteins. In fact, it has previously shown that reelin, a glycoprotein essential for the correct cyto-architecture organisation of developing brain and involved in signalling pathways linked to neuro-degeneration in several human diseases, were increased in the brain from neurological disorders and showed a different glycosylation patterns in plasma from AD [31]. Moreover, acetylcholine esterase from AD samples analyzed by lectin binding activity showed different binding properties when compare with those from controls [43].

Also our data showed a slight but significant difference in the two AD sets. AD 1 showing higher fluorescence intensity in terminal GlcNac and sialic acid than AD 2. Patients belonging to the AD 1 group showed a faster cognitive deterioration rate in a 2 year follow up. Overall our data suggest altered sialic acid content in ACT from AD samples and the potential presence of focuse residues in the denatured ACT from CTR than AD samples.

Conclusion

Altered glycosylation pattern in purified ACT from the peripheral blood of AD maight be ascribed to an increased inflammation of the brain or an altered glycation process of ACT along with several other brain proteins in AD.

Our findings suggest that low content of terminal GlcNac glycans and sialic acid in peripheral ACT might be a marker of diseases progression and it might be used in clinical trials as surrogate marker of clinical efficacy.

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List of abbreviations

AD: alzheimer's disease; ACT: alpha-1-antichymotrypsin; CTR: controls; IL1α: Interleukin 1α; IL-1ß: Interleukin 1ß; IL-6: Interleukin 6; TNF-α: tumor necrosis factor α; Aβ: beta-amyloid; CSF: cerebrospinal fluid; PNGase F: N-Glycanase digestion from Flavobacterium meningosepticum; GlcNac: N-acetyl glucosamine; GalNac: terminal N-acetyl galactosamine; Gal/GalNac: Galactose/N-acetyl galactosamine.

Authors' contribution

MI and MM performed ACT ELISA assay and ACT GlycoArray;
EP,MC and IC purification of ACT and writing article;
GDS and FL Conception and Design and writing article

Competing interests

The authors declare that they have no competing interests.

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Chapter VII

The CALHM1 P86L Polymorphism is a Genetic Modifier of Age at Onset in Alzheimer's Disease: a Meta-Analysis Study

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The *CALHM1* P86L polymorphism is a genetic modifier of age at onset in Alzheimer's disease: a meta-analysis study

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Abstract

The only established genetic determinant of non-Mendelian forms of Alzheimer's disease (AD) is the ε4 allele of the apolipoprotein E gene (*APOE*). Recently, it has been reported that the P86L polymorphism of the calcium homeostasis modulator 1 gene (*CALHM1*) is associated with the risk of developing AD. In order to independently assess this association, we performed a meta-analysis of 7,873 AD cases and 13,274 controls of Caucasian origin (from a total of 24 centres in Belgium, Finland, France, Italy, Spain, Sweden, the UK and the USA). Our results indicate that the *CALHM1* P86L polymorphism is likely not a genetic determinant of AD but may modulate age at onset by interacting with the effect of the ε4 allele of the *APOE* gene.

INTRODUCTION

Although Alzheimer's disease (AD) is the most common cause of dementia in the elderly, its aetiology is still not fully understood. The characterisation of causative factors is thus important for better defining the pathophysiological processes involved. Hereditary, early-onset forms of AD have been linked to disease-causing mutations in three different genes: the amyloidprecursor protein (*APP*) gene on chromosome 21, the presenilin1 (*PSEN1*) gene on chromosome 14 and the presenilin 2 (*PSEN2*)gene on chromosome 1 (1). However, the known mutations in these three genes account for less than 1% of all AD cases (2). Most forms of AD develop after the age of 65 and are considered to be sporadic because they lack an obvious familial aggregation. The term "sporadic" has, however, been gradually replaced by the concept of non-Mendelian (i.e. genetically complex) transmission. Although the importance of the genetic component of these non-Mendelian forms has long been debated, there is now a large body of evidence suggesting that genetic variation plays the major role in determining risk for

this form of AD as well. This evidence is largely based on twin studies which have shown that the heritability of AD in general is high (between 60 and 80%) (3). This latter study has also shown that age at onset (AAO) is significantly more consistent for pairs of monozygotic twins than for dizygotic twins indicating that genetic variants also explain a substantial proportion of AAO variation across AD cases (3). While these observations highlight the importance of genetic factors in the risk for developing AD, at present, only the ε4 allele of the apolipoprotein E (*APOE*) gene has been unequivocally identified as a major determinant for the non-Mendelian forms of AD (4–6). In addition, currently more than two dozen loci show significant risk effects in meta-analyses synthesizing the available data from all published studies in the field. (http://www.alzgene.org) (7).

We recently reported that the gene coding for the newly characterised calcium homeostasis modulator 1 (CALHM1) channel may be a potential genetic risk factor for non-Mendelian forms of AD. The less common allele (L) of a non-synonymous polymorphism (P86L or rs2986017) within this gene was found to be associated with an increased risk for developing AD. Further it was shown that the underlying amino-acid substitution from proline to leucine leads to a loss of Ca^{2+} permeability, modulation of APP metabolism and, ultimately, to an increase in Aβ peptide secretion (8). However, although CALHM1's biological properties make it a plausible AD risk factor (8,9), most of the currently published follow-up studies in Caucasian populations were unable to confirm the association between the P86L polymorphism and the risk of developing AD (10–14) at the exception of one report (15). Despite this contradictory data using affection status as phenotype, three studies, in addition to the original report, showed association between an earlier AAO and homozygosity of the L allele and a marker in the *CALHM1* vicinity (11,15,16).

In this study, we assessed the question whether or not *CALHM1* is a genetic susceptibility factor for non-Mendelian AD, we genotyped a total of 9,662 individuals (2,249 cases and 7,413 controls) not previously tested for *CALHM1* and performed a meta-analysis synthesizing these data with previously published genotypes in a total sample of 7,873AD cases and 13,274 controls of Caucasian origin.

MATERIALS AND METHODS

Case-control samples were obtained from centres in Belgium (1 study) (12,17), Finland (1 study) (10) France (3 studies) (8,18), Italy (10 studies) (14,17), Spain (4 studies) (15,17), Sweden (1 studies) (10), the UK (1 study) (9) and the USA (3 studies) (8,11,13). The main characteristics of the different populations in each country are described in Supplementary Table 3. Clinical diagnoses of probable AD were all established according to the DSM-III-R and NINCDS-ADRDA criteria (19). Controls were defined as subjects not meeting the DMS-III-R dementia criteria and with intact cognitive functions (mini mental status examination score>25). Written informed consent to participation was provided by all subjects or, in cases of substantial cognitive impairment, a caregiver, legal guardian or other proxy. The study protocols for all populations were reviewed and approved by the appropriate institutional review boards in each country. Depending on the centre, a broad range panel of technologies were used to genotype the rs2986017 SNP (8,10–15).

Univariate analysis was performed using Pearson's χ^2 test. Review Manager software release 5.0 (http://www.cc-ims.net/RevMan/) was used to estimate the overall effect (random effect odds ratio). For multivariate analysis, SAS software release 9.1 was used (SAS Institute, Cary, NC) and inter-population homogeneity between was tested using Breslow-Day computation (20). The association of the P86L polymorphism with the risk of developing AD was assessed by a multiple logistic regression model adjusted for age, gender, *APOE* status and centre or country (see Supplementary Table 3 for description of AAO per country). The association

between the P86L polymorphism and AAO was assessed using a mixed model adjusted for gender and using the centre as a random variable. Similar results were obtained when using the country as a random variable (data not shown). The presence or absence of an interaction between *APOE* status and the P86L polymorphism was systematically assessed in all logistic regression or mixed models.

RESULTS

Upon combining all available case-control genotype data for the P86L SNP in allele-based effects meta-analyses, we observed that the population-specific ORs showed significant evidence for heterogeneity across datasets (p=0.003). We thus calculated the summary OR using a random-effects model, where the overall P86L association appeared to be not significant (OR=1.07; 95% confidence interval (CI) [0.97–1.17]; p=0.17; Figure 1). Upon exclusion of the five initial case-control datasets (all part of the initial, positive study)[8], the heterogeneity across population-specific ORs was substantially reduced (p=0.29), but neither meta-analysis showed significant results (OR=1.01; 95% CI [0.95–1.08]; p=0.76).

As we had access to subject-level genotype and phenotype data for all samples, we also tested for association between P86L and AD risk by pooling data across studies and adjusting for age, gender, APOE ε4 status, and centre using an additive logistic regression model. This model is equivalent to the allelic association approach when the conditions for Hardy-Weinberg equilibrium are met (21), which was true for the combined sample (Supplementary Table 1). In this model, the L allele of the P86L polymorphism was weakly associated with AD (OR=1.09; 95% CI [1.03–1.15]; p=0.002). However, this association was mainly driven by the initial case-control datasets of the original report, and was no longer significant after exclusion of these samples (OR=1.02; 95% CI [0.95–1.08], adjusted for age, gender, APOE status and centre; p=0.66).

Finally, we assessed the association of the P86L polymorphism with AAO using a mixed model with centre of origin as a random variable. As previously reported (8,11,15), patients bearing the LL genotype displayed an earlier AAO than carriers of the LP and PP genotype (71.8 ± 8.9 vs. 73.0 ± 8.9 years of age, respectively; $p=8\times10^{-4}$; Table 1 and supplementary Table 2). This association was still observed after exclusion of the initial samples (73.2 ± 8.2 vs. 74.3 ± 8.2 years of age, respectively; p=0.001). Following the detection of an interaction between the P86L, APOE ε2/ε3/ε4 polymorphisms and AAO (p=0.04), we stratified the data according to *APOE* status and observed that the association of the LL genotype with AAO was the strongest in ε4 carriers (70.2 ± 8.5 vs. 72.0 ± 8.2 years; $p = 4\times10^{-5}$ (Table 1 and Supplementary Table 2). Again, this association was still observed after exclusion of the initial samples (71.9 ± 7.4 vs. 73.2 ± 7.5 years of age, respectively; p=0.002).

When taking into account the well characterised *APOE*ε4 allele dose effect on AAO, we observed that the P86L LL genotype was systematically associated with a decrease in AAO in ε3/ε4 and ε4/ε4 carriers (Table 2). Comparison of likelihood ratio between a mixed model including only APOE genotype and a mixed model including both APOE and CALHM1 genotypes indicated that addition of the CALHM1 P86L polymorphism was more informative to explain the AAO variability than the APOE ε4 allele alone ($p=1\times10^{-10}$).

DISCUSSION

Using both novel and previously published genotype data, we performed meta-analyses of 7,873 AD cases and 13,274 controls from 24 centres assessing the potential association between the P86L polymorphism in CALHM1 and risk for AD, but were unable to replicate the initial findings. The discrepancy of risk effects between the independent follow-up data and the data

first published by Dreses-Werringloer et al. (8), may indicates a false-positive finding in the initial report, a situation commonly observed in genetically complex diseases and referred to as "proteus phenomenon" or to as the "winner's curse phenomenon" (22). In addition to chance variation and technical artifacts, this may be caused by population substructure across cases and controls included in the affected association studies. Indeed, this type of difference can lead to spurious associations between diseases and genetic markers (23–26), particularly when low increases in risk are involved (27). This observation may be particularly relevant for the P86L L allele, since its frequency appears to be highly variable (even ranging from 20 to 31% for Caucasian populations) and its association with AD risk was categorized as moderate in the initial report (8).

However, even though our meta-analysis results rather unequivocally refute the initial findings suggesting that CALHM1 is a genetic risk factor for AD, the present work suggests that the CALHM1 P86L polymorphism could modulate AAO and more specifically the *APOE* ε4 allele's dose effect on this phenotype. Interestingly, several studies have shown that AAO in AD is highly heritable (28,29), and (in addition to the strong association of the ε4 allele with AAO) it has been suggested that genes such as GTS1 or GTS2 may have a specific effects on AAO without necessarily modifying the risk for developing AD (30–32), although these findings have not been independently replicated to date. In this context, it is worth noting that AAO data are difficult to acquire reliably reducing the power of such analyses. Although the large overall sample size analyzed in the present study should help to decrease the likelihood of a false-negative outcome, additional genetic studies will be required to further characterize the association between the P86L polymorphism and AAO in ε4-carriers. However, it appeared that the association of the P86L polymorphism with AAO was still observed after exclusion of the initial samples, this supporting a real impact of *CALHM1* on disease progression. It is also worth noting that factors affecting AAO tends to be spuriously associated with disease susceptibility (and the younger the cases the stronger this artefactual association may be) and this confounding effect may explain in part positive results in cross-sectional studies (33).

Furthermore, it would be of particular interest to extend the association analyses to non-Caucasian populations, such as those of South-East Asian (for which conflicting results have already been reported (34–36), or African descent. However, since the P86L L allele frequency is lower in Asian populations than Caucasian populations, particularly large sample sizes will be needed to detect significant risk or AAO effects.

Given that the P86L L allele has been associated with an increase in Aβ production *in vitro* (8), confirmation of this association with AAO may indicate that a variation in Aβ production can modulate AD progression without increasing the AD risk. Interestingly, biological evidence suggests that both the *APOE* gene and the genetic determinants characterised in two recent genome-wide association studies (GWASs) in AD may be primarily involved in Aβ peptide clearance (17,37). Combination of these genetic results and physiopathological data may thus indicate that whereas familial, early-onset forms of AD are mainly linked to genes that are involved in Aβ overproduction, genetic variants of APOE and the GWAS-defined loci may influence susceptibility to late-onset forms of the disease via a role in Aβ clearance (38). In this context, we could hypothesize that the moderate over-production of Aβ peptides associated with the P86L L allele only modifies the AD process when there is a failure in Aβ clearance - a failure that is likely to be particularly exacerbated in ε4 carriers.

In conclusion, the present meta-analysis does not support the notion that CALHM1 is a genetic risk factor for AD. However, we found a significant association between the P86L L-allele and earlier onset for AD, particularly in carriers of the APOE ε4-allele. Therefore, further studies are warranted aimed at investigating whether or not genetic variation at CALHM1 may modify

some of the pathophysiological processes involving Ca^{2+} homeostasis and leading to AD (39–41), in particular in carriers of the APOE ε4 allele.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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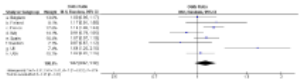

Figure 1.
Association between the P86L L allele and the risk of developing AD in the different case-control studies, according to the country of origin.

Table 1

Association between the CALHM1 P86L polymorphism and age at onset (in years ± SD) for all AD cases and for ε4 or non-ε4 AD cases.

	Whole		ε4 bearers		non ε4 bearers	
	n	age at onset	n	age at onset	n	age at onset
GG	3658	73.0 ± 8.9	1969	72.0 ± 7.9	1673	74.2 ± 9.8
AG	2761	73.1 ± 8.9	1473	71.9 ± 8.3	1277	74.4 ± 9.5
AA	588	71.8 ± 8.9	316	70.2 ± 8.2	271	73.6 ± 9.3
p[1]		0.004		2×10^{-4}		0.78
Δ (AA versus AG+GG)[2]		−1.2		−1.8		−0.7
p[3]		8×10^{-4}		4×10^{-5}		0.54

[1] mixed model adjusted for gender and using centre as a random variable

[2] Δ, the difference in AAO between LL and PL + PP carriers (in years).

[3] the difference in AAO between LL and PL + PP carriers, using a mixed model adjusted for gender and with centre as a random variable.

Table 2

Association between the *APOEε4* allele alone and in combination with the P86L polymorphism with age at onset (in years ± SD).

APOE	n	age at onset[1]	APOE	rs2986017	n	age at onset[2]
ε4−/ε4−	3223	74.2 ± 9.6	ε4−/ε4−	AG+GG	2952	74.3 ± 9.7
				AA	271	73.6 ± 9.3
ε4−/ε4+	3027	72.5 ± 8.1	ε4−/ε4+	AG+GG	2774	72.6 ± 8.1
				AA	253	70.9 ± 8.3
ε4+/ε4+	736	68.4 ± 7.5	ε4+/ε4+	AG+GG	671	69.0 ± 7.5
				AA	65	67.2 ± 7.0

[1] $p=1.1\times10^{-31}$ (mixed model adjusted for gender and using centre as a random variable)

[2] $p=2.6\times10^{-31}$ (mixed model adjusted for gender and using centre as a random variable)

Chapter VIII

Alzheimer's disease gene signature says: beware of brain viral infections

Porcellini et al. Immunity & Ageing 2010, 7:16
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Alzheimer's disease gene signature says: beware of brain viral infections

Elisa Porcellini, Ilaria Carbone, Manuela Ianni, Federico Licastro*

Abstract

Background: Recent findings from a genome wide association investigation in a large cohort of patients with Alzheimer's disease (AD) and non demented controls (CTR) showed that a limited set of genes was in a strong association ($p > 10^{-5}$) with the disease.

Presentation of the hypothesis: In this report we suggest that the polymorphism association in 8 of these genes is consistent with a non conventional interpretation of AD etiology.
Nectin-2 (NC-2), apolipoprotein E (APOE), glycoprotein carcinoembryonic antigen related cell adhesion molecule-16 (CEACAM-16), B-cell lymphoma-3 (Bcl-3), translocase of outer mitochondrial membrane 40 homolog (T0MM-40), complement receptor-1 (CR-I), APOJ or clusterin and C-type lectin domain A family-16 member (CLEC-16A) result in a genetic signature that might affect individual brain susceptibility to infection by herpes virus family during aging, leading to neuronal loss, inflammation and amyloid deposition.

Implications of the hypothesis: We hypothesized that such genetic trait may predispose to AD via complex and diverse mechanisms each contributing to an increase of individual susceptibility to brain viral infections

Background

The incidence of Alzheimer's disease (AD) is rising sharply and a large fraction of the elderly population will ultimately be affected by the disease. Because of an urgent need for effective preventative and therapeutic measures, extensive research has focused on pathogenetic mechanisms of the disease. However, effective therapy is not already available. AD pathology is characterized by neuronal loss leading to brain atrophy and a decrement of the cerebral metabolism. Major neuropathologic lesions are: (i) synapse and neuron loss; (ii) extracellular amyloid deposits and amyloid plaques, principally composed of amyloid beta (Aβ) peptide; (iii) intraneuronal accumulation of hyperphosphorylated Tau proteins leading to neurofibrillary degeneration; (iv) reactive astrogliosis; (v) brain inflammation. Current views of AD pathogenetic mechanisms describe amyloid deposition and neuritic plaque formation as a central mechanisms leading to neuro-degeneration, cognitive impairment and sporadic AD [1]. Therefore, therapeutic approaches have focused on reducing amyloid load and plaque deposition or clearance of brain amyloid. Other mechanisms may be closely related with the etiology and pathogenesis of sporadic AD.

Presentation of the hypothesis

Here we discuss recently published genetic data from a genome wide association (GWA) study including several thousand AD European patients and controls (CTR) [2] and showing that a limited number of genes were highly associated ($p > 10^{-5}$) with the disease even after the inclusion of additional data from control population (stage 3 of GWA replication and statistical evaluation by principal component adjustment [2]). The view presented here supports the notion of an infective etiology for sporadic AD. The first set of genes was located in close vicinity of the APOE locus on the chromosome 19 (see Table 1) and consisted of the poliovirus receptor-related 2 or nectin-2 (NC-2), apolipoprotein E (APOE), the translocase of outer mitochondrial membrane 40 homolog (TOMM-40), the glycoprotein carcinoembryonic antigen related cell adhesion molecule-l6 (CEACAM-l 6) and B-cell/lymphoma-3 (Bcl-3) genes. Genes in the second set were located on different chromosomes: APOJ or clusterin on chromosome 8; the complement receptor

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Porcellini et al. Immunity & Ageing 2010, 7:16
http://www.immunityageing.com/content/7/1/16

Table 1 Genes cluster surrounding the APOE gene on human chromosome 19 Region: 45,120K-45,710 K bp

Start	Stop	Symbol	Cyto	Description
45116956	45138792	LOC147710	19	hypothetical LOC147710
45147098	45169429	PVR	19q13.2	poliovirus receptor
45174724	45187627	CEACAM19	19	carcinoembryonic antigen-related cell adhesion molecule 19
45202358	45213986	CEACAM16	19	carcinoembryonic antigen-related cell adhesion molecule 16
45251978	45263301	BCL3	19q13.1-q13.2	B-cell CLL/lymphoma 3
45281126	45303903	CBLC	19q13.2	Cas-Br-M (murine) ecotropic retroviral transforming sequence c
45312338	45324678	BCAM	19q13.2	basal cell adhesion molecule (Lutheran blood group)
45349393	45392485	PVRL2	19q13.2	poliovirus receptor-related 2 (herpesvirus entry mediator B)
45394477	45406946	TOMM40	19q13	translocase of outer mitochondrial membrane 40 homolog (yeast)
45409039	45412650	APOE	19q13.2	apolipoprotein E
45411802	45431701	LOC100129500	19	hypothetical LOC100129500
45417921	45422606	APOC1	19q13.2	apolipoprotein C-I
45430017	45434450	APOC1P1	19q13.2	apolipoprotein C-I pseudogene 1
45445495	45448751	APOC4	19q13.2	apolipoprotein C-IV
45449243	45452818	APOC2	19q13.2	apolipoprotein C-II
45458638	45496599	CLPTM1	19q13.2-q13.3	cleft lip and palate associated transmembrane protein 1
45504712	45541452	RELB	19	v-rel reticuloendotheliosis viral oncogene homolog B
45542298	45574214	SFRS16	19q13.3	splicing factor, arginine/serine-rich 16
45574758	45579688	ZNF296	19	zinc finger protein 296
45582518	45594782	GEMIN7	19	gem (nuclear organelle) associated protein 7

1 (CR-1), and C-type lectin domain family 16 member A (CLEC-16A) on chromosome 16. Polymorphic variations in each of these genes were individually associated with AD (P values ranging from 10^{-16} to 10^{-5}). However we argue that the concomitant presence of several polymorphisms of these genes in the same individual might represent a genetic signature of AD. In this report we hypothesized that such a genetic trait may predispose to AD via complex and diverse mechanisms each contributing to an increase of individual susceptibility to brain viral infections. The evidence supporting this new notion are briefly listed below.

1) NC-2, also known as herpes virus entrance-B (HveB) or poliovirus receptor-relate protein-2 (PVRL-2 or Prr2), is a member of the immunoglobulin superfamily, is expressed in a variety of cell tissues, including neurons, belongs to the cadherin adhesion molecules [3] and mediates the entry of herpes simplex viruses (HSV) [4]. The glycoprotein D (gD) of HSV is the ligand for NC-2 and one of several HSV binding proteins that are essential for fusion to the human target cell and viral entering [4]. Gene variants in the human NC-2 gene might affect individual susceptibility to HSV infection of the brain by influencing virus cell entry and cell-to cell virus spreading.

2) APOE 4 allele is a well established genetic risk factor for AD and it has been also confirmed in the European GWA study [2]. ApoE protein may affect Aβ deposition. However, APOE 4 allele has been also shown to influence: susceptibility to viral infections [5], human immune deficiency virus (HIV) cell entry *in vitro*, HIV disease clinical progression [6], recurrent genital herpes in patients co-infected by HSV-2 and HIV [7] and progression of experimental ocular lesions induced by HSV-1 [8]. Therefore, APOE 4 allele association with AD might also influence the susceptibility to virus entry and spreading into neuronal cells. On the other hand, APOE 4 allele seems to be protective in the case of liver damage caused by HCV [9].

3) TOMM-40 gene codes for a mitochondrial translocase. It is interesting to note that HSV DNAase such as the UL12.5 enzyme destroys the mitochondrial genome [10] by inducing rapid and complete degradation of mitochondrial DNA [11]. Gene variations at TOMM-40 gene might influence DNA digestion and mitochondrial damages induced by HSV DNAase and other less defined virus dependent mechanisms.

4) CEACAM-16 belongs to a family of gene coding for adhesion molecules related to cancer replication, such as the carcinoembryonic antigen (CEA), and has been recently shown to regulate apoptosis in early tumor development by affecting caspase-1/3 activation [12].

5) Bcl-3 is an oncogene and is also involved in cell replication and apoptosis. Apoptosis may act as a primitive immune response and is a potent host defense mechanism. It is known that HSV is able to both induce

Porcellini et al. Immunity & Ageing 2010, 7:16
http://www.immunityageing.com/content/7/1/16

and suppress apoptosis in infected cells. In particular HSV-1 was shown to inhibit initially induced apoptosis in neuronal cells via a caspase-3 dependent pathway [13]. Moreover, Bcl-2 protein was able to block HSV-1 induced apoptosis in human hepatocytes [14]. Therefore, gene polymorphism in both CECAM-16 and Bcl-3 genes might influence individual susceptibility to apoptosis regulation induced by HSV and favor virus spreading in the central nervous system (CNS).

6) The APOJ, also known as clusterin, is a modulator of complement activation. Complement biosynthesis and activation occurs in neurodegenerative diseases such as AD [15] and the cytolytic activity of complement components is important for virus neutralization. ApoJ is synthesized in the CNS and is present in amyloid plaques [16]. Polymorphism in APOJ gene might influence virus lytic defences by regulation of complement activation.

7) CR1 is a complement receptor which bind different complement components (C3b, C3 d, C2a). Herpes virus family (especially alpha herpes) expresses a member of gC protein family that is able to bind heparan sulphate and the C3b component of the complement system [4]. Genetic variation in CR1 and CR2 receptors might affect individual capacity of virus clearance via C3 activation and C3b binding to the HSV. APOJ and CR1 genes might be illustrated as a synergistic gene cluster and influence brain virus defences such as complement activation, virus lysis and clearance.

8) CLEC-16A gene codes for a C-type lectin domain receptor. Lectin-like receptor, such as mannose receptor, recognizes and binds sugar moieties on pathogen glycoproteins. No data are on record regarding CLEC-16A and HSV or other viruses. However, gene polymorphism in the CLEC-16A gene might influence individual ability to recognize and bind virus glycoproteins.

Implications the hypothesis

The genetic signature here discussed is suggestive of individual susceptibility to pathogen infection of the brain, particularly HSV and related viruses. Recently, an independent investigation in late-onset sporadic AD from Japan also showed that gene variations near the APOE locus (PVRL-2, APOE 4 allele and APOC1) on chromosome 19, were associated with increased risk for the disease [17]. These independent findings appear to reinforce the new notion that individual brain susceptibility to virus infection and/or reactivation may be one complex genetic trait influencing the risk of neurodegeneration leading to clinical AD in old age. Moreover, evidence from other investigators showing HSV infection in AD brains are on record [18-20]. It is of interest that the concomitant presence of the APOE 4 allele and vertical transmission of HSV-1 has been shown to confer a differential risk of brain infection and AD [21]. Moreover, APOE 4 deficient mice had significantly lower virus load in CNS than APOE 4 transgenic mice [22]. In addition, in transgenic mouse model, APOE4 was shown to be a risk factor for ocular herpes favoring increased HSV-1 intra ocular replication [23].

Reactivation of HSV-1 in the brain was also found in patients with familial AD who showed increased viral DNA and protein expression in cortical neurons [24]. HSV-1 has been also related to Down's syndrome, a condition at high risk for AD type dementia [25]. It is of interest that mothers of children with Down's syndrome showed increased serum HSV-2 antibody levels [26]. Viruses of the HSV family are among the most probable pathogen candidates for brain reactivation in old age, since their possess a well known ability to escape peripheral immune responses by invading neurons. It is of interest that during aging a substantial proportion of peripheral CD8 T cytotoxic cells have been found to be directed against Epstein-Barr virus (EBV) and cytomegalovirus (CMV), which belong to the HSV family. Moreover, it has been suggested that aged immune system is no longer able to control EBV or CMV reactivation [27] and virus infection might become chronic in a large proportion of the elderly. Therefore, we speculate that with advancing age an impaired immune system might facilitate virus reactivation in the brain, especially in those subjects showing the above suggested genetic signature. Latent or chronic viral infection by CMV has been indeed found to correlate with the rate of cognitive decline in the Sacramento Area Latino Study on Aging [28]. Another study, focused on elderly with cardiovascular disease, showed that HSV and CMV burden was associated with cognitive impairment [29].

Therefore, brain infection by reactivated latent viruses might be one of the *primus movens* inducing progressive neuronal loss, astro-glia activation, and, by impairing APP transport along the axons [19], APP dis-appropriate metabolism and amyloid deposition.

This hypothesis is partially supported by data from HIV positive patients under protease inhibitor treatment and without encephalitis, where Aβ amyloid brain deposition was a common neuropathological feature [30]. Moreover it has been showed that APP, a putative receptor for the microtubule motor named kinesin, is a major component of viral HSV-1 particles, as abundant as any viral encoded protein [31].

These findings indeed showed that a brain virus infection could induce amyloid deposition. Another GWA in AD from Europe and USA recently confirmed the association of TOMM-40, PVRL-2, APOJ and APOE with AD. This investigation also signaled a significant association of the phosphatidilinositol-binding clathrin assembly protein gene (PICALM) with AD [32]. It is of

interest that clathrin (CLA) mediated endocytosis is involved in internalization and transportation of viruses into the infected cell and to the nucleus. For instance, human rhinovirus is internalized by a CLA dependent mechanism [33] and adenovirus transport into motor-neuron axons is mediated via CLA endocytosis [34]. Insect parvovirus particles were also shown to be rapidly internalized into CLA-coated vesicles and slowly moved within early and late endocytic compartments to the nucleus [35]. Moreover, varicella herpes zoster virus was shown to interfere with intracellular trafficking by interacting with CLA-coated vesicles for subsequent transportation to endosomes [36]. Data from this independent GWA in AD patients also seem to support the presence of a genetic signature suggestive of a viral risk factor in AD. Finally recent data, reporting that Aβ peptide showed an anti-microbial activity [37] and acted as a defense molecule of the innate immunity, is compatible with the hypothesis of viral association with AD etiology and pathogenesis. The accumulation of Aβ and plaque deposit may derive by an over-production of Aβ peptides directed against a viral invader of the brain. Moreover, some evidence is on record showing that HSV1 can directly contribute to the processing of Aβ and to the development of senile plaques and a $Ca^{(2+)}$ dependent APP phosphorylation and Aβ 42 accumulation in rat cortical neurons [38,39].

Two recent meta-analysis from GWA [40,41] confirmed APOE, CLU, PICALM and CR-1 as susceptibility genes for AD risk. Therefore, this genetic trait in association with the other above discussed genes might represent a gene cluster affecting AD risk by influencing virus infection susceptibility. Our hypothesis describes a set of gene upstream of the APOE locus on chromosome 19 spanning from CEACAM-19 to APOE (as shown in Table 1) that may constitute a gene cluster of susceptibility for AD by affecting different mechanism involved in virus entrance or resistance to virus infection. CLU/APOJ, CR-1 and CLEC-16 genes located on different chromosome complement the AD susceptibility gene cluster also by affecting virus entry and cellular defense mechanism. It is interesting to note that SNPs upstream of APOE locus spanning from TOMM-40 to APOE promoter may also play a role in AD risk by affecting APOE expression in AD brain [42]. Moreover a genetic association study also confirmed that PVRL-2 (Nec-2), TOMM-40, APOE and APOC1 predispose to AD and showed that this region is firmly sandwiched between two recombination hotspots [17]. Therefore, the APOE ε4 might represent a genetic beacon of this set of genes located in its proximity on chromosome 19. Our hypothesis confirm and extend to other genes, a recent suggestion indicating that APP, APOE, CR-1, CLU and PICALM genes may be involved in HSV life cycle [43]. In conclusion, present findings suggest that during ageing virus reactivation may be more frequent in the elderly showing a genetic signature predisposing to an increased susceptibility for HSV and other virus infections of the brain. In these subjects the microorganisms are more likely to induce a limited, segmental and chronic sub-clinical pseudo-encephalitis resulting in progressive neurodegeneration. Further investigations will validate or refute this innovative approach to dementia in old age and clarify whether the presence of HSV and/or other infectious agents in the CNS represents a causative factor or a secondary infection in AD.

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Authors' contributions
EP contributed to generate part of genetic data and searched in gene bank to find biological function of candidate genes. IC searched and defined by a detailed perusal in gene bank the biological function of each gene regarding virus pathway. MI contributed to search Medline for virus association in AD. FL designed the hypothesis, supervised gene bank and medline data mining and wrote most of the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Chapter IX

Sharing Pathogenetic Mechanisms between Acute Myocardial infarction and Alzheimer's Disease as Shown by Partially Overlapping of Gene Variant Profiles

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Sharing Pathogenetic Mechanisms between Acute Myocardial Infarction and Alzheimer's Disease as Shown by Partially Overlapping of Gene Variant Profiles

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Abstract. Gene variants that promote inflammation and cholesterol metabolism have been associated with acute myocardial infarction (AMI) and Alzheimer's disease (AD). We investigated a panel of relevant polymorphisms to distinguish genetic backgrounds for AMI and AD: *IL10 –1082G/A*, *IL6 –174G/C*, *TNF –308G/A*, *IFNG +874T/A*, *SERPINA3 –51G/T*, *HMGCR –911C/A*, *APOE ε2/3/4* (280 AMI cases, 257 AD cases, and 1307 population controls, all Italian (presumed risk alleles are shown in bold). Six genetic risk sets I to VI were identified by fuzzy latent classification: I had low risk; II and III had low risk before age 65 (II, III); low risk sets lacked pro-inflammatory alleles for *HMGCR-TNF-APOE*. Pro-inflammatory alleles for *SERPINA3–IL10–IFNG* were found for high risk sets IV to VI. Set IV 'AMI < age 40, AD < age 65' included risk alleles for *HMGCR*. Set V 'AMI over a broad range of age' included risk alleles for *TNF + IL6*. Set VI 'AMI at ages 40 to 55, AD ages 65+' included *APOE ε4*. Close resemblance to the high risk sets, as indicated by membership scores close to one, defined wide relative risks. We conclude that AMI and AD share genetic backgrounds involving cholesterol metabolism and the upregulation of inflammation and that gene-gene interactions in relevant sets of genes may be useful in defining inherited risk for common disorders.

Keywords: Acute myocardial infarction, Alzheimer's disease, *APOE*, cholesterol, gene polymorphism, genetic epidemiology, grade-of-membership analysis, inflammation

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly [1]. Pathologically, it is characterized by a loss of neuronal synapses, extracellular deposits of amyloid protein, termed neuritic plaques, and intracellular formation of degenerate neurofilaments forming neurofibrillary tangles [2]. Inflammatory processes can be observed in the AD brain [3–5]. Cytokines and other inflammatory molecules are secondary plaque components [6–8]. Increased levels of circulating acute phase reactants in middle age portend AD in old age [9]. One acute phase protein, ACT (*SERPINA3*), is specifically increased in the blood of AD and correlated with cognitive impairment or decline in these patients [10–12]. Gene variants that upregulate inflammation or alter cholesterol transport, are often found to be at elevated frequency among AD cases [13–18].

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Cardiovascular disorders are the leading causes of morbidity and mortality in modern western societies [19–21]. Hypercholesterolemia is considered to be the most relevant risk factor among several others for atherosclerosis and atherogenesis. However, more than half of patients with atherosclerotic complications, such as acute myocardial infarction (AMI), do not demonstrate classical risk factors. In fact, these AMI cases have 'normal' cholesterol levels, no hypertension, a negative history of smoking, no diabetes or obesity, and lack a sedentary life style [19].

Abnormal lipid metabolism and inflammatory processes are now both considered to be pathogenetic mechanisms leading to atherogenesis and the clinical manifestations of atherosclerosis complications. For instance, modest elevation of blood C reactive protein (CRP) levels, a marker of inflammation, is associated with an increased risk of cardiovascular events [22]. An elevation of blood level of cytokines and interleukins in patients with cardiovascular disease has also been reported [23, 24]. Promoter polymorphisms with functional relevance in the expression of the cognate inflammatory gene are often found at elevated frequency among patients with AMI [24–37].

Despite these parallels, there is little information directly linking AMI and AD. Most studies do not clearly place vascular damage prior to AD onset, which would support the belief that cardiovascular disease (CVD) and atherosclerosis contribute to AD pathogenesis [38], as opposed to the view that atherosclerosis and AD represent independent converging disease processes possibly sharing common determinants, e.g., *APOE ε4* [39]. For example, cerebral infarcts and large vessel cerebrovascular disease have been associated with plaque deposition [40]. Coronary artery disease was found associated with increased AD neuropathology [41]. Furthermore, atherosclerosis of the circle of Willis [42], which would alter blood flow to the brain, has been correlated with the density of neuritic plaques and neurofibrillary tangles. Pathologically verified AD has also been associated with atherosclerosis of the circle of Willis [43]: AD patients examined using transcranial Doppler ultrasonography systematically demonstrated increased pulse indices which could represent increased rigidity of arterial walls induced by atherosclerotic changes [44].

Longitudinal studies improve this temporal distinction, but do not rule out the possibility that vascular disease and AD develop at differing rates while sharing common determinants. Peripheral arterial disease and non-invasive markers of CVD in late middle age predict an increased risk of AD in late age [45]. CVD and vascular risk factors increased clinical conversion of patients with mild cognitive impairment to AD [46]. These studies suggest that atherosclerotic lesions of brain circulation may induce a chronic brain hypoperfusion leading to neuronal energy defects that later might result in plaques and tangles [47]. It is important to keep in mind that CVD risk factors, such as hypertension, high LDL, increased total cholesterol, low HDL, and diabetes have also been found associated with an increased risk of AD [38].

Selective survival may play a role in determining apparent patterns of causation: Using detailed autopsy information, extensive atherosclerosis at age 75 and death by AMI was frequently associated with a recent diagnosis of possible AD, while little atherosclerosis was found for persons about age 85 who lived to the end-stages of AD. Notably, both sets over-represented *APOE ε4* [48].

Perhaps the best evidence that AMI and AD are linked comes from epidemiological studies showing that control of inflammation via non-steroidal anti-inflammatory drugs or statins decreased the incidence of both AMI and AD [49–54]. Intriguingly, an immune regulatory effect of statins has indeed recently been proposed [55].

No attempt has been made to evaluate whether specific immune genetic risk factors might constitute an important etiologic and pathogenetic link between AD and AMI. The study presented here integrates information on a panel of gene variants that modulate inflammation and cholesterol synthesis (*IL10 –1082G/**A***, *IL6 –174G/**C***, *TNF –308G/**A***, *IFNG +874T/**A***, *SERPINA3 –51G/**T***, *HMGCR –911C/**A***, *APOE ε2/3/**4***) investigated among AMI and AD patients and unaffected persons in order to directly look for over-lapping and/or distinct genetic profiles.

Fuzzy latent classification identified six genetic risk sets I to VI [56]. They represented low intrinsic risk (I), low risk in middle age (II, III), and high intrinsic risk (IV, V, VI). Sets IV and VI described the etiologic overlap of AMI and AD. Set V was most typical of AMI, and distinct from AD. The membership of individuals in these sets varied widely defining a range of genetic predilection related to the investigated gene variants.

MATERIALS AND METHODS

Study subjects

The sample consisted of 280 patients with AMI [31], 257 patients with clinical diagnosis of probable AD

Table 1
The investigated gene polymorphisms

Official symbol*	Locus name	Substitution	Expected Outcome
IL6	Interleukin 6 (interferon, beta 2)	–174 *G→ C*	↑ IL-6
TNF	Tumor necrosis factor (TNF superfamily, member 2), 'tumor necrosis factor alpha'	–308 *G→ A*	↑ TNFα
IL10	Interleukin 10, 'cytokine synthesis inhibitory factor'	–1082 G→ *A*	↓ IL-10
SERPINA3	Serpin peptidase inhibitor, clade A, member 3, alpha(1)-antichymotrypsin (ACT)	–51 *G→ T*	↑ ACT
IFNG	Interferon, gamma	+874 *T→ A*	↑ IFNγ
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	–911 *C→ A*	↑ cholesterol
APOE	Apolipoprotein E; ε2, ε3, ε4 isoforms		Altered cholesterol transport

*HUGO Gene Nomenclature Committee; presumptive risk alleles are displayed in bold.

[31], and 1307 unaffected persons, i.e., 'controls' from a longitudinal population study: "The Conselice Study on Brain aging" [12]. Diagnosis of AD was performed according to the DSM-IV and NINCDS-ADRCDA criteria [57]. To allow for different genetic backgrounds for early and late cases, each patient was assigned to the relevant age group (<40 years, 40–54, 55–64, 65–74, 75–84, and 85+). To allow for changes in genetic composition of the control subjects with increasing age due to selective mortality and the occurrence of AMI or AD, infrequent before age 55, each control was assigned to one of five age groups: <55 years, 55–64, 65–74, 75–84, and 85+. The research protocol was approved by the relevant institutional review boards and all participants or their caregivers gave written informed consent.

Genetic determinations

Genotyping methods by PCR and gel electrophoresis were previously described [11, 15, 16]: *APOE* ε*2/3/4*, 40%; *IL10 –1082G/A*, 38%; *TNF* –308 *G/A*, 49%; *IL6* –174 *G/C*, 49%; *IFNG* + 874 *T/A* 43%; *SERPINA3* –51 *G/T*, 39%; *HMGCR* –911 *C/A*, 54% (Table 1). Presumptive pro-inflammatory alleles or genotypes are shown in bold; the percentage incomplete information is listed. The data analytic approach does not automatically exclude subjects having missing items of information: Missing items are ignored unless the observation is substantially incomplete not contributing to the model likelihood. In each instance, the presumptive risk allele is a minor allele. In order to simplify presentation, the alleles found at each locus are coded as either *a*- or as *b* -yielding genotypes *a*/*a*, *a*/*b*, and *b*/*b* – where "*a*" is the more common and less inflammatory allele (Fig. 1).

The data analytic approach

The goal was to identify extreme pure type risk sets representing high intrinsic risk for AMI and AD and

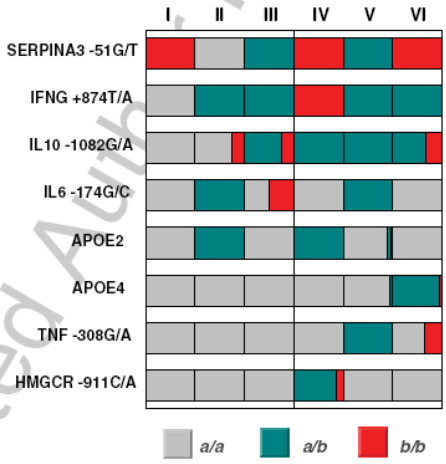

Fig. 1. Inflammatory profiles for risk sets I to VI. Probabilities for each genotype *a/a*, *a/b*, and *b/b* are displayed for each model-based pure type risk set I to VI. Allele *a* denotes the more common and less pro-inflammatory while *b* denotes the less common and more pro-inflammatory allele.

others representing low intrinsic risk for these disorders employing grade-of-membership analysis (GoM) [56, 58, 59]. Membership scores are automatically generated for each subject denoting degree of resemblance in each model-based group.

The GoM model likelihood can be described after first identifying four indices. One is the number of subjects I ($i = 1, 2, ..., I$). Here, $I = 1844$ subjects were identified. Of these, 1577 had sufficient information to be included in the model likelihood. The second index is the number of variables J ($j = 1, 2, ..., J$). There are $J = 8$ variables. Our third index is Lj: The set of response levels for the Jth variable. This leads to the definition of the basic GoM model where the probability that the ith subject has the Ljth level of the Jth

variable is defined by a binary variable (i.e., $y_{ijl} = 0, 1$). The model with these definitions is

$$\text{Prob}(y_{ijl} = 1.0) = \sum_k g_{ik}\lambda_{kjl} \quad (1)$$

where the g_{ik} are convexly constrained scores (i.e., $0.0 \le g_{ik} \le 1.0; \sum_k g_{ik} = 1.0$) for subjects and the λ_{kjl} are probabilities that, for the Kth latent group, the Ljth level is found for the Jth variable. The procedure thus uses this expression to identify K profiles representing the pattern of $J \times Lj$ responses found for I subjects.

The parameters g_{ik} and λ_{kjl} are estimated simultaneously using the likelihood function (in its most basic form).

$$L = \prod_i \prod_j \prod_l \left(\sum_k g_{ik} . \lambda_{kjl} \right)^{y_{ijl}} \quad (2)$$

In the likelihood y_{ijl} is 1.0 if the Ljth level is present and 0.0 if it is not present.

The clinical and genetic variables used to define the risk sets are termed "internal" variables. Information regarding gender was not used to define the risk sets because the control subjects did not necessarily reflect the gender composition of unaffected subjects due to the method of sampling that included medical students and hospital staff. Information on gender was, however, used to further characterize each group as an "external" variable. One option in the likelihood is to separate calculations for "internal" (here, clinical and genetic) and "external" (here, gender) variables. For internal variables, maximum likelihood estimations [MLE] of g_{ik} and λ_{kjl} are generated and the information in internal variables is used to define the K groups. For external variables the likelihood is evaluated (and MLE of λ_{kj}; generated) but the information is not used to redefine the K groups, that is, the likelihood calculations for likelihood equations involving the g_{ik} are disabled for external variables so that the g_{ik}, and the definition of the K groups, is not changed.

The membership of individuals in the sets was then categorized as low, limited, strong, and close resemblance, i.e., < 0.25, 0.25–0.49, 0.50–0.74, 0.75–1.00. Categorized scores for high risk sets were input into logistic models to quantify the relative risks of AMI (< age 40, 40–54, 55+) and AD (< age 65, 65–84, 85+). The 371 unaffected subjects aged 65 and older were the comparison subjects.

RESULTS

Overview

Six genetic risk sets I to VI were identified by fuzzy latent classification (Table 2). Each set had probabilities of being affected and probabilities of occurrence for each genotype at the multiple loci. Low risk for AMI and AD was represented by set I (low risk). Sets II and III were at low risk before age 65. These sets lacked pro-inflammatory alleles for *HMGCR*, *TNF*, and *APOE*. The high risk sets IV to VI included pro-inflammatory alleles for *IL10* + *IFNG* + *SERPINA3*. Disease outcome and onset age were influenced by the co-occurrence of *HMGCR* (IV, AD or AMI), *TNF* + *IL6* (V, AMI), or *APOE* (VI, AD or AMI). Close resemblance to one of the high risk sets, or the high risk sets taken together, denoted very high risk for AMI and/or AD.

'Low risk' (I)

Set I might be considered ideal as it represented unaffected elderly subjects (Table 2). Presumed risk alleles were not found except for *SERPINA3* (*b/b*) implying a positive role on health and, possibly, longevity in this context (Fig. 1). The putative low risk allele is labeled as '*a*' while the putative high risk allele is labeled '*b*' in Fig. 1.

'Low risk before age 65' (II, III)

Sets II and III were at low risk before age 65. These sets represented unaffected middle aged subjects. Pro-inflammatory alleles for *HMGCR, TNF* and *APOE* were not found. Lacking these risk alleles, low risk before age 65 was consistent with other risk alleles: Set II included risk alleles for *IL6* (*a/b*), *IL10* (*b/b* 24% probability) and *IFNG* (*a/b*), and the 'protective' *ε2/3* genotype of *APOE*. Set III included risk alleles for *IL6* (*b/b* 49% probability), *IL10* (*a/b* or *b/b*), *IFNG* (*a/b*) and *SERPINA3* (*a/b*).

'Early AMI and AD' (IV)

Sets IV, V, and VI represented high risk and included pro-inflammatory alleles for *SERPINA3-IL10-IFNG*. Disease outcome and onset age were influenced by the co-occurrence of other risk alleles. Set IV might be regarded as the worst case scenario representing early AMI (<age 40) and early AD (<age 65): The core risk set of *SERPINA3 b/b* + *IL10 a/b* + *IFNG b/b*

Table 2
Disease outcome and gender for risk sets I to VI

Variable			Sample %	I	II	III	IV	V	VI
Outcome	AMI	<40	1.3	0	0	0	46	0	0
$H = 1.35$		40–	2.5	0	0	0	0	0	28
		55–	4.2	0	0	0	0	31	0
		65–	2.3	0	0	0	0	37	0
		75–	2.0	0	0	0	0	27	0
		85+	0.4	0	0	0	0	5	0
	AD	<55	0.4	0	0	0	0	0	3
		55–	2.0	0	0	0	39	0	0
		65–	5.1	0	0	0	0	0	31
		75–	5.7	0	0	0	0	0	39
		85+	1.0	0	0	0	16	0	0
	Unaff.	<65	51.5	0	100	100	0	0	0
		65–	16.4	80	0	0	0	0	0
		75–	3.6	18	0	0	0	0	0
		85+	0.4	2	0	0	0	0	0
Gender	Male		53.7	43	53	55	47	72	42
$H = 0.02$	Female		46.3	57	47	45	53	28	58

Sample frequencies and probabilities for each risk set are displayed. Each set was distinct in terms of disease outcome (summarized by information statistic $H = 1.35$). They were similar in terms of gender ($H = 0.02$).

was supplemented by *HMGCR a/b* or *b/b*, associated with up-regulation of cholesterol synthesis. This set also included the ε2/3 genotype for *APOE*, suggesting that in this context ε2 contributes to risk.

'AMI ages 55 and older' (V)

Set V represents AMI at ages 55 and older (Table 2). The core risk set *SERPINA3 a/b + IL10 b/b + IFNG a/b* was supplemented by permissive expression of *TNF a/b* and *IL6 a/b*. This multi-gene profile appeared to be the risk signature for AMI as opposed to AD (Fig. 1).

'AMI ages 40–54; late onset AD' (VI)

Set VI was consistent with AMI at ages 40 to 54 and late onset AD occurring at age 65 or older. The core risk set *SERPINA3 b/b + IL10 a/b* or *b/b + IFNG a/b* was supplemented by one or two copies of *APOE* ε4 allele, and, possibly *TNF b/b* (36% probability).

Informative gene variants

These six genetic risk sets represent important aspects of risks for AMI and AD. Clearly, no single gene variant was an exclusively relevant risk factor. In order to convey a sense of the importance of each locus, information statistic '*H*' (Shannon, Bell Laboratories) was estimated for each locus. None of the loci had values of *H* close to zero, which would have denoted an uninformative variable having similar genotypic frequencies for each of the six risk sets. Each locus determining the core risk set had high *H* scores: *SERPINA3* ($H = 0.92$) + *IL10* ($H = 1.14$) + *IFNG* ($H = 0.67$). Each locus determining the core protective set had lower *H* score: *HMGCR* ($H = 0.34$) + *TNF* ($H = 0.53$) + *APOE* ($H = 0.68$). Each outcome modifying locus was informative: *HMGCR* ($H = 0.34$) for set IV; *TNF* ($H = 0.53$) + *IL6* ($H = 0.71$) for set V; and, *APOE* ($H = 0.68$) for set VI.

Classification of subjects

Risk sets I to VI are idealized representations akin to stereotypes that define important facets of the data. Subjects may match one risk set, or they may partly resemble two or more risk sets depending on genetic makeup. Membership scores from 0 (no resemblance) to 1 (an exact match) are generated for each subject during the estimation of the risk sets. To describe the resemblance of subjects to the risk sets, membership scores were categorized for each risk set (0–0.24, 0.25–0.49, 0.50–0.74, 0.75–1) (Table 3). For the control subjects, scores for the low risk sets I to III were summed and categorized. For the case subjects scores for the high risk sets IV to VI were summed and categorized.

As might be expected, the majority (81%) of the control subjects resembled the low risk sets taken together (combined membership from 0.50 to 1). Only 1% had very limited resemblance to the low risk sets (membership <0.25), and, instead, were genetically very much like the high risk sets. However, a total of 19% of the control subjects resembled the high risk sets taken

Table 3
Membership of the subjects in risk sets I to VI

Controls ($n = 1307$)	0–0.24	0.25–0.49	0.50–0.74	0.75–1
I	792	225	153	137
II	899	139	94	175
III	674	259	175	199
IV	1132	138	35	2
V	931	287	88	1
VI	1247	59	1	0
Low Risk I + II + III	14	232	327	734
AMI $n = 280$	0–0.24	0.25–0.49	0.50–0.74	0.75–1
I	226	41	7	6
II	226	42	9	3
III	195	65	19	1
IV	219	53	8	0
V	90	93	61	36
VI	212	58	8	2
High Risk IV + V + VI	32	56	73	119
AD $n = 257$	0–0.24	0.25–0.49	0.50–0.74	0.75–1
I	214	25	6	12
II	212	34	9	2
III	213	28	14	2
IV	189	51	17	0
V	151	77	25	4
VI	95	97	48	17
High Risk IV + V + VI	18	42	68	129

together (membership from 0.50 to 1 in sets IV to VI). These subjects might be presumed to be at elevated risk.

Turning to the case subjects, the majority of AMI cases (68%) resembled the high risk sets taken together. A third exclusively resembled the AMI signature risk set V. Only 11% closely resembled the low risk sets. The majority of AD cases (73%) resembled the high risk sets taken together. A quarter resembled the late onset AD set IV. Only 7% closely resembled the low risk sets. Thus the measured risk factors were relevant to most cases.

Relative risks for individuals

Logistic models were then constructed to indicate the relative risk associated with each increment of 0.25 in membership in sets II to VI. For AMI (Fig. 2), relative risk was estimated for three age groups (<40, 40–54, 55+) in separate models. Each increment in set V multiplied the risk of AMI from 3- to 8-fold depending on age: <age 40: OR = 7.8 (95% CI 3.7–16.5); ages 40 to 54: OR = 3.1 (95% CI 1.8–5.1); ages 55+: OR = 8.1 (95% CI 5.5–12.2). Squaring, strong resemblance multiplied risk 10- to 66-fold and close resemblance further multiplied risk at least 30-fold. Even limited resemblance to set IV carried a 40-fold elevated risk of AMI before age 40 (OR = 41, 95% CI 15–109). Limited resemblance to set VI carried a 19-fold elevated risk of AMI at ages 40 to 54 (OR = 18.8, 95% CI 10–36).

Turning to AD, limited resemblance to sets IV and VI multiplied risk for early onset AD before age 65 (set IV: OR = 328, 95% CI 56–1000; set VI: OR = 120, 95% CI 23–637). Odds ratios for sets II, III, and V ranged from 10 to 15. At ages 65 to 84, risk most specifically concerned set VI. Limited resemblance multiplied AD risk 23-fold (OR = 23, 95% CI 14–38). Odds ratios for sets II, III, and V ranged from 2 to 4. An individual's risk can be approximated by multiplication of relevant odds ratios together defining a broad range of risk. Risk at ages 85 and older ($n = 18$ AD), also involved set IV (OR = 31, 95% CI 10–96). Limited membership in other sets multiplied risk from 2-fold to 4-fold. Again, relative risk for an individual is the product of the relevant odds ratios.

DISCUSSION

This study closely defines parallels between risks for AMI and AD by investigating both outcomes in relation to a panel of carefully selected polymorphisms known to differentially modulate inflammation and cholesterol synthesis. We applied a relatively novel data analytic approach, namely, grade-of membership analysis, to integrate information identifying low and high intrinsic risk sets which defined strong gradients

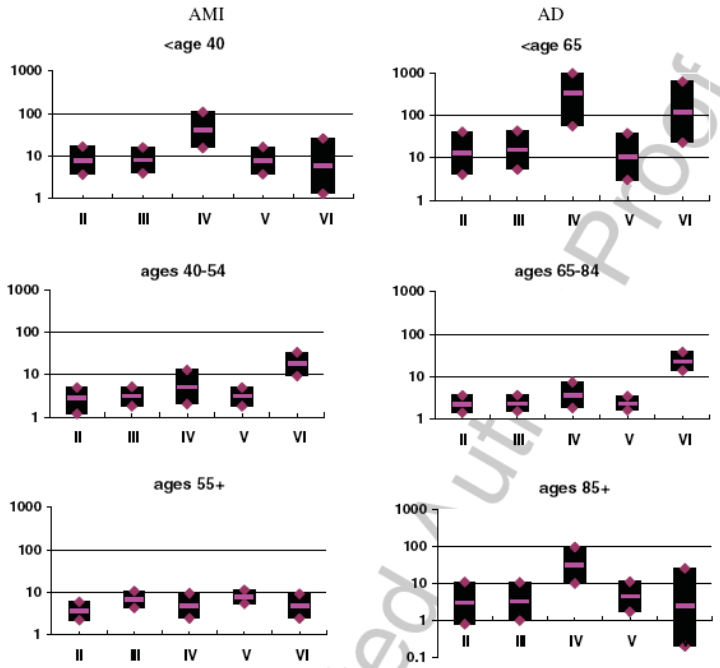

Fig. 2. Odds of disease (95% CI) in relation to membership in risk sets II to VI. Logistic models were constructed to estimate the relative risks of AMI and AD in relation to membership in sets II to V (five predictive ordinal variables coded as shown in Fig. 2). The displayed odds ratios pertain to limited membership. They need to be squared for strong membership and cubed for close membership. Risk for individuals is arrived at by multiplying all relevant odds. An individual's odds of disease is arrived at by multiplying all relevant odds ratios. Statistical significance is denoted by 95% CI that do not include the neutral referent value of one.

of risk for individuals. This approach has previously been used to define stages in melanoma [60], genetic heterogeneity in AD [61], patterns of disability for elderly Americans and trends over time [62], disease subtypes in schizophrenia [63, 64], pathologic stages and subtypes of AD brain pathology [65], sib-pair linkage methods with high statistical power [66], multilocus risk genotypes for AD [67–71], vascular damages in AD patients [72], estrogen-related gene variant profiles for breast cancer *versus* fibroadenoma [73], mitochondrial complex I gene variant profiles robust and vulnerable to pesticides resulting in Parkinson's disease [74], sets of polymorphisms in several genes that together predict AD, and confirmation that low frequency haplotypes for LRRK2 multiply the risk of Parkinson's disease [75].

The identified high risk sets IV, V, and VI carried a core set of pro-inflammatory alleles for *SERPINA3 + IL10 + IFNG*. Set IV included the *HMGCR* mutated allele that, impairing cholesterol metabolism, multiplied risk for AMI before age 40 as well as AD before age 65. Set VI additionally carried *APOE* ε4 allele and showed increased risk for AMI at ages 40 to 54, as well as AD from ages 65 to 84. *APOE* ε4, i.e., set VI, posed limited risk for AD at advanced ages 85 and older. On the other hand, permissive cholesterol synthesis, i.e., set IV, was more relevant from age 85 onward.

None of the high intrinsic risk groups expressed the *IL10 a/a* genotype thought to favor control of TNF-α production and delimit inflammatory responses. Each set at high risk for AMI favored expression of *IFNG* (better interaction with transcription factor NF-KB) and *SERPINA3* (an acute phase protein). These findings present clear parallels and a partial overlapping in the gene risk profile between AMI and AD.

However, etiologic overlap with respect to the investigated gene variants was incomplete: Set V was

represented by additional pro-inflammatory alleles for *TNF+IL6*. These allele or genotype combinations were specifically relevant to AMI across a broad range of age. The propensity to up-regulate TNF-α production, in parallel with a poor suppression by IL-10, was the primary background for AMI especially from age 55 onward, i.e., when influence on cholesterol synthesis (*HMGCR*) or transport (*APOE ε4*) was less evident. This genetic make-up might promote atherosclerosis and destabilization of plaque via abnormal synthesis regulation of IL-6 and other cytokines.

Conversely, the low risk sets I to III lacked pro-inflammatory alleles for *HMGCR*, *APOE*, and *TNF*. Set I, that we proposed as the 'long life and low risk' genetic makeup, represented the favored few who carried a very low load of pro-inflammatory alleles other than *SERPINA3* (an acute phase protein). In the absence of other investigated risk alleles, these non-mutated alleles appeared to facilitate survival to a ripe old age. Sets II and III can be considered to be incomplete risk sets consistent with good health with respect to AMI and AD until at least age 65.

Membership in these sets defined truly high and low risk persons and an intervening gradient of risk to be better characterized by other factors. Figure 2 uses a log scale to express these findings.

We were aware that this is a partial list of gene variants. Moreover, environmental factors might as well play a role in the diseases, since they influence gene expression level. For instance, other factors, e.g., low folate and vitamin B12 plasma levels of homocysteine, contribute to risk for dementia [76].

On the other hand, other candidate genes for AD have been recently described in a genome wide association study, and other inflammatory genes, such as clusterin, complement receptor 1, and 2 were highly associated to dementia [77].

Moreover, a fraction of both AMI and AD did not resemble the high risk sets, i.e., they carried few of the investigated pro-inflammatory alleles. Assuming that the identified high risk sets approximate sufficient risk sets, very few cases matched any one sufficient risk set. This situation implies a more complex causation and the present gene profiles are indicative but incomplete. Further studies will add other relevant gene variations in diverse candidate genes to complete and build up more selective risk profiles for these diseases. Set V had an extremely broad range of age at onset for AMI. These findings may suggest that AMI risk is modulated by other non-investigated factors. In fact, about 9% of the elderly controls who resembled set V were not yet affected by AMI [78].

On the whole, the model-based risk sets recapitulate and substantially extend findings derived from association studies of single gene variants modulating inflammation and have been discussed in previous work [37, 79]. This model identifies partially overlapping multi-gene risk profiles associated with AMI or AD. The overlapping describes an emerging picture showing that an abnormal regulation of inflammation is implicated in the pathogenesis of atherosclerosis and its complications [80, 81] and neurodegenerative processes leading to AD [82, 83]. On the other hand, the implication of vascular factors in AD is an emerging reality [84].

This profile may be used to identify among healthy individuals those with intrinsic high risk of developing with age these diseases.

Present findings imply the possibility of personalized medicine based on intrinsic risk factor profile. For instance, statins may be helpful for some persons and not others, e.g., those who carry the permissive allele for *HMGCR*, in avoiding AMI and AD. Potentially, interventions to modulate inflammation would limit the deleterious effects of cholesterol with respect to these outcomes without compromising immune responses.

In conclusion, data presented here represent an approach to define individual risk profiles that may be applied to healthy subjects of different ages to predict intrinsic risk of AMI or AD. These risk profiles might then be used to define further diagnostic procedures which might indicate specific early therapeutic interventions aimed at prevention or significantly delay of the clinical manifestations of these two diseases. Finally, these findings may contribute to the goals of predictive diagnostics and personalized medicine.

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General discussion

Elevated plasma levels of α-1-antichymotrypsin in age-related cognitive decline and Alzheimer's disease: a potential therapeutic target

Alzheimer's Disease (AD) is a chronic neurodegenerative disorder and the clinical disease develops when the neuronal loss is already advanced. The clinical diagnosis of AD is performed by excluding other forms of dementia and formal diagnosis is done only post mortem after the identification of senile plaques. Amyloid deposit consists in extracellular deposition of beta amyloid protein and neurofibrillary tangles consists of intracellular aggregates of tau. Microglia and astrocites surrounding brain areas with neurodegeneration showed activated phenotypes and expressed inflammatory molecules and several of these molecules are found in brains of patients affected by AD and associated with senile plaques.

Many studies support the hypothesis that the inflammation plays a pivotal role in AD since the routine use of non-steroid anti-inflammatory drugs was associated with a decreased incidence of AD. Alpha 1 antichymotrypsin (ACT) is a serum acute phase glycoprotein belonging to the Serin Protease Inhibitor family called SERPIN. ACT is mainly produced by the liver, but is also widely distributed in the central nervous system. In the brain, ACT is produced by astrocytes and it is a secondary component of senile plaques in the AD brain (Abraham CR, 2001). It has been proposed that ACT and

APOE co-localized with Abeta amyloid plaques. Moreover, in animal model, ACT and also APOE, affected amyloid deposition and cognitive performances. ACT plasma levels have been correlated by many authors with the cognitive decline and dementia therefore data emerging from the association of ACT levels and the developing of AD are conflicting: some studies reported high serum ACT levels in AD patients (Licastro F et al., 1995; Licastro F et al., 2000) while other showed normal ACT values (Furby A et al., 1991; Lanzrein AS et al., 1998)

In this work we proposed a new method to measure ACT plasma levels in a large cohort of cognitively healthy subjects, in subjects with cognitive impairment but not dementia (CIND) and two different AD populations (mild and severe AD).

Circulating levels of ACT were higher in patients with mild or severe AD than in CIND or in controls.

We found also an age dependent increase of ACT levels: the oldest elderly showed higher ACT levels than younger. In a previously paper (Licastro et al., 2005) we demonstrated that a polymorphism in the promoter region at position -51 was associated to AD and to the rate of cognitive deterioration.

Patients with mild AD were also followed up for 2 years and the cognitive performances were monitored. MMSE score were recorded and patients were stratified according to the rate of cognitive decline as described by Doody (Doody RS, 2001). ACT levels were higher in AD patients with fast cognitive deterioration than those in patients with an intermediate or a slow decline. A small group of CIND were also monitored for 4 years. ACT

levels were, once again, higher in CIND subjects that developed dementia then in those who did not developed AD. These association was also showed in APOE e4 carrier subjects.

Our data confirm that high ACT levels were associated with Alzheimer's disease and with the rate cognitive decline.

We reinforce also the hypothesis that the association APOE and ACT play a pivotal role in AD and in its the clinical progression.

Multivariable network associated with cognitive decline and dementia

AD is a multifactorial complex disease and several risk factors may differentially contribute to the clinical history of the disease, therefore powerful statistically algorithm are needed to evaluate interactions among variables and their association with the pathogenetic mechanisms involved in the disease.

We just discussed the importance of the epistasis in complex disease like AD where genetic and environmental factors interact to lead to neuropathological features typical of AD. Genetic studies try to explain the complexity of this disease but with poor results since the use of classical statistical methods does not allow to combine a large number of variables of different nature.

The use of Artificial Neuronal Network (ANN) might be very useful to understand how the interaction between genetic and phenotipic variables could be associated to Alzhimer's disease and cognitive decline.

ANNs could be used to find connection between variables that normally are hidden or normally difficult to find. This approach aims to create a semantic connectivity map in which non linear association were preserved, connections schemes are explicit and the complex dynamics of adaptive interactions is captured.

The final graphic results is a map with all the variable connected and with some aggregation points called hubs that represent the convergence point of group of variables.

For these reasons we applied this new statistical method to analyzed the database generate during the Conselice Study of Brain Aging.

This study is a population-based prospective investigation focused on an homogeneous elderly population from Northern Italy (Ravaglia G et al., 2001).

The principal aim of this investigation was to explore environmental, epidemiological, genetic and phenotypic risk factors for dementia in the elderly.

The study start in 1999 where about 1200 elderly 65 years old or older, living in Conselice a little county in northern Italy were enrolled. All participants were interviewed and underwent medical examination for cognitive evaluation. A blood sample and a computerized radiogram scan of the brain from each subject was taken. After 5 years subjects underwent to medical and cognitive re-evaluation years subjects underwent medical and cognitive re-evaluation and 937 elderly completed the follow-up. (Ravaglia, G et al. 2001.).

From this study a biological and clinical database during the 5-year follow-up has been generated and biological markers have been found individually associated with the AD risk, cognitive decline and incident AD (Ravaglia G et al., 2006; Ravaglia G et al., 2007a; Ravaglia G et al., 2007b).

Here we showed very interesting results from the application of this ANNs statistical methods regarding 35 different variables (both genetic, phenotypic and environmental) in the Conselice Study.

In this article data recording during the follow up allowed to build a map where three major biological hubs connected variables with the three different cognitive conditions: no cognitive decline, CIND and dementia were identified.

The three hubs have been identified in hydroxyl-methyl-gutaryl-CoA reductase enzyme (HMGCR), plasma cholesterol levels and age.

Related dependent variables converge to these hubs, that in turn are considered as relevant biological variables in the connectivity map.

Among variables, several gene variants of different inflammatory genes and the plasma levels of their cognate phenotypic factors showed a variable degree of relevance to brain aging and development of dementia.

Everyone knows that age is a risk factor for AD in fact it is important to keep in mind that AD is the main pathological disease associated with ageing. being less than 5% of AD cases affect people under the age of 65 years.

HMGCR is the rate limiting enzyme in cholesterol synthesis and controls cholesterol availability by affecting the synthesis of mevalonate and isoprenoid compounds which are necessary for the attachment of several proteins to biological membranes (Zhang FL and Casey PJ, 1996). HMGCR genotype has been recently described as a genetic risk factor for AD and affect the rate of cognitive decline in AD patients (Porcellini et al., 2007).

Our data from The Conselice Study confirmed that this enzyme was a relevant factor for the developing of dementia.

This enzyme is also very important since it is the molecular target for statins and for this HMGCR enzyme could be pharmacological target for AD prevention.

In fact, data regarding the possible preventive effect of statins in AD were on record (Jick H et al., 2000), although another investigation did not confirm these observations (Rea TD et al.,2005).

There was a strong linkage of the HMGCR genotype hub with the second hub, i.e. serum cholesterol. There are many evidences showing that cholesterol is associated to Alzheimer's disease. Moreover the most common risk factor for AD is the Apolipoprotein E, the main cholesterol transporter in the brain. In our map also a link of other lipidic variables with cholesterol and AD is shown.

In conclusion, the connectivity map presented here on incident dementia extended previous observations from case/control investigations and confirmed that some immune factors could indeed play a role in the pathogenesis of age associated dementia. Our findings also showed a new link between immunity, cholesterol metabolism and age in relation with cognitive deterioration.

Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease

Three decades of genetic research in Alzheimer disease (AD) have substantially broadened our understanding of the pathogenetic mechanisms leading to neurodegeneration and dementia.

Genetic studies have led to the consistent identification of the ε4 allele of *APOE* as a susceptibility locus for late-onset Alzheimer's disease. Twin studies suggest that genes may have a role in more than 60% of Alzheimer's disease susceptibility (Gatz M. et al., 2006.) and that *APOE* may account for as much as 50% of this genetic susceptibility (Ashford JW. and Mortimer JA, 2002).

More than 550 other genes have been proposed as candidates for Alzheimer's disease susceptibility, but thus far none have been consistently confirmed to have a role in Alzheimer's disease pathogenesis (Bertram L et al., 2007).

To identify other risk loci, we conducted first a large genome-wide association study of 2,032 individuals from France with Alzheimer's disease (cases) and 5,328 controls.

Then, the study was extended to AD and control samples from Belgium, Finland, Italy and Spain (AD cases 3,978 and 3,297 controls).

In this GWA about 500.000 SNPs have been analyzed by a new generation sequencing in AD and control samples.

As expected, APOE ε4 resulted associated to AD. Moreover other two loci showed a strong association with AD ($p=9x10^{-8}$). The first of these loci encompasses *CLU* on 8p21-p12, and the second spans the gene encoding complement component (3b/4b) receptor 1 (*CR1*) on 1q32.

CLU also called APOJ, like APOE is the most abundantly expressed apolipoproteins in the central nervous system (Roheim PS et al., 1979; May PC & Finch CE, 1992), with strong analogies in terms of possible impact on the AD physiopathological process. Like APOE, CLU is present in amyloid plaques (May PC. et al., 1990; Calero M. et al., 2000) and can bind Aβ (Ghiso J et al., 1993. ; Zlokovic BV. et al.. 1996)

In the CLU gene three SNPs (rs2279590, rs11136000, rs9331888) showed statistically significant association with Alzheimer's disease in both stages of the study. The marker that showed the highest association was CLU rs 11136000 with an OR for the minor allele of 0.86 (95% CI 0.81–0.90, $P = 7.5 \times 10{-}9$). We detected a statistical interaction between the *APOE* ε4 status and the *CLU* SNPs. For rs11136000, although the association was significant in both ε4 carriers and non-carriers, it was more significant in APOE ε4 carriers.

Then, a linkage disequilibrium test was applied to these three SNPs to investigate if certain CLU haplotypes could be correlated to AD. We found three common haplotypes (TTC, CCC and CCG) all associated with a statistically significant increased disease risk.

The other locus associated to AD emerging from this GWA study was CR1. CR1 is the main receptor that binds the complement protein C3b; plays an important role in the removal of immune complexes coated with C3b and C4b. It also regulates the complement cascade activation by preventing formation of classical and alternative pathway convertases.

Several observations suggest that pathways involving C3b and CR1 are involved in the Alzheimer's disease process, particularly in Aβ clearance. According to this, APP transgenic mice with an inhibition or deficiency of C3 display increased Aβ accumulation and neurodegeneration (Wyss-Coray T et al., 2002)

Like in CLU, also in CR we found SNPs that seems to be associated to AD: rs6656401 with an OR of 1.21 ($p<10^{-9}$) and rs3818361 with an OR of 1.19($p<10^{-8}$); this association was also confirmed in APOE 4 carriers.

Also in CR1 we identified three possible haplotypes correlated to the risk of Alzheimer's disease (GG, GA and AA).

The odds ratio was highest for the AA haplotype compared to the GG haplotype.

In addition to the previously known *APOE* locus, we have identified loci at *CLU* and *CR1* that are potentially associated with the risk of late-onset Alzheimer's disease. Biological evidence suggests that the genes at these loci, along with *APOE*, are involved in Aβ clearance. These data may indicate that whereas familial early-onset forms of Alzheimer's disease are mainly linked to genes implicated in Aβ overproduction, genetic variants at

APOE and these newly defined loci may influence susceptibility to late-onset forms of the disease as a result of roles in Aβ clearance.

Multi factorial interactions in the pathogenesis pathway of Alzheimer's disease: a new risk charts for prevention of dementia

Alzheimer's disease (AD) is the most common form of dementia. Severe memory loss, confusion, and impaired cognitive abilities characterize AD. Since a dramatic increase in mean life span and life expectant leading to a substantial increment of elderly population in West society, AD has also become a globally important health issue and the treatment of AD is a challenge for modern medicine.

Neuropathological hallmarks of AD are extracellular amyloid deposits (neuritic plaques) and intracellular deposition of degenerate filaments (neurofibrillary tangles) (Selkoe DJ, 2001).

The presence of these characteristic features are no sufficient to explain such a complexity of the disease. Other factors are then involved in the pathogenesis of AD like chronic inflammation and vascular damage. In fact, it has been demonstrated that all age related disease like Alzheimer have an important inflammatory component as an increased levels of circulating inflammatory mediators (Baggio G et al., 1998; Dumont Pet al., 2000)

Genetic studies had also underlined the importance of specific genetic background leading to a probable risk to develop AD (Licastro et al., 2007; Chiappelli M et al., 2006; Licastro F et al., 2010)

In this article we showed a second application of the Artificial Neuronal Network (ANN) in the Conselice Database. The first one, as just described before, regarding 35 variable dataset, three main hubs were represented by HMGCR enzyme, plasma cholesterol levels and age.

Here we extended the data set increasing the numbers of variables and adding prevalent AD, VD and CIND cases. In this new connectivity map we found four major biological hubs: 1) low blood cholesterol, 2) high BMI index, 3) low blood HDL, 4) low blood folate.

Different genotypic, phenotypic, clinical, pharmacological or habit variables converged to these hubs or cluster of connectivity. Age was closely correlated to prevalent AD cases confirming that age is the major factor in AD pathogenesis.

Variables as APOEε4 allele, increased Vit B12 and ACT levels, presence of mutated allele of several inflammatory genes were related to the main hubs underlining the implication of all these factors with the disease.

As expected, cognitive healthy status, in the map, is far from AD, VD and CIND condition.

Our findings showed four major connecting nodes from the Conselice data base; these hubs linked apparently different factors to cognitive impairment and dementia via cholesterol, cholesterol gene dependent pathway, BMI and age. A new association among different immunological factors and lipid metabolism with incident dementia has also emerged.

With this article, we confirmed that some immune factors could play a role in the pathogenesis of age-associated dementia by modifying metabolic and

lipid variables and also show a new link between immunity, cholesterol metabolism and age related cognitive deterioration.

Altered glycosylation profile of purified plasma ACT from Alzheimer's disease

As we just described before, the main pathological features of AD are the presence of extracellular senile plaques formed of β amyloid peptide and the presence of intracellular deposition of neurofibrillary tangles.

ACT, an inflammatory glycoprotein may be involved in the pathogenesis of Alzheimer's disease. In fact, it has been suggested that ACT binds Ab peptide and affects the rate of amyloid fibril formation in vitro (Eriksson S et al., 1995; Fraser PE et al., 1993) and it has been also shown to influence TAU protein phosphorylation and apoptosis in neuronal cells (Padmanabhan J et al., 2006).

ACT levels have been associated to AD and to cognitive decline. However, conflicting results are on record. Different techniques for ACT detection, different criteria for the selection of controls and AD patients or small numbers of cases and controls included in the studies may account for contradictory results regarding the association of abnormal ACT plasma levels with AD.

Moreover, alterations in molecular forms of ACT present in tissues and/or blood might also account for increased variability of ACT detection in AD and controls.

In this paper we focused our attention to the different molecular form of ACT in AD patients and controls.

Inflammatory states are usually associated with changes in the glycosylation pattern of acute phase proteins like ACT (Chavan MM et al., 2005; Gornik O and Lauc G, 2008).

ACT in fact is a glycoprotein composed mainly by six N-glycosylation sites and shows four oligosaccharide side-chains with disialyl diantennary and trisialyl triantennary type glycan structures with traces of disialylated triantennary oligosaccharides.

Many disease as myocardial infarction and some kind of cancers, are correlated to changes in sugar content of ACT protein, but no studies on plasma ACT glycosylation patterns in AD are on record.

In AD altered glycosilation pattern of presenilin-1, a molecule forming the catalytic core of the γ-secretase complex and able to generate amyloidogenic peptides (Farquhar MJ et al., 2003) and an abnormal glycosylation of reelin, a glycoprotein essential for the correct cyto-architectonic organization of the developing CNS, were previously shown (Botella-Lopez A et al., 2008).

Here we show results on different glycosilation pattern of ACT protein in AD patients and controls.

ACT blood levels were measured as previously described with some modifications (Porcellini et al., 2008), ACT was performed by affinity chromatography using Hitrap NHS-activated HP columns and the glycan profile of purified ACT samples was obtained by using the Qiagen Qproteome™ GlycoArray.

From Western Blot analysis we showed that ACT resulted in three bands both in AD patient and in controls.

On the contrary, when PNGase F treatments was performed on denatured purified ACT, four bands were detected in controls, whereas ACT from AD samples resolved again into three bands showing differences in protein composition between two groups of samples after denaturation.

As we just said, glycosylation is a versatile biochemical mechanism and changing in sugar chains composition could be strongly associated to several diseases including AD.

For this reason we further analyzed the sugar composition of purified ACT from controls and AD samples using an Array technique. This kit allowed to evaluate different glycan epitopes on ACT protein: Bi Antennary, Tri/Tetra Antennary, High Mannose, Sialic Acid, Terminal GlcNAc, Terminal GalNAc, Bisecting GlcNAc and O-Glycans.

The analysis of the results obtained from the scan of the array produced a detailed profile of ACT glycosylation status and a glycan epitope prediction pattern.

This analysis resulted in a partially different pattern of glycan profiles between ACT from AD and controls; sialic acid content being different between AD and CTR.

AD samples were further stratified in to two groups called AD 1 and AD 2 according to the differential rate of cognitive decline in a two years of follow up. Differences were found in GlcNac residues between AD 1 and AD 2 group where AD 1 showed a faster cognitive deterioration than AD 2.

In conclusion, in this article we confirm that ACT is a protein involved in Alzheimer's disease and in the cognitive impairment. Moreover, our

findings suggest that low content of terminal GlcNac glycans and sialic acid in peripheral ACT might be a marker of diseases progression and it might be used in clinical trials as surrogate marker of clinical efficacy.

The CALHM1 P86L Polymorphism is a Genetic Modifier of Age at Onset in Alzheimer's Disease: a Meta-Analysis Study

Although Alzheimer's disease (AD) is the most common cause of dementia in the elderly, its etiology is still not fully understood. The characterization of causative factors is thus important for better defining the patho-physiological processes involved.

Although the importance of the genetic component of these non-Mendelian forms of dementia has long been debated, there is now a large body of evidence suggesting that genetic variations play a major role in determining risk for this form of AD as well.

Recently, a novel gene on chromosome 10 (10q24.33) was reported to modulate the risk for late-onset sporadic AD (Dreses-Werringloer U et al., 2008). In that study, several independent case-control cohorts were genotyped for a Pro to Leu alteration at codon 86 (*P86L*; rs2986017) in the gene for calcium homeostasis modulator-1 (*CALHM1*), a transmembrane glycoprotein.

Perturbations in calcium homeostasis were observed in several neurodegenerative disorders including Alzheimer's disease. CALHM1 is a component of a novel cerebral calcium channel family involved in Aβ metabolism. The identification of CALHM1 as a key modulator of calcium

homeostasis and Aβ levels provides strong support for the calcium hypothesis of AD.

Moreover, *CALHM1* polymorphisms may influence AD risk even if some results are conflicting.

Many studies in fact did not shown an association of P86L polymorphism with AD (Bertram L et al, 2009) whereas other studies found it (Boada M et al, 2010; Li H et al, 2008).

Despite this contradictory data using cognitive status as phenotype, three studies showed association among an earlier age at onset (AAO) the homozygosity of the L allele and a marker in the *CALHM1* vicinity (Minster RL et al., 2009; Boada M et al., 2010; Li H et al., 2008).

In this article we presented a meta analysis on the CALHM1 P86L polymorphism conducted on 7.873 AD cases and 13.274 controls.

Using both novel and previously published genotype data the P86L polymorphism in CALHM1 seems not to be associated to the risk of Alzheimer's disease.

The discrepancy of risk effects between the independent follow-up data and the data first published by Dreses-Werringloer et al. (Dreses-Werringloer U et al, 2008), may indicates a false-positive finding in the initial report, a situation commonly observed in genetically complex diseases and referred to as "proteus phenomenon" or to as the "winner's curse phenomenon" (Kraft P, 2008).

However, even though our meta-analysis results rather unequivocally refute the initial findings suggesting that CALHM1 is a genetic risk factor for AD,

the present work suggests that the CALHM1 P86L polymorphism could modulate AAO and more specifically the *APOE* ε4 allele's dose effect on this phenotype.

Alzheimer's disease gene signature says: beware of brain viral infections

AD represents one of the most important cause of disability in the elderly and therefore one of the major age associated health and social problem.

As above reported, AD is a chronic neurodegenerative disorder clinically merging when the progressive neuronal death is in an advanced state and up to date no effective medication is available. Unfortunately, AD is still a non curable human disorder.

Several experimental findings showed that in the non familial form of the disease representing over 95% of cases genetic factors might be involved in the disease. A recent of genome wide association (GWA) study conducted by many European research laboratories on 5000 patients and 7000 controls reported that the allele 4 of APOE gene and single nucleotide polymorphism (SNP) in other genes regulating inflammation pathways were strongly associated with AD.

It can not be excluded that environmental factors may also contribute to brain inflammation and degeneration associated to AD. In particular, some initial observations indicated that the DNA of Herpes simplex virus type 1 (HSV-1) was found more frequently in the cerebral cortex of patients with AD than non-demented controls (Itzhaki RF et al., 1997).

The presence of viral DNA was particularly frequent in AD patients carrying the allele e4 of APOE gene.

In the recent GWA by Lambert et al. (Lambert et al., 2009) the gene CLU and CR1 have been strongly correlated to AD with a very low association probability ($p<10^{-10}$). Moreover, in this report, also a limited number of genes were highly associated ($p > l0^{-5}$) with the disease.

In this article we hypothesized that a gene cluster may predispose to AD via complex and diverse mechanisms each contributing to an increase of individual susceptibility to brain viral infections.

The first set of genes was located in close vicinity of the APOE locus on the chromosome 19 and consisted of the poliovirus receptor-related 2, APOE gene, the translocase of outer mitochondrial membrane 40 homolog (TOMM-40), the glycoprotein carcinoembryonic antigen related cell adhesion molecule-l6 (CEACAM-l 6) and B-cell/lymphoma-3 (Bcl-3) genes. Genes in the second set were located on different chromosomes: CLU on chromosome 8; CRl, and C-type lectin domain family 16 member A (CLEC-16A) on chromosome 16.

All this gene, as reported in the paper, are linked to different herpes simplex viral mechanisms and we argue that the concomitant presence of several polymorphisms of these genes in the same individual might represent a genetic signature of AD

Such hypothesis discussed here, where individual susceptibility to pathogen infection of the brain, particularly HSV and related viruses seems to be associated to Alzheimer's disease, is supported also by other different papers (Itzhaki RF et al., 2008; Carter CJ, 2008; Wozniak MA et al., 2009).

In fact brain infection by reactivated latent viruses might be one of the *primus movens* inducing progressive neuronal loss, astro-glia activation, and, impaired APP transport along the axons.

In conclusion, present findings suggest that during ageing virus reactivation may be more frequent in the elderly showing a genetic signature predisposing to an increased susceptibility for HSV and other virus infections of the brain. In these subjects the microorganisms are more likely to induce a limited, segmental and chronic sub-clinical pseudo-encephalitis resulting in a progressive neurodegeneration.

Sharing pathogenetic mechanisms between acute myocardial infarction and alzheimer's disease as shown by partially ovelapping of gene variant profiles

Alzheimer's disease (AD) is the most frequent form of dementia in the elderly (Nussbaum MD and Christopher E, 2003) characterized by a loss of neuronal synapses, deposits neuritic plaques and formation of neurofibrillary tangles (Katzman RN, 1996). Increasing levels of cytokines have been associated to dementia (Schmidt R et al., 2002; Licastro F and Chiappelli M, 2003; Shepherd CE et al., 2005) such as a large number of genetic variants (like single nucleotide polymorphism-SNPs) regulating inflammatory pathways or cholesterol methabolism (Licastro F et al., 2005; Corder EH et al., 1993; Grimaldi LM et al., 2000).

Cardiovascular disorders and mainly Myocardial Infarction (AMI) are the leading causes of morbidity and mortality in modern western societies. (Weir RA et al., 2006; Gupta R and Kaufman S, 2006).

The classical risk factor for these disease are: high cholesterol levels, hypertension, positive history of smoking, diabetes, obesity or sedentary life style. Unfortunately , more than half of patients with myocardial infarction do not demonstrate classical risk factors (Braunwald E, 1997).

Like in AD, many polymorphisms with functional relevance in the expression of inflammatory gene are often found at elevated frequency among patients with AMI (Lio D et al., 2004; Licastro F et al., 2002).

No attempt has been made to evaluate whether specific immune genetic risk factors might constitute an important etiologic and pathogenetic link between AD and AMI.

In this paper, we applied a relatively novel data analytic approach, namely, grade-of membership analysis (GoM) an alternative statistical approach to connect a large number of variables.

The study presented integrates information on a panel of gene variants that modulate inflammation and cholesterol synthesis (*IL10 -1082G/A, IL6 -174G/C, TNF -308G/A, IFNG +874T/A, SERPINA3 -51G/T, HMGCR -911C/A, APOE ε2/3/4*) investigated among AMI, patients with AD and healthy controls, in order to directly look for hypothetical over-lapping and/or distinct genetic profiles.

This methods allow to integrate information identifying low and high intrinsic risk sets which defined strong gradients of risk for individuals.

We applied GoM to our dataset to verify if groups of variables (genetic variations in this case) could be associated to controls, AD, or AMI.

Six genetic risk sets (I to VI) were identified by fuzzy latent classification. In the first group we localized mainly controls. In this group all the alleles are present in wild form (excepted for ACT -51).

Sets II & III were at low risk before age 65. These sets lacked pro-inflammatory alleles for *HMGCR, TNF* & *APOE*. The high risk sets IV to

VI included pro-inflammatory alleles for *IL10* + *IFNG* + *SERPINA3*. Disease outcome and onset ages were influenced by the co-occurrence of *HMGCR* (IV, AD or AMI), *TNF*+ *IL6* (V, AMI) or *APOE* (VI, AD or AMI). Close resemblance to one of the high risk sets, or the high risk sets taken together, denoted very high risk for AMI and/or AD.

The partial overlapping of the genetic risk profile between AMI and AD describes an emerging picture showing that an abnormal regulation of inflammation is implicated in the pathogenesis of atherosclerosis and its complications and neurodegenerative processes leading to AD.

In conclusion, data presented in this article, represent an approach to define individual risk profiles that may be applied to healthy subjects of different ages to predict intrinsic risk of AMI or AD. These risk profiles might then be used to define further diagnostic procedures which might indicate specific early therapeutic interventions, like statins and anti inflammatory drugs, aimed at prevention or significantly delay of the clinical manifestations of these two diseases. Finally, these findings may contribute to the goals of predictive diagnostics and personalized medicine.

Conclusion

AD is the most common neurodegenerative disease and one of the most common diseases in the industrialized world.

Clinically AD is defined as a slowly progressing loss of cognitive functions, altered behavior, loss of social appropriateness and a progressive decline in language function ultimately leading to dementia and death.

In Italy, AD affected subjects are between 800.000 and 1 million and unfortunately the number of new cases/year (incidence) is going to dramatically increase as a consequence of the progressive increase of the mean age and life expectancy in our population

Neuropathologically, AD is characterized by the aggregation and deposition of mis-folded proteins, in particular aggregated b-amyloid (Ab) peptide in the form of extracellular senile (or neuritic) plaques, and hyperphosphorlylated tau protein in the form of intracellular neurofibrillary tangles (NFTs).

These neuropathological hallmarks are often accompanied by abundant microvascular damage, including vascular amyloid deposits, and pronounced inflammation of the affected brain regions.

Moreover, microglia and astrocytes surrounding brain areas with neuro-degeneration showed activated phenotypes and expressed inflammatory molecules, and several of these molecules are found associated with senile plaques in brains of patients affected by AD (Chiappelli M et al., 2006). The presence of these kind of molecules and the presence of an activated phenotype confirms that inflammation plays a pivotal role in AD. This notion is also reinforced by the hypothesis that the routine use of non-

steroid anti-inflammatory drugs was associated with a decreased incidence of AD (Breitner JC et al., 1994; Cohen HJ et al., 2003).

Alzheimer's disease is a very complex and multifactorial disease where clinical factor as inflammation, pathogens infections, environmental factors and genetics underlines the pathogenesis of the disease.

AD is commonly divided in two forms: one sporadic AD that involve about the 99% of cases and familial AD with an age of onset before 65 years (approximately 0.5% of cases) where autosomal dominant mutations in the APP, PSEN1 or PSEN2 genes are present.

Several genetic studies have shown that the presence of apolipoprotein E e4, the main carrier of cholesterol in the brain was associated with an increased risk of developing late-onset AD (Corder EH, 1994).

More than 550 other genes have been proposed as candidates for Alzheimer's disease susceptibility, but thus far none have been confirmed to have a role in Alzheimer's disease pathogenesis (Gatz M. et al., 2006).

Two studies of Genome Wide Association (GWA) were recently published in Nature Genetics (Lambert JC et al., 2009; Hardold D et al.. 2009), in which independent groups have studied thousands of patients with AD and control subjects with the aim of identifying a set of single nucleotide polymorphisms (SNPs) associated with AD.

In this thesis we investigated several aspects of Alzheimer focusing mainly on the genetic aspect of the disease.

The first approach that we applied was to analyze a single protein involved in inflammatory pathway and verify if this protein and its levels were associated to the pathogenesis of AD.

In a large cohort of cognitively healthy subjects, in subjects with cognitive impairment and in two independent AD populations we confirmed that Alpha 1 antichymotrypsin (ACT), an acute phase protein, was associated to AD subjects, being ACT plasma levels higher in AD cases than controls.

Moreover ACT protein from AD showed different glycosylation pathway.

Even if these genetic studies gave positive results, this kind of approach is unfortunately very limited, since it is unlikely that a single genetic or phenotypic biomarkers may provide sufficient information for the potential risk of such a complex disease as dementia.

The usual approach of focusing on one or a few candidate genes or one or few SNPs limits our ability to identify novel genetic factors associated with disease.

Genome-wide association studies have been proposed as a solution to these problems: by new sequencing, technologies and microarray platform, in fact it is now possible analyze a large number of genes and SNPs in thousand of samples.

Here we presented a GWA study where about 500.000 SNPs have been analyzed in 5.800 AD patients and 8.500 healthy controls.

In this paper we demonstrated that two different gene, Clusterin and Complement receptor 1 were strongly associated to AD, independently to APOE ε4 allele.

Another gene found associated to AD is CALHM1: we found a significant association between the P86L L-allele and earlier onset for AD, particularly in carriers of the APOE ε4-allele.

My studies presented in this thesis showed several biological markers individually associated with AD risk and cognitive decline, but results could not be conclusive or completely satisfactory because of the limited power of classical statistical analysis used.

The goal should be to created a network of genetic, phenotypic and clinical data that allows to combine different type of variables.

To analyze many variables in a large population are necessary statistical models capable of analyzing the relationship between factors and disease and the degree of interaction of all these factors together and with the disease.

We used a new algorithm, the ANNs, aimed to map variables and search for connectivity. This method is based on an artificial adaptive system able to define the association strength of each variable with all the others in database, named the Auto Contractive Map (AutoCM).

AutoCM generates a map of main connections between and among variables and the principal hubs of the system. These hubs can also be defined as variables with the maximum amount of connections in the map.

In this new method non-linear associations were preserved, explicit connection schemes were investigated and the complexity of dynamic interactions were preserved.

We tested this new approach using Conselice database and we found specific variables associated to AD like cholesterol levels, the presence of variation in HMGCR enzyme and the age underlining the importance of cholesterol in the pathogenesis of the disease.

A second application of ANNs was tested to an extended dataset in which other clinical and phenotypical variables were added. New factors such as the BMI, the amount of HDL and blood folate levels were associated to AD. We just strussed the complexity of Alzheimer's underlining the importance of the interaction between genetic and environmental factors.

Pathogen infections, above all viral infections, have been previously associated to the pathogenesis of AD (Sequiera LW et al., 1979).

The hypothesis suggests that virus and in particular herpes virus could enter the brain when an individual becomes older, perhaps because of a decline in the immune system. Brain invasion by virus triggers various mechanisms that lead to AD.

Based on GWA results published on Nature Genetics, (Lambert et al., 2009) we suggest that a genetic cluster on chromosome 19, close by to the

APOE gene, was strongly associated with AD.

All these genes are involved in the entry and/or in the replication of herpes simplex viruses, in the cell-to cell virus spreading and also in the host immune resistance to virus infection.

Our new hypothesis is that the presence of allelic polymorphisms in these genes results in a genetic signature that might affect individual brain

susceptibility to infection by herpes virus family during aging, leading to neuronal loss, inflammation and amyloid deposition.

This thesis presents data on emerging disease that affects more and more people in all industrialized and developing countries and is becoming an important social and economic problem.

Unfortunately there are no effective therapies for this disease and for this reason it is important to find new strategies for early intervention in the age-associated cognitive decline and the prevention of the occurrence of clinical AD.

If Alzheimer's disease is the main type of dementia, cardiovascular disorders are the leading causes of morbidity and mortality in modern western societies.

With an epistatic statistical approach, we identified a partial overlapping multi-gene risk profiles associated to Acute myocardial infarction (AMI) and AD. This overlapping describes an emerging picture showing that an abnormal regulation of inflammation is implicated in the pathogenesis of atherosclerosis and its complications and neurodegenerative processes leading to AD (Licastro et al., 2010).

These risk profiles might then be used to define further diagnostic procedures which might indicate specific early therapeutic interventions, like statins and anti inflammatory drugs, aimed at prevention or significantly delay of the clinical manifestations of these two diseases. Finally, these findings may contribute to the goals of predictive diagnostics and personalized medicine.

The epistatic approach suggested here, might help us to identify unaffected subjects with high risk of developing AD to be selected for early intervention trials focused on the prevention of cognitive decline and dementia.

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