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Human Herpes Virus

and

Alzheimer's disease

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Introduction

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disorder and the most common neurodegenerative disorder in the industrialized world. In 1906, Aloise Alzheimer, a German Psychiatrist, identified and presented the first case of a fifty-year-old woman called Auguste D.

In the early stages of AD, the most commonly recognized symptom is the 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. Neurodegeneration associated with 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 AD are not well understood. When AD is suspected, the diagnosis is usually confirmed by behavioural assessments and cognitive tests, often followed by a brain scan.

An internationally agreement upon standard of tests for AD clinical diagnosis includes a detailed history, functional measurement of cognitive decline such as instrumental activity of daily living scales, Mini Mental Status Examination test (MMSE), Clinical Dementia Rating (CDR), Disability Assessment for Dementia (DAD), neuropsychological evaluation, neurological and psychiatric examination.

The MMSE test, for example, allows classifying 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 provides a semi quantitative evaluation of the degree of cognitive impairment (Folstein et al. 1975).

The cognitive evaluation obtained from this 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, fronto- temporal 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 were defined as mild cognitive impairment (MCI) (Figure 1) (DeCarli 2003).
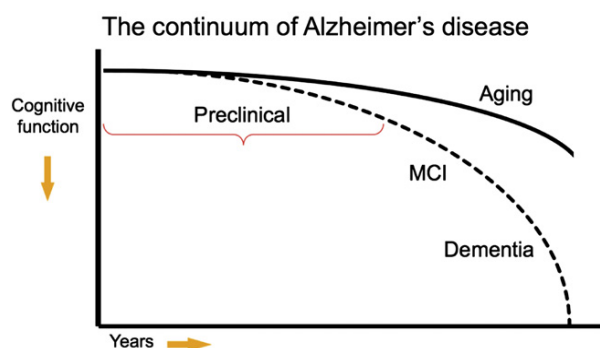

Figure 1. Hypothetical model for the pathological-clinical continuum of AD (Sperling et al. 2011).

The diagnosis of MCI is performed according to the following criteria: memory complaint, normal activities of daily living, normal general cognitive function, abnormal memory for age, and absence of dementia (Petersen 2004). It was been observed that MCI are likely to progress to AD at a rate of approximately 12% per year compared to 1 to 2% for cognitively normal people at the same age (Petersen et al. 1999).

Because not all individuals with MCI progress to clinical dementia, it is critical to identify risk factors and biomarkers for the development of AD in this cohort. So, Aggarwal et al. showed that the presence 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 et al. 2005).

Cerebral atrophy can be detected during life with structural imaging techniques, such as magnetic resonance imaging (MRI) which allows the quantification of atrophy due to its ability to acquire high resolution images with good soft tissue contrast properties. Studies using MRI have identified different patterns of cerebral atrophy in different degenerative disorders. Subjects with AD typically show patterns of atrophy involving the medial temporal lobe, particularly the hippocampus and entorhinal cortex, and the posterior cingulate, precuneus and the tempo-parietal neocortex, with concurrent expansion of the ventricles. Subjects with MCI typically show similar, although less severe, patterns of atrophy such as those observed in AD (Whitwell 2010).

Epidemiology

According to the World Health Organization it is estimated that 35.6 millions of people worldwide currently suffer AD. This number is expected to double by 2030 and more than triple by 2050. Dementia affects people in all countries; with more than half living in low- and middle-income countries and by 2050, this is likely to rise to more than 70% (http://www.who.int/mediacentre/news/releases/2012 /dementia_ 20120411).

North America and Western Europe have the highest prevalence of dementia (6.4% and 5.4% of the population at age 60), followed by Latin America (4.9%) and China and its developing western-Pacific neighbours (4.0%). The prevalence rates for AD also rise exponentially with age, increasing markedly after 65 years. The annual incidence rates (per 1000 individuals in the population) for Western Europe was estimated at 8.8, increasing exponentially with age, especially through the seventh and eighth decades of life (Reitz et al. 2011).

In Italy, AD patients are almost 1 million and the annual incidence is going to increase as a consequence of the progressive increase of the mean age and life expectancy in our population.

Moreover AD is more prevalent in women than in men. The higher prevalence of AD in women is explained in part by differences in life expectancy between the genders (Vest et al. 2012).

Pathogenesis

AD is characterized by a progressive decline in cognitive functions which typically begins with memory deterioration. In the final stage of the disease, individuals with this disorder have usually become dependent on caregivers. AD patients develop severe impairments in memory and executive cortical functions. Although living patients can be diagnosed clinically with possible or probable AD, a confirmed diagnosis requires *post-mortem* histological identification of neuropathological hallmarks. In figure 2 are shown time points at which preventive, disease modifying, and symptomatic interventions, respectively, are likely to be most effective, while in the upper bar are shown the identified milestones (genetic mutation, Aβ (amyloid-β peptide) and tau misfolding, oxidative and inflammatory stress and cell death) in the patho-biology of AD that culminates in death and autopsy confirmation of AD.
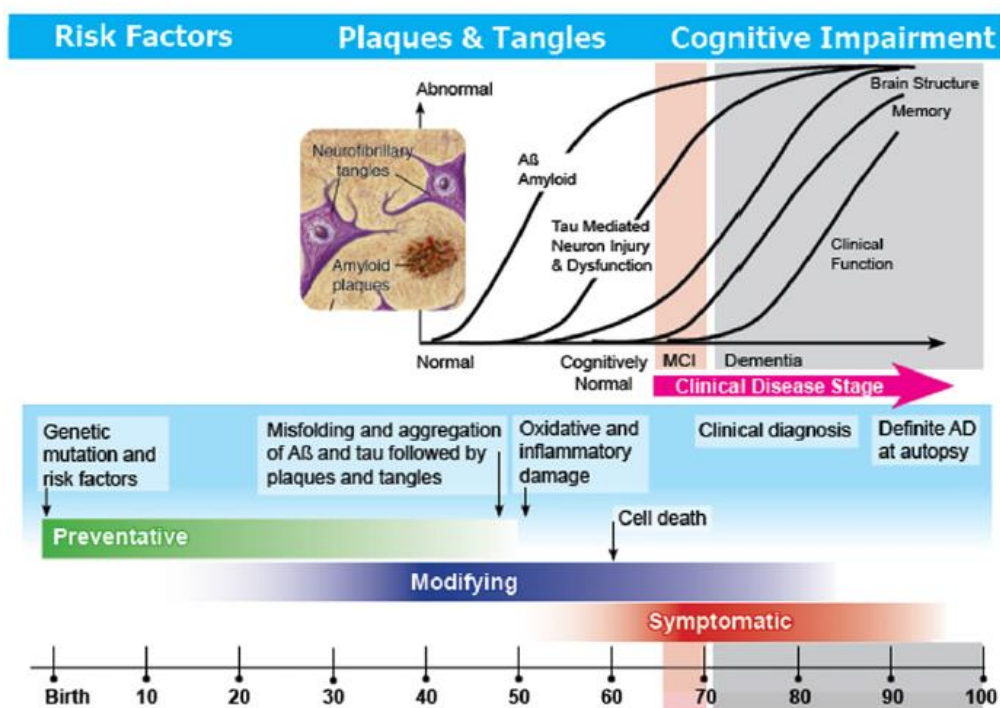
Figure 2. Hypothetical time line for the onset and progression of AD, neurodegeneration and cognitive impairments progressing from normal control to MCI and to AD (Trojanowski et al. 2010).

AD begins with abnormal processing of amyloid precursor protein thereby increasing brain Aβ which leads to neuron dysfunction and death. The model also assumes a lag phase between Aβ deposition and neuron loss, and differences in brain resiliency, plasticity, cognitive reserve or other factors likely account for the variable duration of this lag phase (Trojanowski et al. 2010).

The key pathological changes that are observed in AD brain tissue are increased levels of both the amyloid-β (Aβ) peptide, which is deposited extracellular in diffuse and neuritic plaques, and hyperphosphorylated tau (p-tau), a microtubule assembly protein that accumulates intracellular as neurofibrillary tangles (NFTs) (Figure 2). These neuropathological hallmarks are often accompanied by the presence of reactive microgliosis and widespread loss of neurons and synapses (Reitz et al. 2011).

The current view of AD pathogenetic mechanisms describes amyloid deposition and neuritic plaques formation as a central mechanism leading to neuro-degeneration, cognitive impairment and AD (Figure 2). APP (amiloid precursor protein) is the Aβ peptide precursor and is a trans-membrane glycoprotein widely expressed, produced by the endoplasmatic reticulum and involved in the neuronal and dendritic growth and synapses formation (Steuble et al. 2012).

The metabolic cleavage of APP involves three different enzymes called α, β and γ secretase. APP gene encodes the amyloid precursor protein, which gives rise to Aβ, composed of 40-42 amino acids, through serial cleavage by β-secretase and γ-secretase. Proteolitic cleavage by α secretase prevents Aβ release and results in the so called non amyloidogenic pathway (Thinakaran et al. 2008).

γ-secretase is a complex of four proteins in which the enzymatic components are encoded by the early-onset familial AD genes presenilin 1 (PSEN1) and presenilin 2 (PSEN2). Aβ 40-42 peptide is the major form of Aβ produced during βAPP metabolism. In the pathogenesis of AD, accumulation of Aβ in the brain, particularly Aβ42, is considered to be an important step (Small et al. 1999). Aß40 is the major form of secreted Aβ. However, Aß42 aggregates more readily and is thought to seed amyloid fibril polymerization during the early stages of plaque formation (Jarrett et al. 1993).
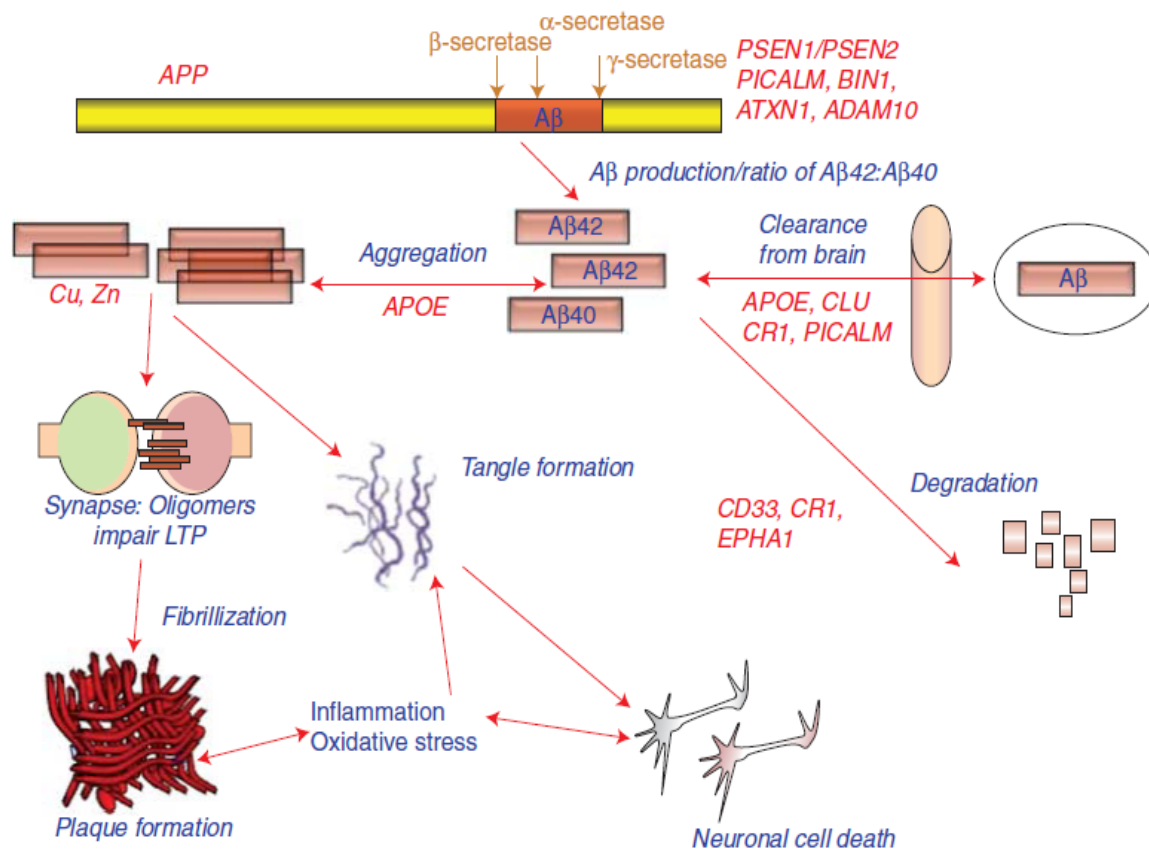
Figure 3. Model of amiloidogenic cascade (Tanzi 2012).

Once it has been generated, Aβ can be transported out of the brain with the assistance of ApoE. Alternatively, Aβ can undergo proteolytic degradation. As Aβ 40-42 accumulates it can aggregate, and this is influenced by ApoE. Aggregation into oligomers (for example, dimers and trimers) can lead to impairment of long-term potentiation (LTP). Aβ oligomers can further aggregate into fibrils, which might ultimately be deposited in senile plaques. Senile plaques and Aβ aggregates can induce inflammatory responses and oxidative stress. Transferrin (TF) regulates the metabolism of iron, a reactive metal that is involved in free-radical generation and, thus, in oxidative stress. Oxidative stress and iron have been associated with abnormal tau phosphorylation and aggregation, and

with the formation of NFTs. The principal component of NFTs is tau. Aβ oligomers have been reported to induce NFT formation. NFTs induce neuron death, which can result in further inflammation and oxidative stress. In turn, inflammation and oxidative stress can enhance further Aβ deposition, resulting in a vicious cycle (Bertram et al. 2008).

This model of AD views Aβ peptide accumulation as a key early event in the pathophysiological process of AD. However, the etiology of AD remains uncertain, and some investigators have proposed that synaptic, mitochondrial, metabolic, inflammatory, neuronal, cytoskeletal, and other age-related alterations may play an even earlier, or more central, role than Aβ peptides in the pathogenesis of AD.

Classification

AD is usually classified according to its age of onset. The majority (>95%) of patients who develop this disease are aged >65 years (sporadic or late-onset AD), with 1–5% of AD cases exhibiting an earlier onset, typically in the late 40s or early 50s (familiar or early-onset AD).

Familiar form of early onset AD (EOFAD)

Approximately 5% of cases of AD are familial (autosomal dominant) and with an early age onset. In these cases, AD is caused by mutations in three genes: APP on chromosome 21, PSEN1 on chromosome 14, and the PSEN2 on chromosome 1 (Table 1) (Tanzi 2012).

Gene	Protein	Chromosome	Mutations	Molecular phenotype
APP	Amyloid β (A4) protein precursor	21q21	24 (duplication)	Increased $A\beta_{42}/A\beta_{40}$ ratio Increased $A\beta$ production Increased $A\beta$ aggregation
PSEN1	Presenilin 1	14q24	185	Increased $A\beta_{42}/A\beta_{40}$ ratio
PSEN2	Presenilin 2	1q31	14	Increased $A\beta_{42}/A\beta_{40}$ ratio

Table 1. Early-onset familial Alzheimer disease genes and their pathogenic effects

Sporadic form of late onset AD (LOAD)

Late-onset Alzheimer is the most common form of the disease and it is defined by the age at onset after 65 years. Whereas EOFAD is characterized by classic Mendelian inheritance usually in an autosomal-dominant manner, LOAD is associated with a genetically complex pattern of inheritance in which genetic risk factors work together with environmental factors and life exposure events to determine lifetime risk for AD.

It is well established that the inheritance of the apolipoprotein E (APOE) ε4 allele is the single most potent and common genetic risk factor for LOAD (Tanzi 2012). The APOE gene is polymorphic, with three common alleles (ε 2, ε 3, ε 4), and in studies of the general population six genotypes are observed: 3/3, 4/3, 3/2, 4/4, 4/2, and 2/2. The three major alleles of the APOE locus, ε2, ε3, and ε4, correspond to combinations of two amino acid changes at residues 112 and 158 (ε2: Cys112/Cys158; ε3: Cys112/Arg158; ε4: Arg112/Arg158). APOE ε 4/4 increases the AD risk more than about 12-fold, and APOE ε3/4 about 3-fold when compared with the APOE ε 3/3 genotype (Koffie et al 2012). In contrast, the APOE ε2 allele exerts "protective" effects (Corder et al. 1994). Synapse loss is a strong correlate of cognitive decline in Alzheimer's disease and imaging studies have suggested the occurrence of synaptic dysfunction decades before AD onset in APOE ε4 patients. However, the mechanistic link between apoE isoform and these effects in AD remains unknown (Koffie et al 2012).

Moreover many other genes, involved in different pathways, have been proposed as candidates for AD susceptibility. Our previews studies showed the association of several SNPs (single nucleotide polymorphisms) in the promoter region of genes involved in the inflammatory pathway with AD.

IL-10 gene, located on chromosome 1 is synthesized in central nervous system and its function is to limit the inflammatory response. Many SNPs have been identified in this gene; the most informative one is at position -1082 (Tagore et al. 1999). 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 et al. 2003). Tumor necrosis factor α (TNF- α) is an inflammatory cytokine involved in the local immune response occurring in the

central nervous system of AD patients. TNF-α SNP in the promoter region −308 G/A is associated with AD (Lio et al. 2006).

IL-1β gene by affecting brain immune responses may influence the age at onset of the disease, AD progression and survival. This cytokine is expressed by activated microglia in AD (Sheng et al. 1996). A polymorphism at position -511 in the promoter region of IL-1 beta gene is present and the TT genotype has been associated with an increased risk of AD (Licastro et al. 2000, Chiappelli et al. 2006).

Hydroxy-methylglutaryl-coenzyme A reductase (HMGCR) is the rate limiting enzyme in the synthesis of cholesterol. A SNP in the promoter region (-911) of this gene was investigated in a case-control study and appears to be linked to both AD risk and disease progression (Porcellini et al. 2007).

Even if it is very important to find new SNPs associated to AD, the study of SNP on limited sets of patients is not very informative. This situation has changed to some degree since the advent of massively parallel genotyping techniques that now allow the sequencing of the genomes of a large number of subjects at varying degrees of resolution. In the last few years, the most popular genetic approach has been based on genome-wide association studies (GWAs) where up to one million genetic markers are simultaneously genotyped and assessed for potential correlations with the disease risk and other phenotypic variables.

Genome wide association studies

Between 2007 and 2009, several GWAs in AD were performed and they improved our knowledge of the genetics link with AD (Lambert et al. 2011).

The two large GWAs from the UK (Harold et al. 2009) and France (Lambert et al. 2009) found sets of genes located in different chromosome as shown in Table 2.

Gene		Chromosome	GWA studies
CEACAM 16	Carcinoembryonic antigen-related cell adhesion molecule 16	19	Lambert et al 2009, Harold et al 2009
BCL3	B-cell CLL/lymphoma 3	19	Lambert et al 2009, Harold et al 2009
PVRL2	Poliovirus receptor-related 2 (herpesvirus entry mediator B)	19	Lambert et al 2009, Harold et al 2009
TOMM40	Translocase of outer mitochondrial membrane 40 homolog (yeast)	19	Lambert et al 2009, Harold et al 2009
APOE	Apolipoprotein E	19	Lambert et al 2009, Harold et al 2009
APOC1	Apolipoprotein C1	19	Lambert et al 2009, Harold et al 2009
CLU	Clusterin	8	Lambert et al 2009, Harold et al 2009
CR1	Complement receptor 1	1	Lambert et al 2009, Harold et al 2009
CLEC 16A	C-type lectin domain family 16 member A	16	Lambert et al 2009, Harold et al 2009
PICALM	Phosphatidylinositol binding clathrin assembly protein	11	Lambert et al 2009, Harold et al 2009

Table 2. Cluster of genes from GWA studies associated with AD risk.

The first set of genes was located in close vicinity of the APOE locus on chromosome 19 and consisted of the poliovirus receptor-related 2 (PVRL2) or nectin-2 (NC-2), apolipoprotein E (APOE), the translocase of outer mitochondrial membrane 40 homolog (TOMM40), the glycoprotein carcinoembryonic antigen related cell adhesion molecule-16 (CEACAM-l6), and Bcell/lymphoma-3 (Bcl-3) genes. Genes in the second set were located in different chromosomes: APOJ or clusterin on chromosome 8; the complement receptor l (CR-l) on chromosome 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}).

Moreover, three novel AD genes, i.e., CLU (clusterin or apolipoprotein J), CR1 (complement component (3b/4b) receptor 1), and PICALM (phosphatidylinositol binding clathrin assembly protein) were also confirmed to be associated with AD risk. These loci have since received overwhelming support from independent follow-up studies (Carrasquillo et al. 2010, Jun 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 effect exerted by the new GWA SNPs is 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. It is important to note that the presence of the APOE ε4 allele is associated with a nearly 4-fold increase in AD risk.

A third new set of genes has emerged in a more recently GWA studies (Hollingworth et

al. 2011, Naj et al. 2011) as summarized in Table 3.

Gene		Chromos ome	GWA studies
ABCA7	ATP-bonding cassette, sub family A, member 7	19	Hollingworth et al 2011, Naj et al 2011
MSA4	Membrane spanning A4	11	Hollingworth et al 2011, Naj et al 2011
CD2AP	CD2 associated protein	6	Hollingworth et al 2011, Naj et al 2011
CD33	Cluster of differentiation 33	19	Hollingworth et al 2011, Naj et al 2011
EPHA1	Ephrin receptor A1	7	Hollingworth et al 2011, Naj et al 2011

Table 3. Second cluster of genes from GWA studies associated with AD risk.

Interestingly, the one overarching common finding emerging from all published AD GWAs to date is the highly significant association between increased AD risk and the presence of the APOE ε4 allele (Bertram 2011).

In fact, GWAs approach is different from the classical genetic studies, because the majority of today's genetics projects are not aimed at the identification of rare, disease-causing mutations, but instead at finding common DNA variants or SNP that can modify susceptibility to high-prevalence disorders. The GWA approach is solved by genotyping hundreds of thousands SNPs on a genome wide scale (eg, in affected cases and healthy controls). Genotyping is followed by statistical analyses of data base, which probe for significant differences in allele or genotype frequencies. In addition to not being limited to some predefined set of candidate genes, the GWAs approach has several other advantages that increase the validity of the emerging results, eg, the ability to adjust for otherwise difficult-to-detect population substructure, to perform in silico

fine -mapping based on genotype imputation, and to serve as a "replication engine" for proposed associations from other datasets without having to perform additional experiments. Instead, owing to the very large number of markers tested in typical GWAs, many SNPs are bound to show significant effects by chance alone, which has to be taken into account when reporting and interpreting GWAs results (Bertram 2011).

Environmental factors and genetic interactions

AD is a complex multifactorial disease resulting from the interaction of several determinants: principally genetic and environmental, all mostly unknown.

Environmental factors might play a role in this complex disease. Environmental risk factors may accumulate with advancing age and play the role of multiple triggers of the disease in the susceptible brain; however these factors are still largely unravelled in AD. It is important to know that environmental factors may also interact with genetic risk factors in a complex interplay that might be the central node to a correct interpretation of pathogenic mechanisms leading to age-related neurodegeneration and dementia. However, it is essential to start or refine the search for environmental components associated to AD, in order to reveal such a complex cross talk with the partially known genetic risk factors predisposing to the disease.

A viral etiology, involving in particular herpes virus in AD, has been already investigated (Wozniak et al. 2009, Itzhaki et al. 2008, Carter 2008, Burgos et al. 2006, Mori et al. 2004).

We suggest that infective agents of CNS, such as viruses of the herpes family, are the probable link for all SNPs found associated with AD from GWA studies and the view presented here supports the notion of an infective etiology of sporadic AD (Porcellini et al. 2010, Licastro et al. 2011).

• NC-2, also known as herpes virus entrance-B (HveB) or poliovirus receptor-relate protein-2, is a member of the immunoglobulin super family, is expressed in a variety of cell tissues, including neurons, belongs to the cadherin adhesion molecules (Takai et al.

2003) and mediates the entry of herpes simplex viruses (HSV) (Spear 2004). 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 (Spear 2004). 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.

• APOE 4 allele is a well established genetic risk factor for AD and it has been also confirmed in the European GWA study (Lambert at al. 2009). ApoE protein may affect Aβ deposition. However, APOE 4 allele has been also shown to influence: susceptibility to viral infections (Mahley et al. 2009), human immune deficiency virus (HIV) cell entry in vitro, HIV disease clinical progression (Burt et al. 2008), recurrent genital herpes in patients co-infected by HSV-2 and HIV (Jayasuriya et al. 2008) and progression of experimental ocular lesions induced by HSV-1 (Bhattacharjee et al. 2008). Therefore, APOE 4 allele association with AD might also influence the susceptibility to virus entry and spreading into neuronal cells.

• TOMM-40 gene codes for a mitochondrial translocase. It is interesting to note that HSV DNAase such as the UL12 enzyme destroys the mitochondrial genome (Corcoran et al. 2009) by inducing rapid and complete degradation of mitochondrial DNA (Saffran et al. 2007). Gene variations at TOMM-40 gene might influence DNA digestion and mitochondrial damages induced by HSV DNAase and other less defined virus dependent mechanisms.

• 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-l/3 activation (Nittka et al. 2008).

• 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 and suppress apoptosis in infected cells. In particular HSV was shown to inhibit initially induced apoptosis in neuronal cells via a caspase-3 dependent pathway (Wang et al. 2005). Moreover, Bcl-2 protein was able to block HSV induced apoptosis in human hepatocytes (Galvan et al. 2000). 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).

• The APOJ, also known as clusterin, is a modulator of complement activation. Complement biosynthesis and activation occurs in neurodegenerative diseases such as AD (Francis et al. 2003) and the cytolytic activity of complement components is important for virus neutralization. ApoJ is synthesized in the CNS and is present in amyloid plaques (Calero et al. 2000). Polymorphism in APOJ gene might influence virus lytic defences by regulation of complement activation.

• CR1 is a complement receptor which binds different complement components (C3b, C3d, and C2a). HSV 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 (Spear 2004). 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.

• 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 polymorphisms in the CLEC-16A gene might influence individual ability to recognize and bind virus glycoproteins.

• ABCA7 is highly expressed in the brain, especially values in hippocampal CA1 neurons (Kim et al. 2006) and microglia (Jehle et al. 2006) and regulates the efflux of lipids from cells to lipoproteins. Moreover ABCA7 influences the quality of lipoprotein by interacting with APOA1 molecules especially in female, since this gene is involved in the assembly reaction of high density lipoprotein (HDL) (Ikeda et al. 2003) and controls heterogeneity of HDL (Hayashi et al. 2005). It is known that certain viruses can circulate in biological fluids bound to lipoproteins, for instance hepatitis C virus particles circulates associated with plasma lipoproteins (André et al. 2005). Therefore, the type of lipoproteins and lipids might influence virus transport to a given tissue and its circulation within the brain especially in women; incidence and prevalence of AD being higher in women. This gene also affects the efficiency of phagocytosis of apoptotic cells by monocytes cell lineage (Kim et al. 2005) and clearance of apoptotic virus infected cells (Jehle et al. 2006). Therefore, ABCA7 variants might also influence clearance of infected cells from the brain.

• MSA4 gene belongs to a genetic cluster located on chromosome 11 and encodes for the beta sub-unit of high affinity IgE receptor (Kinet et al. 1988, Crocker et al. 2007); this molecule is a component of an oligomeric cell surface complex involved in signal transduction in different cell lineages (Liang et al. 2001). The MSA4 cognate protein has been involved in antiviral responses in human plasmocytoid or lung dendritic cells

(Gill et al. 2010, Grayson et al. 2007). Furthermore, CD23 or Fc-epsilon IIIR play a role in astrocyte inflammatory response during HIV-1 encephalitis (Dugas et al. 2001) and HIV-1 infection induces an impaired regulation of the IgE Fc-epsilon RI network (Marone et al. 2001). Therefore, this gene might influence virus entrance in neuronal cells and virus infectivity might in turn affect the expression of this membrane complex.

• CD2AP gene is located on chromosome 6 and codes for a member of a novel family of scaffold/adaptor proteins, expressed on several cell types and regulates the actin cytoskeleton (Lynch et al. 2003). CD2AP plays an important role in antivirus defences, since it regulates transportation and fusion of cytoplasmic granules in NK cells (Ma et al. 2010). Moreover this molecule also affects selective activation of survival pathways and repression of apoptosis signalling by TGF-beta (Schiffer et al. 2004). CD2AP might play multiple roles by regulating defence mechanisms against virus infectivity and cell sensitivity to apoptosis induced by the virus infection. Finally, CD2AP, by affecting early endosome morphology and traffic between early and late endosomes (Cormont et al. 2003), might disturb APP metabolism and amyloid deposition.

• CD33 gene is on chromosome 19 and codes for a member of the sialic-acidic-binding immunoglobulin like lectin or SIGLEC family that promotes cell-cell interactions and regulates immune functions of both innate and adaptive immunity (Tateno et al. 2007). Human cytomegalovirus latent infection induced the up-regulation of the MCP-1 molecule in a restricted subset of CD33 positive myeloid progenitor cells and this mechanism may contribute to virus dissemination (Stern et al. 2008, Hahn et al. 1998). Human herpes virus 7 also induced an up-regulation of CD33 in cultured human cells (Mirandola et al. 2000). Moreover, microarray analysis of blood mononuclear cells from HIV-1 positive patients on retroviral therapy showed an over expression of CD33

molecule (Wu et al. 2008). In liver Kupffer cells infected by HCV over-expressed the CD33 molecule (Dolganiuc et al. 2007). Once again one gene might affect multiple steps involved in herpes infectivity and individual susceptibility to virus infection.

• EPAH1 is a member of the ephrin receptor sub-family of tyrosine-kinases and mediates cell and axon guidance, synaptic development and plasticity (Coulthard et al. 2001, Yamazaki et al. 2009). This molecule is also implicated in apoptosis (Duffy et al. 2008) and inflammatory response regulation (Ivanov et al. 2006). EPAH1 might be implicated in antivirus resistance by affecting both apoptosis impairment induced by the virus infection, the efficiency of the host immune responses and synaptic plasticity of infected neurons.

Our hypothesis describes sets of genes (as shown in Table 1 and 2) that may constitute a gene cluster of susceptibility for AD by affecting different mechanisms involved in virus entrance or resistance to viruses infection.

We suggest that infective agents of CNS, such as viruses of the herpes family, are the probable link for all SNPs found associated with AD from recent GWA studies and the view presented here supports the notion infective factors are associated with the clinical history of sporadic AD.

We argue that the concomitant presence of several SNPs in these genes in the same individual might represent a genetic signature of AD and further reinforce our hypothesis that such genetic trait predisposes to AD via complex and diverse mechanisms each contributing to the differential individual brain susceptibility to viral infections.

Herpes viruses

The Herpesviridae are a large family of DNA viruses that cause diseases in animals and humans. Herpes viruses share a common structure: are composed of relatively large double-stranded, linear DNA genomes encoding 100-200 genes encased within an icosahedral protein cage called capsid, which is itself wrapped in a protein layer called tegument containing both viral proteins and viral mRNAs and a lipid bilayer membrane called envelope. This whole particle is known as a virion. The name herpes comes from the Latin herpes which, in turn, comes from the Greek word herpein which means to creep. This reflects the creeping or spreading nature of the skin lesions caused by many herpes virus types. Once a patient has become infected by herpes virus, the infection remains for life. The initial infection may be followed by latency with subsequent reactivation. Herpes viruses infect most of the human population and persons living past middle age usually have antibodies to most of the above herpes viruses. The Herpesviridae are currently divided into three sub-families, as shown in the Table 3 (Hunt, Virology, chapter eleven, HERPES VIRUSES, Microbiology 5th Ed).

Human herpes type	Name	Sub Family	Target cell type	Latency	Transmission
1	Herpes simplex-1 (HSV-1)	α-herpesvirinae	Mucoepithelia	Neuron	Close contact
2	Herpes simplex-2 (HSV-2)	α-herpesvirinae	Mucoepithelia	Neuron	Close contact usually sexual
3	Varicella Zoster virus (VSV)	α-herpesvirinae	Mucoepithelia	Neuron	Contact or respiratory route
4	Epstein-Barr Virus (EBV)	γ-herpesvirinae	B lymphocyte, epithelia	B lymphocytes	Saliva

5	Cytomegalovirus (CMV)	β-herpesvirinae	Epithelia, monocytes, lymphocytes	Monocytes, lymphocytes and possibly others	Contact, blood transfusions, transplantation, congenital
6	Herpes lymphotropic virus (HHV-6)	β-herpesvirinae	T lymphocytes and others	T lymphocytes and others	Contact, respiratory route
7	Human herpes virus-7 (HHV-7)	β-herpesvirinae	T lymphocytes and others	T lymphocytes and others	Unknown
8	Human herpes virus-8 (HHV-8) Kaposi's sarcoma-associated herpes virus (KSHV)	γ-herpesvirinae	Endothelial cells	Unknown	Exchange of body fluids?

Table 3. Herpes virus family.

It is interesting to note that many herpes viruses share the ability to become latent in the infected host and eventually latently infect neurons. However, investigations focused on different viruses of the herpes family, such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) or Human herpes virus 6 (HHV-6), in AD are scarce.

CMV is ubiquitously distributed in human population and is the most frequent infection of the brain in infants with the congenital virus transmission or in immune compromised patients (Tsutsui et al. 2008). Postnatal acute CMV infection is usually asymptomatic, but once established, the virus remains latent in blood monocytes (Pawelec et al. 2009). CMV has also been associated with other chronic diseases of aging, including cardiovascular disease, cognitive decline and cancer. The specific mechanisms responsible for these associations have not been fully understood, but they are likely to have an immune and inflammatory component (Simanek et al. 2011). The serological-

conversion to CMV may vary over the years, ranging between 0.5 to 1.5% per year, and CMV is responsible of the age-associated immune changes in the elderly which lead to a reduction in the number of naive T cells (Rymkiewicz et al. 2012). An increased rate of cognitive decline over a 4-year period in subjects with elevated CMV antibody levels has been reported (Aiello et al. 2006). Previous work upon brain frontal and temporal cortex samples found that both AD patients and elderly healthy subjects were positive for CMV with not statistically significant difference (Lin et al. 2002). CMV was found in the brain of a greater proportion of patients with vascular dementia than normal elderly; these findings suggested a role for this virus in the disease (Itzhaki et al. 2004).

EBV infects more than 95% of human beings within first decades of life. The virus causes acute infectious mononucleosis in a minority of immune competent subjects, while the majority develops a lifelong asymptomatic infection and the virus remains latent in B-lymphocytes. EBV is involved in the development of several diseases such as Burkitt lymphoma, Hodgkin lymphoma and nasopharyngeal carcinoma (Kutok et al. 2006).

Moreover, EBV seems to be involved in the pathogenesis of various neurological diseases, such as encephalitis, neuritis, myelitis, cerebellitis, acute disseminated encephalomyelitis, or central nervous system (CNS) lymphoma in patients with the immunodeficiency virus (HIV) infection (Kleines et al. 2011). EBV has been also associated with the pathogenesis of the multiple sclerosis, but its role in the disease is still unclear (Lassmann et al. 2011). To our knowledge no data regarding the presence of EBV in the AD peripheral blood are on records. HHV-6 is a neurotropic virus and has been associated with multiple neurological diseases including seizures, encephalitis, mesial temporal lobe epilepsy and multiple sclerosis (Yao et al. 2010). HHV-6 has been

found in a higher proportion of AD brains than age-matched control (CTR) brains (Lin et al. 2002). However, these findings were not confirmed by another investigation (Hemling et al. 2003). Moreover we were also interested in the immune response of the host to infections, since with aging the immune system undergoes changes following a process called immunosenescence, leading to an increased susceptibility of older adults to develop, not only infectious disease, but also AD, osteoporosis, cancer and autoimmunity (Lang et al. 2012).

Inflammation

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.

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, prostaglandin and also some hormones (McGeer and McGeer 1998).

Epidemiological investigations also support the notion that inflammation may play a significant role in AD. In fact, head injuries in early adulthood may be associated with increased risk of AD in late life, and the routine use of non steroid anti inflammatory drugs is associated with a decreased incidence of AD (Plassman et al. 2000). Inflammation is associated with the degeneration of brain areas; in fact, senile plaques in AD brains are associated with reactive astrocytes and activated microglial cells and cytokines and acute phase proteins are over expressed 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 (McGeer and McGeer 1998, Licastro 2002). 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 (Licastro et al. 2010).

In AD brain, moreover, inflammatory response appears to be altered: levels of cytokines such as TNF, IL-1, IL-6, IL-8 IL-10 and some interferons seem to be elevated (Baumann et al. 1994).

Inflammation clearly occurs in pathologically vulnerable regions of the AD brain, and it does so with the full complexity of local peripheral inflammatory responses. 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 as α-1-antichymotrypsin (ACT) in patients with clinical AD (Licastro et al. 2000, Licastro et al. 2003b). Therefore, altered immune responses in the brain and the peripheral blood appeared to be associated with the disease. Finally, ACT plasma levels also correlated with the degree of cognitive impairment in AD patients from a case-control study (Licastro et al. 2000), 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 (Licastro et al. 2003). These findings suggested that an important, but still largely unknown, interplay between brain and peripheral immune responses existed in the diseases.

Moreover, our previous investigations also showed that SNPs in the promoter region of several genes controlling for different cytokines synthesis and release, such as IL-1α, IL-1β, IL-6, IL-10, interferon-γ (IFN-g) and TNF-α were differentially associated with the risk of AD (Licastro et al. 2007). These results may also be explained by the virus infection hypothesis, since individual differential ability to mount an effective immune

response can influence the control of the virus latency and individual susceptibility to virus re-infection both in peripheral tissues and brain.

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Multi factorial interactions in the pathogenesis

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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.

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].

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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

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.

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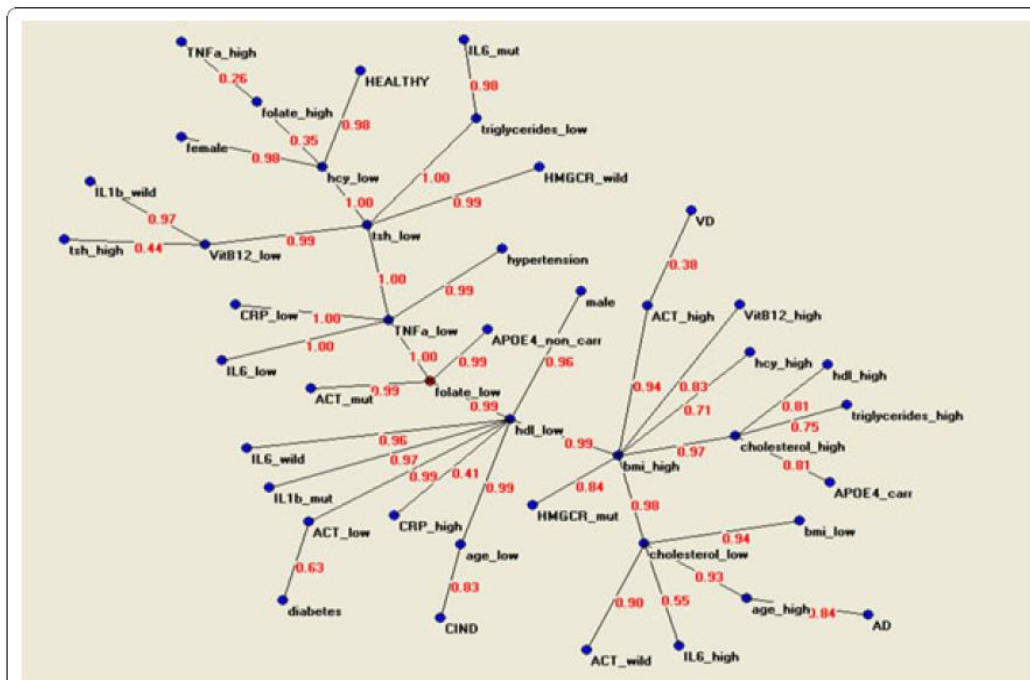
Figure 1 Connectivity map of 42 epidemiological, genetic and clinical variables showing different output such as prevalent AD, prevalent VD, prevalent CIND and control cases

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.

Abbreviations
ACT: alpha-1 antichymotrypsin; APOE: Apolipoprotein E; HMGCR: hydrossil-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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Alzheimer's disease gene signature says:

beware of brain viral infections

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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-l6) 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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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-l/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

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

Scientific production

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 motorneuron 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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Gene Signature in Alzheimer's disease

and Environmental Factors:

the Virus Chronicle

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Gene Signature in Alzheimer's Disease and Environmental Factors: The Virus Chronicle

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Abstract. Genome wide association investigations from large cohorts of patients with Alzheimer's disease (AD) and non demented controls (CTR) showed that a limited set of genes were associated ($p > 10^{-5}$) with the disease. A very recent study from our group showed that an additional limited group of SNP in selected genes were associated with AD. In this report we argue that the association of these genes with AD is suggestive of a pivotal role of environmental factors in the pathogenesis of the disease and one of these factors is virus infection. In other words, the genetic signature revealed by genome wide association (GWA) studies discloses a network of genes that might influence the ability of the central nervous system to cope with and fight against the invasion by virus of the herpes family. In fact, 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-l); APOJ or clusterin and C-type lectin domain A family-16 member (CLEC-16A); Phosphatidyl inositol- binding clathrin assembly protein gene (PICALM); ATP-bonding cassette, sub family A, member 7 (ABCA7); membrane spanning A4 (MSA4); CD2 associated protein (CD2AP); cluster of differentiation 33 (CD33); and ephrin receptor A1 (EPHA1) result in a genetic signature that might affect individual brain susceptibility to infection by the herpes virus family during aging, leading to neuronal loss, inflammation, and amyloid deposition.

Keywords: Alzheimer's disease, genetic background, GWA studies, herpes-virus

INTRODUCTION

Alzheimer's disease (AD) pathology is characterized by neuronal loss leading to brain atrophy and to 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-β (Aβ) peptide; (iii) intraneuronal accumulation of hyper-phosphorylated tau proteins leading to neurofibrillary degeneration; (iv) reactive astrogliosis; and (v) brain inflammation. The incidence of AD is rising sharply

and an increased number of elderly will ultimately be affected by the disease. Because of the urgency for effective preventive and therapeutic measures, extensive research has focused on pathogenetic mechanisms of the disease. Current views of AD pathogenetic mechanisms describe amyloid deposition and neuritic plaque formation as central mechanisms leading to neurodegeneration, cognitive impairment, and sporadic AD. Therefore, therapeutic approaches have focused on reducing amyloid load and plaque deposition or clearance of brain amyloid. However, a therapy is not already available.

Other mechanisms may be closely related with the etiology and pathogenesis of sporadic AD. This disease in fact is one of the most heritable common complex diseases with a heritability ranging 60–80%, as simplified by the association of the APOE gene with the

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disease, where the presence of 1 or 2 APOE4 alleles considerably increase the risk of AD. However, concordance rate for AD in monozygous twins is no higher than 61% and AD heritability decreases with increasing age [1]. Environmental risk factors are still largely unrevealed in AD, even if they may accumulate with advancing age and play the role of multiple triggers of the disease in the susceptible brain. Here, we discuss recently published genetic data from genome wide association (GWA) studies on several thousand AD patients and controls (CTR) [2, 3] showing that a limited number of genes were highly associated ($p > 10^{-5}$) with the disease. The effect of a single SNP or gene, with the exception of APOE, was small; therefore, the urgent challenge is to take into consideration genetic risk factors in the context of environmental risk factors or protective variables.

GWA DATA, THEIR INTERPRETATION, AND ENVIRONMENTAL FACTORS

One key question is: how can we make mechanistic sense of a collection of weak associations between SNPs and a diseases phenotype, i.e., AD? This question is perhaps the most difficult to solve and it stems from the heart of GWA studies focused upon common complex diseases. In fact, in a complex trait, as is the case in AD, several of the loci with weak effects might code for proteins that would interact in common pathways to yield a synergistic mechanism of action in AD pathogenesis. This is exactly the situation applicable to our previous GWA investigation and to other similar independent GWA studies confirming the association data in AD. In fact, in spite of the elevated numbers of patients and controls from AD GWA studies, each single SNP showed a modest OR for the diseases, usually <2.0. These findings are suggestive of the following considerations: 1) Interactions among different SNPs in diverse genes might be more informative than a single SNP. 2) None of these genes alone is causative for the diseases. 3) All described genes are however involved in different aspects of AD pathogenesis and/or clinical history. 4) Environmental factor(s) might trigger several of these genes. 5) Many of these genes upon activation by environmental factor(s) would turn on or influence other genes that would affect secondary pathogenetic mechanisms in the brain such as apoptosis, immune responses, cholesterol synthesis and transportation, and oxidative stress. Here we suggest that infective agents of CNS, such as viruses of the herpes family, are the probable link for all SNPs

found associated with AD from recent GWA studies and the view presented here supports the notion of an infective etiology of sporadic AD.

SNPs ASSOCIATED WITH AD AND VIRUS INFECTIONS

The first set of genes was located in close vicinity of the APOE locus on chromosome 19 and consisted of the poliovirus receptor-related 2 or nectin-2 (NC-2), apolipoprotein E (APOE), the translocase of outer mitochondrial membrane 40 homolog (TOMM40), the glycoprotein carcinoembryonic antigen related cell adhesion molecule-16 (CEACAM-1 6), and B-cell/lymphoma-3 (Bcl-3) genes. Genes in the second set were located in different chromosomes: APOJ or clusterin on chromosome 8; the complement receptor l (CR-l) on chromosome 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}). We already discussed in another publication the relevance of these genes along with PICALM gene association for the virus susceptibility and AD pathogenesis [4] as summarized in Table 1.

Table 1
First set of genes GWA studies resulted associated with AD risk

Gene		Chromosome	GWA studies
CEACAM16	Carcinoembryonic antigen-related cell adhesion molecule 16	19	[2, 3]
BCL3	B-cell CLL/lymphoma 3	19	[2, 3]
PVRL2	Poliovirus receptor-related 2 (herpesvirus entry mediator B)	19	[2, 3]
TOMM40	Translocase of outer mitochondrial membrane 40 homolog (yeast)	19	[2, 3]
APOE	Apolipoprotein E	19	[2, 3]
APOC1	Apolipoprotein C-1	19	[2, 3]
CLU	Clusterin	8	[2, 3]
CR1	Complement receptor 1	1	[2, 3]
CLEC 16A	C-type lectin domain family 16 member A	16	[2, 3]
PICALM	Phosphatidylinositol binding clathrin assembly protein	11	[2, 3]

Table 2
Second set of genes GWA studies resulted associated with AD risk

Gene		Chromosome	GWA studies	Functions
ABCA7	ATP-bonding cassette, sub family A, member 7	19	[5, 6]	[7–13]
MSA4	Membrane spanning A4	11	[5, 6]	[14–20]
CD2AP	CD2 associated protein	6	[5, 6]	[21–24]
CD33	Cluster of differentiation 33	19	[5, 6]	[25–30]
EPHA1	Ephrin receptor A1	7	[5, 6]	[31–34]

FURTHER GWA DATA SUPPORTING THE INFECTION HYPOTHESIS

A third new set of genes has emerged by a very recent GWA AD studies [5, 6] as summarized in Table 2. The association of these five genes with AD also appears to support the virus infection hypothesis in AD. This last group consisted of the following genes: ATP-bonding cassette, sub family A, member 7 (ABCA7), membrane spanning A4 (MSA4), CD2 associated protein (CD2AP), cluster of differentiation 33 (CD33) and ephrin receptor A1 (EPHA1), and here we suggest their potential relevance in virus infection and AD.

ABCA7 is highly expressed in the brain, especially values in hippocampal CA1 neurons [7] and microglia [8] and regulates the efflux of lipids from cells to lipoproteins. Moreover ABCA7 influences the quality of lipoprotein by interacting with APOA1 molecules especially in females, since this gene is involved in the assembly reaction of high density lipoprotein (HDL) [9] and controls heterogeneity of HDL [10]. It is known that certain viruses can circulate in biological fluids bound to lipoproteins; for instance, hepatitis C virus particles circulation is associated with plasma lipoproteins [11]. Therefore, the type of lipoproteins and lipids might influence virus transport to a given tissue and its circulation within the brain especially in women; incidence and prevalence of AD being higher in women. This gene also affects the efficiency of phagocytosis of apoptotic cells by monocyte cell lineage [12] and clearance of apoptotic virus infected cells [13]. Therefore, ABCA7 variants might also influence clearance of infected cells from the brain.

The MSA4 gene belongs to a genetic cluster located on chromosome 11 and encodes for the beta sub-unit of high affinity IgE receptor [14, 15]; this molecule is a component of an oligomeric cell surface complex involved in signal transduction in different cell lineages [16]. The MSA4 cognate protein has been involved in

antiviral responses in human plasmocytoid or lung dendritic cells [17, 18]. Furthermore, CD23 or Fc-epsilon IIIR play a role in astrocyte inflammatory response during HIV-1 encephalitis [19] and HIV-1 infection induces an impaired regulation of the IgE Fc-epsilon RI network [20]. Therefore, this gene might influence virus entrance in neuronal cells and virus infectivity might in turn affect the expression of this membrane complex.

CD2AP gene is located on chromosome 6 and codes for a member of a novel family of scaffold/adaptor proteins, expressed on several cell types and regulates the actin cytoskeleton [21]. CD2AP plays an important role in antivirus defenses, since it regulates transportation and fusion of cytoplasmic granules in NK cells [22]. Moreover this molecule also affects selective activation of survival pathways and repression of apoptosis signaling by TGF-beta [23]. CD2AP might play multiple roles by regulating defense mechanisms against virus infectivity and cell sensitivity to apoptosis induced by the virus infection. Finally, CD2AP, by affecting early endosome morphology and traffic between early and late endosomes [24], might disturb AβPP metabolism and amyloid deposition.

CD33 gene is on chromosome 19 and codes for a member of the sialic-acidic-binding immunoglobulin like lectin or SIGLEC family that promotes cell-cell interactions and regulates immune functions of both innate and adaptive immunity [25]. Human cytomegalovirus latent infection induced the upregulation of the MCP-1 molecule in a restricted subset of CD33 positive myeloid progenitor cells and this mechanism may contribute to virus dissemination [26, 27]. Human herpes virus 7 also induced an upregulation of CD33 in cultured human cells [28]. Moreover, microarray analysis of blood mononuclear cells from HIV-1 positive patients on retroviral therapy showed an overexpression of CD33 molecule [29]. In liver

Kupffer cells infected by HCV overexpressed the CD33 molecule [30]. Once again one gene might affect multiple steps involved in herpes infectivity and individual susceptibility to virus infection.

EPAH1 is a member of the ephrin receptor sub-family of tyrosine-kinases and mediates cell and axon guidance, synaptic development and plasticity [31, 32]. This molecule is also implicated in apoptosis [33] and inflammatory response regulation [34]. EPAH1 might be implicated in antivirus resistance by affecting both apoptosis impairment induced by the virus infection, the efficiency of the host immune responses and synaptic plasticity of infected neurons.

In conclusion we argue that the concomitant presence of several SNPs in these genes in the same individual might represent a genetic signature of AD and further reinforce our hypothesis that such genetic trait predisposes to AD via complex and diverse mechanisms each contributing to the differential individual brain susceptibility to viral infections.

Evidence from other investigators showing HSV-1 infection in AD brains is on record [35–37]. 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 [38]. Moreover, APOE 4 deficient mice had significantly lower virus load in CNS than APOE 4 transgenic mice [39, 40]. Other studies also showed an association of HSV-1 with AD and influence of APOE allele [41–43] 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 [44]. HSV-1 has been also related to Down's syndrome, a condition at high risk for AD type dementia [45]. It is of interest that mothers of children with Down's syndrome showed increased serum HSV-2 antibody levels [46]. Moreover, HSV-1 induces the intracellular accumulation of Aβ in autophagic compartments of neuroblastoma cells [47] and in rat cortical neurons [48]. It has also been recently suggested that AD plaques and tangles might represent a cemetery of a partially unsuccessful immune response against herpes simplex infection [49]. It is important to keep in mind that herpes simplex glycoprotein B generated peptide fragments with high homology with Aβ peptide, forming fibrils and inducing neurotoxicity [50] and HSV-1 infection induced AβPP processing resulting in Aβ peptides formation in rat neuronal cells [51]. Moreover, findings showing that intracerebral infusion of AD brain extracts induced neurodegeneration in human AβPP transgenic mice is compatible with an infective etiology of dementia [52].

FURTHER SUPPORT TO VIRUS INVOLVEMENT IN THE DISEASE, THE OLFACTORY VECTOR HYPOTHESIS OF AD

As we already discussed, the cause(s) of AD is(are) still obscure. Olfactory dysfunction in the early history of the diseases is well documented [53]. The presence of smell loss and olfactory bulb pathology in the early stages of AD together with the evidence that airborne xenobiotics, representing AD risk factors, can enter the brain via the olfactory mucosa has led to the hypothesis that the disease may be caused or activated by agents that enter the brain via the nose. Moreover, the olfactory nerve is uniquely vulnerable to virus penetration. In fact, the dendritic knobs and protruding cilia of millions of olfactory receptor cells provide an exposed surface area of about $23\,cm^2$. These cells are widely distributed throughout the rostral nasal cavity, embedded in a specialized neuroepithelium and are first order neurons projecting axons directly to the brain. It is of historical relevance to note that olfactory receptor cells were the major route of entry for poliomyelitis viruses into the brain. HSV-1 placed intranasally in mice is detected in the olfactory bulbs after several days; thereafter, it infects cholinergic neurons of several brain regions [54]. Approximately 90% of AD patients in the early stage of the disease exhibits olfactory dysfunction and longitudinal studies suggest that olfactory deficit in AD precedes cognitive impairment by several years [55]. Moreover, tau-related pathology within olfactory bulb and anterior olfactory nucleus was detected [56]. Virus may access the brain via olfactory bulb and become latent in several brain areas connected to the olfactory nucleus. Therefore, the investigation of these target areas may give crucial information regarding the relevance of virus infection, latency and transmission of virus vector to the brain cortex.

SNPs IN OTHER GENES REGULATING INFLAMMATORY RESPONSES SIGNALED BY CASE/CONTROL INVESTIGATIONS MAY ALSO PLAY A ROLE BY INFLUENCING VIRUS LATENCY AND INFECTION SUSCEPTIBILITY

Our previous work showed that alpha-1-antichymotrypsin (ACT), a protease inhibitor and acute phase protein, was elevated in plasma, cerebrospinal fluid, and brains from AD patients [57–60]. ACT plasma levels correlated with cognitive decline [57, 60] and SNP

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in the promoter gene of the ACT gene was associated with increased risk of AD, fast cognitive deterioration and elevated levels of plasma levels of the cognate protein [58]. Elevated plasma ACT has also been found in non demented elderly with decreased cognitive performances [61]. It of interest that elevated serum ACT from HIV-1 positive women has been found and its levels correlated with the viral load [62]. Moreover, ACT containing globules within hepatocytes in patients with chronic hepatitis C and cirrhosis have also been reported [63]. Therefore, data from ACT gene association with AD and increased levels of ACT blood protein with cognitive decline and the disease progression might be compatible with an infective etiology of dementia.

Our previous investigations also showed that SNPs in the promoter region of several genes controlling for different cytokines synthesis and release, such as interleukim-1α (IL-1α), IL-1β, IL-6, IL-10, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were differentially associated with the risk of AD [64]. These results may also be explained by the virus infection hypothesis, since individual differential ability to mount an effective immune response can influence the control of the virus latency and individual susceptibility to virus re-infection both in peripheral tissues and brain. Finally, we and other authors also showed an association of SNP in the promoter region of the VEGF gene with an increased AD risk [65–67]. These findings suggested that critical factors such as VEGF that are implicated in neo-angiogenesis, neurogenesis, and glia activation in adult brain, might also influence the clinical manifestation of cognitive impairment and AD. Impairment of neurovascular mechanisms leading to brain hypoperfusion, vessel regression, and neurovascular inflammation have indeed been suggested in AD, and micro-vascular pathology are frequent neuropathology features of the AD brain [68, 69]. HHV-6 during latency expresses the U94/rep latency associated gene and it has been recently shown that U94/rep protein inhibited the formation of *in vitro*-like capillary structures, the migration of endothelial cells and *in vivo* angiogenesis [70]. Finally, VEGF in mammals affects both angiogenesis and neurogenesis in the hippocampus [71]. Therefore, virus infection of brain vessels might impair angiogenesis and neurogenesis, ultimately affecting neuronal repair and survival in critical brain areas such as the hippocampus and contributing to neurodegeneration processes in individuals with low intrinsic capacity to produce angiogenic factors such as VEGF. This notion is indirectly supported by our recent pub-lication showing a partial overlapping of the genetic background between AD and a classical vascular disease such as acute myocardial infarction [72].

The above mentioned SNPs did not show up in the recent GWA studies based upon highly statistically association with the disease, and we might conclude that they are secondary linked to AD. However, it is important to note that even SNP in genes such as ABCA7, MSA4, CD2Ap, CD33, and EPAH1 individually appear to play a limited role in AD pathogenesis, since their OR values are between 1.1 and 1.4. Therefore, it is unlikely that they are causative for the disease.

However, the concomitant presence of several SNPs in many of the above discussed genes in the same individual by impairing body resistance to microorganism infection and/or favoring virus latency and re-infection in the brain over a time interval of several years might results in a genetic signature predisposing to AD.

CONCLUSIONS

Viruses of the herpes family are among the most probable pathogen candidates to CNS neurodegeneration in old age, because of their well known ability to escape peripheral immune responses by invading neurons. The relevance of herpes virus in aging is supported by a recent investigation showing that during aging a substantial proportion of peripheral CD8 T cytotoxic cells of elderly have been found to be directed against EBV and CMV [73].

Moreover, the aged immune system may be no longer able to control virus reactivation [74]. Therefore, viral infection becomes chronic in a large proportion of the elderly. Finally highly pathogenic H5N1 influenza virus has been shown to enter the brain and induce neuroinflammation and neurodegeneration, and this virus has been suggested to be involved in Parkinson disease [75]. It is important to note that up to now most of the investigations have shown an association of HSV1 with AD [35–43]. However, CMV and HSV-2 might play a role in cognitive decline during aging or dementia in Down syndrome patients [46, 76]. Moreover HHV6 was found in brain specimens of control elderly and AD patients although HHV-6 did not appear to be specifically associated with dementia [77]. Therefore, viruses may play multiple and unsuspected role in neurodegeneration of CNS and be the initial hit starting a vicious cycle leading after several years to irreversible brain decline. A flow chart representing the complex interplay among epidemiological, genetic, virus, and inflammatory factors inducing sub-clinical
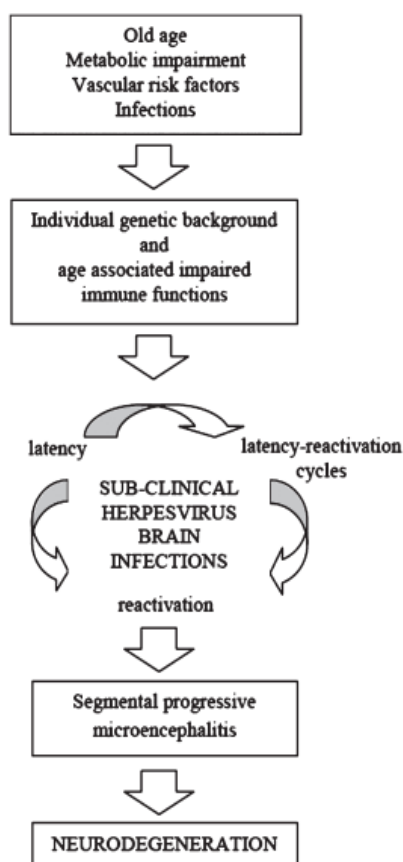
Fig. 1. Complex relationship among risk factors related to brain infections during aging leading to AD.

and chronic neuronal loss is reported in Fig. 1. With advancing age an impaired immune system might facilitate virus reactivation in the brain, especially in those subjects showing the suggested genetic signature. It is important to stress that studies on HLA polymorphisms association appear to support a viral infection involvement in AD pathogenesis [78].

Latent or chronic viral infection has been indeed found to correlate with the rate of cognitive decline in the Sacramento Area Latino Study on Aging [73]. Therefore, brain infection by reactivated latent viruses might induce progressive neuronal loss, astroglia activation, and, by impairing AβPP transport along the axons [36], AβPP misappropriate metabolism and amyloid deposition.

The concomitant presence of several SNPs in many of the above discussed genes in the same individual might results in a genetic signature predisposing to AD, since they contribute to facilitate virus entrance, and latency and impair mechanisms of defense and resistance to microorganism infection.

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Herpes viruses in Alzheimer's disease:

relations with the progression of the disease.

(Submitted)

Herpes viruses in Alzheimer's disease: relation with the progression of the disease.

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Abstract

Studies regarding different viruses of herpes family, such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) or Human herpes virus 6 (HHV-6) in Alzheimer's disease (AD) are scarce. DNA from peripheral blood leukocyte (PBL) samples and brain samples were analyzed by nested PCR for CMV, EBV or HHV-6 presence. All samples for CMV were negative. EBV positivity in PBL samples was 31% in CTR and 45% in AD patients (p=0.05). EBV positivity in AD brain was 6%; the frequency of HHV-6 positivity in PBL samples was 4.4% in CTR and 23.1% in AD (p=0.002). 17% of AD brains were HHV-6 positive. In a group of elderly, followed-up for five years, EBV or HHV-6 positivity in PBL samples was increased in those subjects who developed clinical AD. Seropositivity to CMV and EBV was also investigated and IgG for CMV antigens and specific EBV antigens were increased in those subjects who developed AD. Our findings suggest that EBV and HHV-6 are risk environmental factors for cognitive deterioration and progression to AD in the elderly.

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1. Introduction

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disorder. According to the World Health Organization it is estimated that nearly 35.6 millions of people worldwide currently suffer AD. This number is expected to double by 2030 and more than triple by 2050. Dementia affects people in all countries; with more than half patients living in low- and middle-income countries and by 2050, this figure is likely to rise to more than 70% (http://www.who.int/mediacentre/news/ releases/2012/dementia_20120411/en/). Because of the urgency for effective preventive and therapeutic measures, extensive research has focused on pathogenetic mechanisms of the disease, but up to now, no therapy has been found. In our recent publications (Porcellini et al. 2010, Licastro et al. 2011) we discussed genetic data from four recently genome wide association (GWA) studies on AD (Lambert et al. 2009, Harold et al. 2009, Hollingworth et al. 2011, Naj et al. 2011). From to these investigations a set of single-nucleotide polymorphisms (SNPs) associated with the AD emerged and we suggest that the concomitant presence of these SNPs might results in a genetic signature predisposing to AD, via complex and diverse mechanisms each contributing to an increase of individual susceptibility to herpes virus infection (Porcellini, et al. 2010). A viral etiology, involving in particular herpes virus in AD, has been already investigated and most investigations have shown an association of Herpes simplex virus type 1 (HSV-1) with AD (Wozniak et al. 2009, Itzhaki el al. 2008, Carter 2008, Burgos et al. 2006, Mori et al. 2004). It is interesting to note that others herpes virus share the ability to become latent in the infected host and eventually latently infect neurons. However, investigations focused on different viruses of the herpes family, such as human cytomegalovirus (CMV), Epstein-Barr virus (EBV) or Human herpes virus 6 (HHV-6) in AD are scarce.

CMV is ubiquitously distributed in human population and is the most frequent infection of the brain in infants with the congenital virus transmission or in immune compromised patients (Tsutsui et al. 2008). Postnatal acute CMV infection is usually asymptomatic, but once established, the virus remains latent in blood monocytes (Pawelec et al. 2009). CMV has also been associated with other chronic diseases of aging, including cardiovascular disease, cognitive decline and cancer. The specific mechanisms responsible for these associations have not been fully understood, but they are likely to have an immune and inflammatory component (Simanek et al. 2011). The seroconversion to CMV positivity may vary over the years, ranging between 0.5 to 1.5% per year. It has been recently

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suggested that CMV is responsible of the age-associated immune changes in the elderly which lead to a reduction in the number of naive T cells (Rymkiewicz et al. 2012). An increased rate of cognitive decline over a 4-year period in subjects with elevated CMV antibody levels has also been reported (Aiello et al. 2006). Previous work upon brain frontal and temporal cortex samples found that both AD patients and elderly healthy subjects were positive for CMV with not statistically significant difference (Lin et al. 2002). CMV was found in the brain of a greater proportion of patients with vascular dementia than normal elderly; these findings suggested a role for this virus in the disease (Itzhaki et al. 2004).

EBV infects more than 95% of human beings within first decades of life. The virus causes acute infectious mononucleosis in a minority of immune competent subjects, while the majority develops a lifelong asymptomatic infection and the virus remains latent in B-lymphocytes. EBV is involved in the development of several diseases such as Burkitt lymphoma, Hodgkin lymphoma and nasopharyngeal carcinoma (Kutok et al. 2006).

Moreover, EBV seems to be involved in the pathogenesis of various neurological diseases, such as encephalitis, neuritis, myelitis, cerebellits, acute disseminated encephalomyelitis, or central nervous system (CNS) lymphoma in patients with the immunodeficiency virus (HIV) infection (Kleines et al. 2011). EBV has been also associated with the pathogenesis of the multiple sclerosis, but its role in the disease is still unclear (Lassmann et al. 2011). To our knowledge no data regarding the presence of EBV in AD are on records.

HHV-6 is a neurotropic virus and has been associated with multiple neurological diseases including seizures, encephalitis, mesial temporal lobe epilepsy and multiple sclerosis (Yao et al. 2010). HHV-6 has been found in a higher proportion of AD brains than age-matched control (CTR) brains (Lin et al. 2002). However, these findings were not confirmed by another investigation (Hemling et al. 2003).

Moreover we were also interested in the immune response of the host to infections, since with aging the immune system undergoes changes following a process called immunosenescence, leading to an increased susceptibility of older adults to develop, not only infectious disease, but also Alzheimer's disease, osteoporosis, cancer and autoimmunity (Lang et al. 2012). The seropositivity to CMV, EBV or HHV-6 is very high worldwide. However, no data regarding the association of seropositivity to these viruses with AD are on record. Overall data regarding a possible association of CMV, EBV or

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HHV-6 with AD are scarce and conflicting. Therefore, we decided to investigate the presence of CMV, EBV and HHV-6 in DNA samples extracted from PBL of a large cohort of patients with clinical diagnosis of AD and aged matched CTR and DNA samples from frontal cortex of patients with neurological diagnosis of definitive AD. Samples were analyzed by nested PCR using specific primers for each virus and/or quantitative Real time PCR (qPCR).

We also investigated virus DNA presence in PBL samples and serological positivity in same patients and healthy elderly subjects. Our findings show an association of DNA positivity and serological data with the progression of the disease.

2. Materials and Methods

2.1 PBL samples. Patients with clinical diagnosis of AD and elderly CTR were enrolled from the longitudinal "Conselice study" (Ravaglia et al. 2001), as described elsewhere (Licastro et al. 2010a). Cognitive performances were measured according to MMSE at the baseline of the study (1999) and at the end of the five year follow up (2004). Clinical diagnosis of AD followed the DMS IV and NINCS-ADRDA criteria, as previously reported (Forti et al. 2001). Genomic DNA from circulating leukocytes was purified according to the previously described procedures (Licastro et al. 2010a).

2.2 Serology. Plasma samples were collected from AD and CTR enrolled in the "Conselice study". The serological assays included ELISAs for CMV IgG, EBV Epstein-Barr nuclear antigen (EBNA) IgG, EBV viral capsid antigen (VCA) IgG and HHV-6 IgG plasma levels. We tested 80 samples, according to the manufacture's recommendations, by commercially available assays:

- CMV IgG, ETI-CYTOK-G PLUS (DiaSorin, Saluggia, Italy), quantitative assay (antigens, inactivated HCMV AD 169);

- EBV EBNA IgG, ETI-EBNA-G (DiaSorin, Saluggia, Italy) quantitative assay (antigens, EBNA-1 synthetic peptides);

- EBV VCA IgG, ETI-VCA-G (DiaSorin, Saluggia, Italy) quantitative assay (antigens, mainly p18 synthetic peptide);

- HHV-6 IgG, HHV-6 IgG ELISA kit (PANBIO, Waltham, MA, USA) qualitative assay (antigens, inactivated HHV6).

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We tested two different antigens specific for EBV (EBNA and VCA), to improve EBV detection sensibility.

2.3 Brain samples. Autoptic brain samples were collected from the Brain Bank of the Department of Neurosciences and Pathology at the University of California, San Diego (UCSD). Neuropathological diagnosis of AD was performed as previously described (Hansen et al. 1993) and followed the National Institute on Aging (NIA) and the Consortium to Establish a Registry for Alzheimer's disease (CERAD) criteria. AD also met the criteria of the DMS III-R and NINCDS-ADRDA (McKahnn et al. 1984). Autopsy was performed within 8 h of death, as previously described (Corey-Bloom et al. 2000). Left hemibrain was fixed for 5–7 days and right hemibrain was frozen at -80°C. Each brain was staged for the degree of neuro pathology according to modified Braak and Braak criteria (Braak et al. 1997). Tissue samples were taken from midfrontal area of neocortex. Genomic DNA was obtained from frozen hemibrain samples and purified according to phenol-clorophorm standard extraction after overnight incubation with proteinase K.

2.4 Genotype assessment. Apolipoprotein E (APOE) genotyping for the ε alleles from leukocytes or brain DNA samples was assessed, as previously described (Licastro et al. 1999, Licastro et al. 2007).

2.5 Detection of CMV DNA. DNA from PBL and brain samples were analyzed by nested PCR DNA amplification in two PCR steps. The external (ext) PCR was carried out in a 25 μl mix containing 1μM of each primers, 200 μM of dNTP (Fermentas), reaction Buffer 1X (Euroclone), MgCl$_2$ 2,5 mM (Euroclone), 2 U of Thermus aquaticus polymerase (EuroTaq, Euroclone) and 250ng/5μl of DNA template. The specific amplification conditions and primers are shown in Table1. For the internal (int) PCR 1 μl of the previously PCR reaction was added to 24μl mix at the same condition of external (ext) PCR containing 1 μM of each internal primers (Table 1). To assess the right length of the amplicon, 15 μl of the PCR product were load on 2% agarose gel. As internal control a positive and a non template control (NTC) samples were amplified each time.

To verify results from nested PCR, 64 random samples (32 DNA from blood leukocytes and 32 from brain samples) were also tested by a commercially available assay (Quantification of Cytomegalovirus genomes Advanced kit, Primer Design, UK) using a Real Time PCR method according to the manufacture's recommendations.

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2.6. Detection of EBV DNA. a) Nested PCR: DNA samples from PBL were analyzed by nested PCR amplification in two PCR steps. For the ext PCR 250ng/5μl of DNA template was added in a 25 μl mix containing 1 μM of each ext primers, 200 μM of dNTP (Fermentas), reaction Buffer 1X (Euroclone), MgCl$_2$ 1,5 mM (Euroclone), and 2 U of Thermus aquaticus polymerase (EuroTaq, Euroclone). For the int PCR 1 μl of the previously PCR reaction was added to 24μl mix at the same condition of ext PCR containing 1 μM of each int primers. Primer sequences and PCR cycling conditions for both int and ext PCR are listed in Table1. To assess the right length of the amplicon 15 μl of the external PCR product were load on 2% agarose gel. A positive and a NTC samples were amplified as internal control each time.

b) Quantitative PCR (qPCR): brain samples were analyzed by qPCR. For the standard curve we performed a nested PCR with an EBV positive sample at the same conditions as described above. The PCR products were load on 2% agarose gel and purified with QIAquick gel extraction kit, Qiagen. Concentration of DNA was determined using a Beckman DU 460 spectrophotometer where purified PCR product was placed onto the apparatus and the OD measured (λ=260 nm). OD260 values were converted to the appropriate concentration (ng/μl). The following equation was used to calculate the copy numbers from a known PCR product concentration: weight of PCR fragment (gr/μl)/ (660 g per mol × the number of base pairs of the PCR fragment) × (6.023 × 10(23)) = the number of genomic copies/μl. (Malorny et al. 2003). 10-fold dilutions were made on this cleaned-up DNA. In each run we added virus-specific standards (10^2, 10^3, 10^4, 10^5 copies/5 μl) which were used to generate the reference curve to quantify viral DNA in individual sample.

2.7. Detection of HHV-6 DNA. Samples from peripheral leukocytes and brain specimens were analyzed by qPCR. Standard curve for HHV-6 was made as described before for EBV standard curve. For the ext PCR 250ng/5μl of DNA template was added in a 50 μl mix containing 1 μM of each ext primers, 200 μM of dNTP (Fermentas), reaction Buffer 1X (Euroclone), MgCl$_2$ 2 mM (Euroclone), and 2 U of Thermus aquaticus polymerase (EuroTaq, Euroclone). For the int PCR 10 μl of the previously PCR reaction was added to 50 μl mix at the same condition of ext PCR. Primer sequences and PCR cycling conditions are listed in Table1.

2.8. Detection of β-actin DNA. Samples analyzed for EBV and HHV-6 by qPCR were also analyzed for the reference gene β-actin. For the β-actin standard curve a PCR in a 50 μl mix containing 1μM of

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each primers, 200 μM of dNTP (Fermentas), reaction Buffer 1X (Euroclone), MgCl$_2$ 1,5 mM (Euroclone) and 2 U of Thermus aquaticus polymerase (EuroTaq, Euroclone) was performed. Primer sequences and PCR cycling conditions are listed in Table1. To assess the right length of the amplicon 15 μl of the PCR product were load on 2% agarose gel. As described previously for EBV standard curve, a standard curve of β-actin was performed.

All qPCR were performed in 96-well plates using a Bio-Rad CFX 96 platform. Reaction volume (12μl) included QuantiTect SYBR Green PCR Master Mix (6μl), forward and reverse specific primers for EBV or β-actin 20 nMol, UNG (Uracil-DNA glycosylase) 0.5U (Fermentas) and 250ng/5μl template DNA. Each sample was done in duplicate and in the same runs analyzed for EBV or HHV-6 and for β-actin. To minimize false positive results always a NTC sample was analyzed. The melting curve data were utilized in every experiment to verify that the appropriate PCR product was amplified. Linearity of all log standard curves for all assays was high, with $r^2 > 0.99$. All samples with a Cycling data (Cq) higher than 5 Cq of the last log standard are considered negative according to the MIQE Guidelines for qPCR (Bustin et al. 2009).

2.9. Statistical analysis

Statistical analysis between the mean value of different quantitative variables from CTR and AD was performed by one way ANOVA test or t test. Frequencies analysis was performed by χ^2 test and odd ratio calculation. Statistics package for social science (SPSS, version 20.0, Chicago, IL) for the analysis was use.

3. Results

Data regarding primers, PCR conditions and assays sensitivity for the detection of CMV, EBV and HHV-6 nucleic acid positivity are reported in Table 1.

DNA from PBL samples (123 CTR and 72 AD) and 100 brain samples were analyzed by nested PCR for the presence of CMV. All samples were negative for virus. To confirm negative results, 59 random samples (27 brain samples and 32 PBL samples) were also tested by a commercial kit (Quantification of Cytomegalovirus genomes Advanced kit, Primer Design, UK) and they were confirmed negative (data not shown).

In Table 2 data regarding EBV positivity in peripheral blood leukocytes samples are shown. 31% of CTR and 45% of AD patients were positive for EBV DNA (p=0.05; OR 1.843 CI 0.976-3.480). CTR

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and AD patients were then stratified according to the presence or absence of the ε4 allele of the APOE gene (Table 2). The presence of the APOE ε4 allele appeared to effect EBV positivity only in CTR, since 55% of the APOE ε4 carriers and only 26 % in APOE ε4 non carriers were EBV positive. Data regarding the frequency of HHV-6 positivity in PBL samples were shown in Table 3. The difference between CTR (4.4%) and AD (23.1%) for the presence of HHV-6 DNA was statistically significant (p=0.002; OR=6.5 CI 1.728-24.455). Moreover, the presence of the APOE ε4 allele did not significantly affect HHV-6 positivity in both CTR and AD (Table 2).

EBV and HHV-6 positivity was confirmed by the melting curve data; however, cycling data (Cq) were at the lower limit of the standard curve, therefore, viral load quantification was not possible.

The frequency of EBV and HHV-6 positivity in AD brain tissue samples were reported in Table 4. 6% of brain samples were positive for EBV DNA. However, all EBV positive brains were APOE ε4 carrier. In AD brain samples 17% of patients resulted HHV-6 positive. No difference for HHV-6 positivity in APOE ε4 carriers and non carriers subjects (16.7% vs. 18.2%) was found.

For the first time, we related data on herpes virus latent infection in PBL samples to the progression of the disease (Table 5). We divided our samples in two groups CTR-CTR and CTR-AD based on the cognitive performances at the baseline of the study and at the end of the five year follow up where CTR-CTR remain cognitively healthy, while CTR-AD represented elderly that developed clinical AD at the end of the follow up. A significative association between the positivity of both EBV and HHV-6 with the progression of the disease was found, since DNA virus positivity was increased in elderly developing AD (EBV: p= 0.020 OR=1.458 CI 1.009-2.108; HHV-6: p= 0.007 OR=1.224 CI 1.011-1.480).

Moreover, we investigate the seropositivity to CMV, EBV and HHV-6 in plasma samples from 80 patients by measuring IgG specific levels for one CMV antigen, two different EBV antigens and one HHV-6 antigen.

As shown in figure 1, IgG specific titers for CMV antigens in plasma from CTR and AD at the beginning of the five year follow up were measured (fig. 1, panel A) and no difference between two groups was found. At the end of the follow up 42 subjects remained cognitively healthy (CTR-CTR) and 24 subject suffered cognitive deterioration and developed clinical AD (CTR-AD). IgG CMV titers were significantly increased in those subjects who developed AD (p= 0.014; panel B).

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IgG specific levels for EBNA and VCA antigens for EBV from CTR and AD at the beginning of the five year follow up were measured (Fig. 2; panels A and C) and no differences between CTR and AD were found. At the end of the five year follow up both IgG levels specific for EBNA and VCA were significantly increased in those subjects who developed AD (EBNA p= 0.014; VCA p=0.05; Fig2 panels B and D).

The ELISA kit for the detection of HHV-6 specific IgG was not a quantitative assay and detected only positivity or negativity in each sample. 89% CTR plasma samples and 100% AD plasma samples were positive and the difference was not significantly different. No difference between CTR-CTR and CTR-AD groups after the five year follow up was present (data not shown).

4. Discussion

AD is a multifactorial disease and infectious agents able to escape immune responses, may play a role in the pathogenesis of neurodegenerative processes associated with the disease. Virus of the herpes virus family show features relevant to AD, since they infect a large proportion of human population, develop a latent form persisting for several years, are difficult to eliminate by immune responses especially when latency has been established and are able to infect neurons.

All peripheral blood samples analyzed for the presence of CMV were negative, while serological data showed that almost 90% of samples were positive to CMV IgG assay. It has been shown that peripheral blood polymorphonuclear leukocytes and lymphocytes are not latently infected by CMV, being monocytes the true site of virus latency (Gerna et al. 2004). Moreover, latently infected monocytes are few and virus detection required the analysis at least 1×10^6 monocytes (Gerna et al. 2004). Genomic DNA in our study was isolated from whole white blood cells, where monocytes represented less than 5% of total leukocytes. Therefore, the very low number of latently infected monocytes may explain why we were not able to find any CMV positive samples. Consistently, all AD brain specimens were also negative for CMV DNA. In order to detect CMV DNA positivity a different commercially available assay on 64 samples (32 from blood leukocytes and 32 from brain samples) was also used. This assay consisted of a real time PCR kit specifically developed for CMV detection. Once again no CMV DNA was detected. Our data did not confirm previous finding reporting that DNA extracted from frontal and temporal cortex showed positivity for CMV (36% AD and 34% CTR) (Lin et al. 2002). In our study we used only 250 ng of DNA for the nested PCR

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methods and 25 ng for the commercial kit as recommended by the manufacturer's datasheet, while Lin WR and co-worker used 1 μg of DNA. We concluded that CMV might be present at very low concentrations that are below the detection limit of molecular biology techniques currently used. No results regarding CMV positivity in the peripheral leukocytes in AD are on record.

As far as we know, no report has investigated the presence of EBV DNA in association with AD. Here we found that a high proportion of AD patients showed EBV positive blood leukocytes. The presence of APOE ε4 allele appeared to increase the EBV positivity only in CTR. For the first time we found that almost the 6% of AD brain tissues were positive to EBV and all were APOE ε4 carriers. Our findings suggest that the presence of EBV in the peripheral blood was a risk factor for AD (OR= 1.843) and the APOE ε4 allele increased EBV positivity both in the blood and in the brain.

HHV-6 is a neurotropic virus associated with different neurological diseases (Yao et al. 2010). No data regarding HHV-6 DNA positivity in peripheral blood of AD have been reported. We showed that a higher proportion of AD patients than CTR were positive for this virus. The presence of the APOE ε4 allele did not appear to increase the positivity for HHV-6 in both AD and CTR groups. A high positivity (17%) for HHV-6 was also detected from midfrontal cortex specimens of AD patients. However, in this latter situation the presence of the APOE ε4 allele did not influence the positivity for the virus. Other two reports showed the presence of HHV-6 in AD brains. The first one (Lin et al. 2002) reported a HHV-6 positivity of 72% in frontal and temporal cortex samples from AD patients. The second one found that 88% of AD mixed brain samples (hippocampus, frontal cortex, temporal cortex and anterior cingulated gyrus) was HHV-6 positive (Hemling et al. 2003). However, the number of samples from different brain regions was not specified. Both the above quoted investigations also showed that the APOE ε4 allele increased HHV-6 positivity in AD brains, but they concluded that HHV-6 was not a risk factor for AD. A decreased percentage of positivity for HHV-6 DNA in our brain samples may be ascribed to methodological differences. In fact, we used a low DNA concentration, i.e. 250 ng vs. 1 μg. Moreover, Hemling et al. used a commercial assay to purify virus DNA from human brain samples obtaining a higher efficiency.

Moreover, we described an association between the presence of EBV and HHV-6 DNA from PBL positivity with the cognitive deterioration and progression to AD. In fact, EBV and HHV-6 DNA positivity was higher in subjects who developed AD during a five year follow up.

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Herpes viral replication occurs intermittently during life and, despite this apparent quiescence, virus persistency represent a challenge for the immune system (Vogl et al. 2012). Krstic D et al. showed that chronic inflammatory conditions induce an age-associated development of an AD-like phenotype in wild type mice, suggesting that systemic infections could represent a risk factor for developing AD (Krstic et al. 2012). To asses systemic immune responses in the healthy elderly and AD patients to these viruses, CMV, EBV and HHV-6 IgG plasma levels were tested. CMV and EBV IgG plasma levels were higher in elderly subjects that developed clinical AD at the end of the five year follow up. Our findings are in accordance with a different report showing an association between elevated CMV IgG and cognitive deterioration in elderly followed up for four years (Aiello et al. 2006).

Systemic chronic inflammation has been associated with cognitive impairment and AD; for instance, increased blood levels of cytokines and acute phase protein such as alpha-1-antichimotripsin were also correlated with the age associated cognitive impairment and AD (Licastro et al. 2010b, Ravaglia et al. 2007, Porcellini et al. 2008).

In the present report we showed that both EBV and HHV-6 DNA positivity along with CMV and EBV IgG plasma levels were associated with cognitive deterioration and progression to clinical AD.

Our findings supported the notion that persistent cycles of latency and reactivation phases of these viruses may contribute to impair systemic immune response and induce altered inflammatory process that in turn may affect cognitive decline during aging. These infections agents appear to enter the brain of elderly subjects and induce brain chronic inflammation that contribute to neurodegeneration.

Further studies are needed to clarify the primary or secondary role of herpes virus infection in AD and improvement of methodology focused to investigate viral latency will help in better understanding the role of these pathogens in the AD pathogenesis.

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6. Disclosure statement. The authors declare no actual or potential conflicts of interest.

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8. Illustrations

Figure1. IgG plasma levels specific for CMV from CTR and AD at the beginning of the follow up (panel A) and at the end of five years follow up (panel B). CTR-CTR remained cognitively healthy, CTR-AD represented subject that developed AD.

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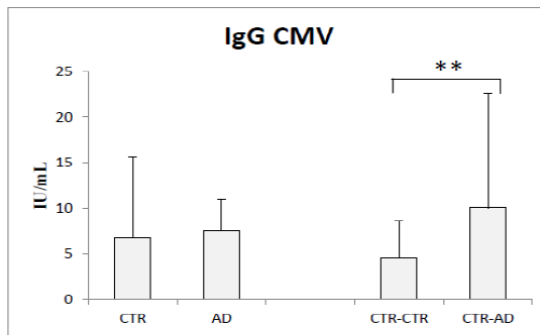
** p<0.001

Figure 2. IgG plasma levels specific for 2 different EBV antigen from CTR and AD at the beginning

of the follow up (panel A and C) and at the end of five years follow up (panel B and D). CTR-CTR

remained cognitively healthy, CTR-AD represented subject that developed AD.

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* p<0.05

** p<0.001

23
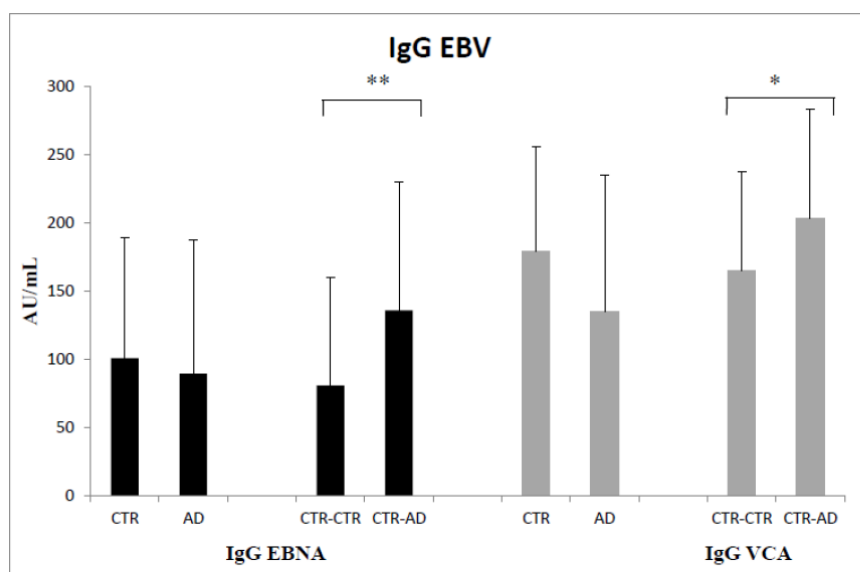
9. Tables.

Table 1. Set of herpes virus primers used in nested PCR and/or qPCR reactions.

Virus	Techniques	Primers sets (Nucleotide sequence 5'→3')	Ref.	Nested PCR	Amplicon size/ protein	PCR cycle conditions	LOD[a]
CMV	Nested PCR	AAGCGGCCTCTGATAACCAAGCC AGCACCATCCTCCTCTTCCTCTGG	(Fenner et al. 1991)	ext	435 bp/ UL122	94°C:5 min, 35 cycles of 94°C:30 sec, 57° C:30 sec,72° C:30 sec, 72° C:10 min, 4°C.	2 copies/ reaction
		AGTGTGGATGACCTACGGGCCATCG GGTGACACCAGAGAATCAGAGGAGC		int	110 bp	94°C:5 min, 25 cycles of 94° C:30 sec, 59°C:30 sec,72°C:30 sec, 72°C:10 min, 4°C.	2 copies/ reaction
EBV	Nested PCR	GAGACCGAAGTGAAGGCCCT GGTGCCTTCTTAGGAGCTGT	(Wakefield et al. 1992)	ext	173 bp EBNA 3B/3C	95°C:4 min, 35 cycles of 95° C:60 sec, 56°C: 1 min,72°C:1 min, 72°C:5 min, 4°C.	2 copies/ reaction
		GCCAGAGGTAAGTGGACTTTAAT GAGGGGACCCTGAGACGGGT		int	96 bp	95°C:2 min, 30 cycles of 95° C:1 min, 56°C: 1 min,72°C:60 sec, 72°C:5 min, 4°C.	2 copies/ reaction
	qPCR[b]	GCCAGAGGTAAGTGGACTTTAAT GAGGGGACCCTGAGACGGGT			96 bp	50°C:2 min, 95°C:15 min, 45cycles of 94°C: 2 min, 60°C:30 sec, 72°:30 sec, melt curve 65°C to 95°C, 4°C.	33 copies/ reaction
HHV-6	Nested PCR	AAGCTTGCACAATGCCAAAAAACAG CTCGAGTATGCCGAGACCCCTAATC	(Wakefield et al. 1992)	ext	223 bp	95°C:12 min, 35 cycles of 94° C:30 sec, 50°C: 30 sec,72°C:30 sec, 72°C:10 min, 4°C.	2 copies/ reaction
		TCCATTATTTTGGCCGCATTCGT TGTTAGGATATACCGATGTGCGT		int	173 bp UL95	95°C:12 min, 35 cycles of 94° C:30 sec, 50°C: 30 sec,72°C:30 sec, 72°C:10 min, 4°C.	2 copies/ reaction
	qPCR[b]	TCCATTATTTTGGCCGCATTCGT TGTTAGGATATACCGATGTGCGT			173 bp	50°C:2 min, 95°C:15 min, 45cycles of 94°C: 2 min, 60°C:30 sec, 72°:30 sec, melt curve 65°C to 95°C, 4°C.	33 copies/ reaction
β-	PCR	GTTAGAAGCCTCTTCATGGACAACG TCAGGCATGCAACACAAGTT			126 bp	95°C:4 min, 30 cycles of 95° C:1 min, 56°C: 1 min,72°C:1	

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actin				min, 72°C:5 min, 4°C.	
	qPCR[b]	GTTAGAAGCCTCTTCATGGACAACG	126 bp	50°C:2 min, 95°C:15 min, 45	33 copies/
		TCAGGCATGCAACACAAGTT		cycles of 94°C: 2 min, 60°C:30	reaction
				sec, 72°:30 sec, melt curve 65°C	
				to 95°C, 4°C.	

[a]Limit of detection

[b]Quantitative Real-Time PCR

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Table 2. Presence or absence of EBV DNA in PBL from CTR and AD patients before and after stratification according to the presence or the absence of the ε4 allele in the APOE gene.

					APOE ε4 carr				APOE ε4 non carr				
	Positive		Negative		pos		neg		pos		neg		
	N	%	N	%	N	%	N	%	N	%	N	%	
CTR	33	31.1	73	68.9	11	55	9	45	22	25.9	63	74.1	χ2=6.369 p=0.012 OR= 3.5 CI (1.280-9.569)
AD	30	45.5	36	54.5	9	52.9	8	47.1	21	42.9	28	57.1	χ2= 4.11 p=0.472

χ^2=3.595 p=0.050

OR= 1.843

CI (0.976-3.480)

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Table 3. Presence or absence of HHV-6 DNA in PBL from CTR and AD patients before and after stratification according to the presence or the absence of the ε4 allele in the APOE gene.

					HHV-6							
	\multicolumn{2}{c}{Positive}	\multicolumn{2}{c}{Negative}	\multicolumn{4}{c}{APOE ε4 carr}	\multicolumn{4}{c}{APOE ε4 non carr}	
					\multicolumn{2}{c}{pos}	\multicolumn{2}{c}{neg}	\multicolumn{2}{c}{pos}	\multicolumn{2}{c}{neg}	
	N	%	N	%	N	%	N	%	N	%	N	%	
CTR	3	4.4	65	95.6	1	6.2	15	93.8	2	3.8	50	96.2	$\chi2= 0.168$ p=0.682
AD	12	23.1	40	76.9	3	30	7	70	9	21.4	33	8.6	$\chi2= 0.334$ p=0.563

$\chi^2=9.386$ p=0.002

OR= 6.5 (CI 1.728-24.455)

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Table 4. Presence or absence of EBV or HHV-6 DNA in frontal cortex samples from AD patients before and after stratification for the APOE ε4 allele.

	EBV						HHV-6					
	Frequency		APOE ε4				Frequency		APOE ε4			
			carr		non carr				carr		non carr	
	N	%	N	%	N	%	N	%	N	%	N	%
Positive	5	5.9	5	100	0	0	14	17.3	8	16.7	6	18.2
Negative	80	94.1	47	58.8	33	41.2	67	82.7	40	83.3	27	81.8
	$\chi^2=3.371$ p=0.066						$\chi^2=0.031$ p=0.859					

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Table 5. Presence or absence of EBV or HHV-6 DNA in PBL from 150 elderly followed up for five year according to the end stage cognitive evaluation.

	EBV				HHV-6			
	+	%	-	%	+	%	-	%
CTR-CTR	33	31.1	73	68.9	3	4.4	65	95.6
CTR-AD	19	52.8	17	47.2	7	21.9	25	78.1
Tot		142				100		
	p=0.020 OR=1.458				p=0.007 OR=1.224			
	(CI 1.009-2.108)				(CI 1.011-1.480)			

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General discussion

Multi factorial interactions in the pathogenesis pathway of Alzheimer's disease:

a new risk charts for prevention of dementia.

AD is a chronic and progressive neurodegenerative disorder and it is the most common neurodegenerative disorder in the industrialized world.

Up to now, the incidence of AD is rising sharply and extensive research has focused on the pathogenetic mechanisms of the disease; however, despite the urgency for effective preventive and therapeutics measures many questions remain unanswered.

The pathophysiological process of AD begins years, if not decades, before the diagnosis of clinical dementia (Morris 2005), so it is critical to better define the biomarker and/or cognitive profile that best predicts progression from the preclinical to the clinical stages of MCI or cognitive impairment not dementia (CIND) and AD dementia.

In this innovative article, a new statistical approach was used to evaluate the relationship among genetic, clinical and classical risk factors in AD: the Auto Contractive Map algorithm (AutoCM). This method is based on an artificial adaptive system able to define the strength of the associations of each variable with all the others in any dataset. This novel data mining, the AutoCM algorithm, (Hamsten et al. 2008) was aimed to explore the concomitant association of different variables with AD and the potential relationships among variables in a multi factor network relevant for the disease. The ultimate goal of this data mining model was to discover hidden trends and new associations among variables, since this algorithm was able to create a semantic

connectivity map in which non linear association were preserved and explicit connection schemes were described.

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. A simple filter (minimum spanning tree by Kruskal) to the matrix of AutoCM system was introduced; this approach shows the map of relevant connections between and among variables and the principal hubs of the system. Hubs can be defined as variables with the maximum amount of connectivity in the map (Campisi et al 2009). The AutoCM was applied to elaborate biological and clinical data (in particular different genotypic, phenotypic, clinical, pharmacological or habit variables) to find relationship between variables and a possible hierarchy in the relevance of each variable with AD, CIND and vascular dementia (VD) (Figure 3).
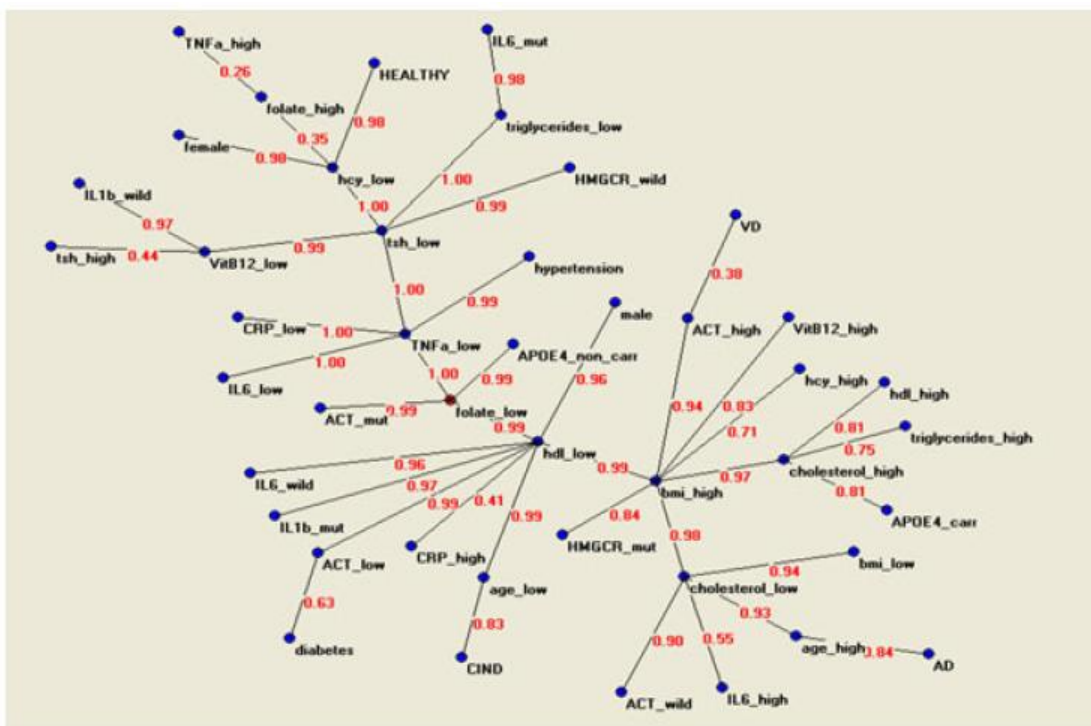
Figure 3. Connectivity map of 42 epidemiological, genetic and clinical variables showing different output such as AD, CIND, VD and control case.

For these reasons we applied this new statistical method to analyze the database generate during the Conselice Study of Brain Aging. This study is a population-based prospective investigation focused on a homogeneous elderly (65 year old or older) population from Northern Italy (Ravaglia et al. 2001).

Participants were interviewed, medically examined and cognitively evaluated in 1999 and divided in 4 groups: AD, VD, CIND subjects and controls (CTR).

CIND, as MCI, is a clinical situation that defines the transitional period between normal aging and dementia and is used to define impairments of any objective cognitive domains by neuropsychological testing in the absence of dementia.

As shown in the figure, data presented here found four major biological hubs: 1) low blood cholesterol, 2) high BMI index, 3) low blood HDL and 4) low blood folate. CIND, as expected, were associated with a lower age at onset, however age was closely correlated to prevalent AD cases confirming that age is the major risk factor in AD.

Variables as APOEε4 allele, increased Vitamin B12 and ACT levels, and 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.

This statistical analysis is innovative because, for the first time, it takes in consideration that AD is a multi-factorial disease and allowed us to look at the AD, CIND and VD with a more complex approach than the classical statistical methods.

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

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

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

Recent GWA studies conducted by many European research laboratories reported that the allele 4 of APOE gene CLU and CR1 have been strongly correlated to AD with a very high association probability ($p \sim 10^{-10}$). Moreover, in this report, also a limited number of genes were highly associated ($p \sim 10^{-5}$) with the disease (Lambert et al. 2009, Harold et al. 2009).

Genetic and environmental factors interact in a complex interplay that might be the central node to a correct interpretation of pathogenic mechanisms leading to age-related neuro-degeneration and dementia.

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 (Itzhaki RF et al. 2006).

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 (Table 4). Genes in the second set were located on different chromosomes: clusterin (CLU) on chromosome 8, complement receptor 1 (CRl) on chromosome 1, and C-type lectin domain family 16 member A (CLEC-16A) on chromosome 16.

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

Table 4. Gene cluster surrounding the APOE gene on human chromosome 19.

These genes, as reported in the introduction, maybe 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.

This hypothesis is supported also by other investigations focused on HSV infection in AD brains (Itzhaki et al. 2008; Carter 2008; Wozniak et al. 2009).

HSV1 encodes a glycoprotein (glycoprotein B, gB) that contains a sequence that is highly homologous to a segment of Aβ (Cribbs et al. 2000) and HSV1 associates with APP during axonal transport, and might thereby affect APP cellular distribution, leading to altered APP processing (Satpute-Krishnan et al. 2003). Moreover, Wozniak et al. 2009 have demonstrated a co-localization of herpes simplex virus DNA within amyloid plaques in temporal and frontal cortices (Wozniak et al. 2009).

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 (Mori et al. 2004).

Therefore, brain infection by reactivated latent viruses might be one of the primus movens inducing progressive neuronal loss, astroglia activation, and, impaired APP transport along the axons.

Moreover, viruses of the HSV family are the most probable pathogen candidates for brain reactivation in old age, since they have a well known ability to escape peripheral immune responses by invading neurons. Recently has been reported that Aβ peptide showed an anti-microbial activity and acted as a defense molecule of the innate immunity, and this observation is compatible with the hypothesis of viral association with AD etiology and pathogenesis (Soscia et al. 2010). The accumulation of Aβ and

plaque deposit may derive by an over-production of Aβ peptides directed against a viral invader of the brain.

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 limited, segmental and chronic sub-clinical pseudo-encephalitis resulting in a progressive neurodegeneration.

Gene signature in Alzheimer's disease
and environmental factors:
the virus chronicle.

The main pathological features of AD are the presence of extracellular senile plaques formed of Aβ peptide and the presence of intracellular deposition of neurofibrillary tangles as central mechanism leading to neurodegeneration and cognitive impairment. However, therapeutic approaches focused on reducing amyloid load and plaques depositions have been unsuccessful. Other mechanisms might be closely related with the etiology and pathogenesis of sporadic AD.

Two years after the publication of Lambert et al. and Harold et al. GWAs other groups published a new set of genes associated with AD risk: ABC7A, MSA4, CD2AP, CD33, EPHA1(Hollingworth et al. 2011, Naj et al. 2011). Their potential relevance in virus infection and AD has already been discussed in the introduction.

Despite of the elevated numbers of patients and controls from AD GWA studies, each single SNP showed a modest OR for the disease, usually less than 2.0. These findings are suggestive of the following considerations:

1) Interactions among different SNPs in diverse genes might be more informative than a single SNP;

2) None of these genes alone is causative for the diseases;

3) All described genes are however involved in different aspects of AD pathogenesis and/or clinical history;

4) Environmental factor(s) might trigger several of these genes;

5) Many of these genes upon activation by environmental factor(s) would turn on or influence other genes that would affect secondary pathogenetic mechanisms in the brain such as apoptosis, immune responses, cholesterol synthesis and transportation, and oxidative stress.

It might be that those other genes may also play a role in AD by influencing virus latency and infection susceptibility.

SNP in the promoter gene of the ACT gene and ACT plasma levels correlated with cognitive decline (Porcellini et al. 2008) was associated with increased risk of AD, fast cognitive deterioration and elevated levels of plasma levels of the cognate protein (Licastro et al. 2005).

Elevated serum ACT from HIV-1 positive women has been found and its levels correlated with the viral load (Friis et al. 2003).

Moreover previous investigations also showed that SNPs in the promoter region of several genes controlling for different cytokines synthesis and release, such as IL-1α, IL-1β, IL-6, IL-10, interferon-γ (IFN-γ) and TNF-α were differentially associated with the risk of AD (Licastro et al. 2007). These results may also be explained by the virus infection hypothesis, since individual differential ability to mount an effective immune response can influence the control of the virus latency and individual susceptibility to virus re-infection both in peripheral tissues and brain.

It is also important to note that, up to now, most of the investigations have shown an association of HSV1 with AD (Burgos et al. 2006, Carter 2008, Itzhaki et al. 1997, Itzhaki et al. 2008, Wozniak et al. 2009). However, CMV and HSV-2 might play a role

in cognitive decline during aging or dementia in Down syndrome patients (Anneren et al. 1986, Aiello et al. 2006).

Moreover HHV-6 was found in brain specimens of control elderly and AD patients, even if did not appear to be specifically associated with dementia (Hemling et al. 2003).

Viruses may play multiple and unsuspected role in neurodegeneration of CNS and be the initial hit starting a vicious cycle leading after several years to irreversible brain decline. A flow chart representing the complex interplay among epidemiological, genetic, virus, and inflammatory factors inducing sub-clinical and chronic neuronal loss is reported in Figure 4.
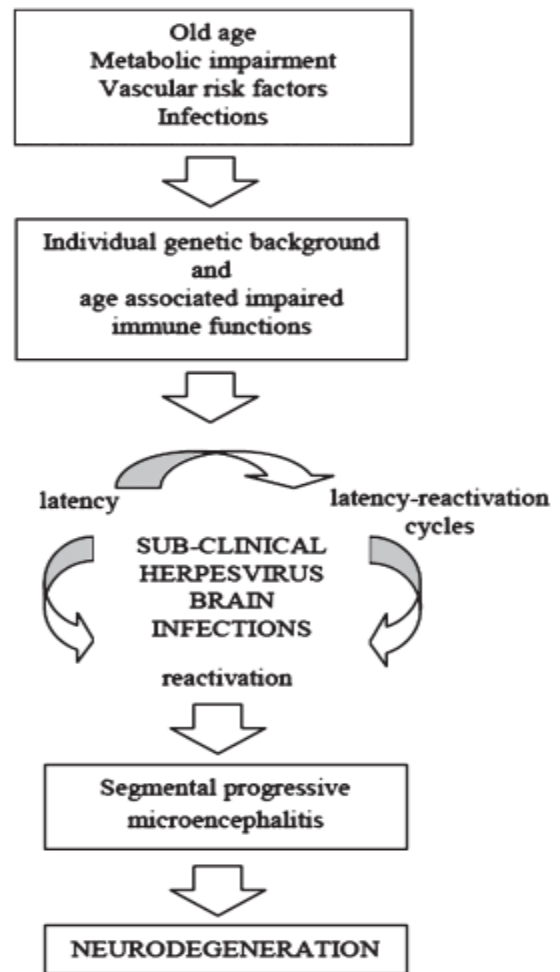

Figure 4. Schematic relationship among risk factors related to brain infections during age.

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Herpes viruses in Alzheimer's disease:
relation with the progression of the disease.

AD is a multifactorial disease where genetic factors and environmental factors interact in a complex interplay that might be the central node to a correct interpretation of pathogenic mechanisms leading to age-related neurodegeneration and dementia.

Recently published genetic data from GWA studies from several thousand AD patients and controls (CTR) (Lambert et al. 2009, Harold et al. 2009, Hollingworth et al. 2011, Naj et al. 2011) indeed showed that a limited number of genes were highly associated (p>10-5) with the disease. However, the effect of a single SNP or gene, with the exception of the APOE gene, even in those above quoted large population studies was small. As discussed elsewhere, we argue that the concomitant presence of several SNPs in these genes in the same individual might represent a genetic signature of AD and further reinforce our hypothesis that such genetic trait may predispose to AD via complex and diverse mechanisms each contributing to a differential individual susceptibility for brain viral infections (Porcellini et al. 2010, Licastro et al. 2011).

Viruses of the herpes virus family show features relevant to AD, since they infect a large proportion of human population, develop a latent form persisting for several years, are difficult to eliminate by immune responses especially when latency has been established and are able to infect neurons.

In this article, we investigated the association between AD and herpes viruses infection, in particular we focused on CMV, EBV and HHV-6 both in peripheral blood and in brain tissue samples.

Patients with clinical diagnosis of AD and elderly CTR were enrolled from the longitudinal "Conselice study" (Ravaglia et al. 2001), cognitive performances were measured according to MMSE at the baseline of the study (1999) and at the end of the five year follow up (2004). Clinical diagnosis of AD followed the DMS IV and NINCS-ADRDA criteria, as previously reported (Forti et al. 2001).

Autoptic brain samples were collected from the Brain Bank of the Department of Neurosciences and Pathology at the University of California, San Diego (UCSD). Neuropathological diagnosis of AD was performed as previously described (Hansen et al. 1993).

All samples analyzed for CMV DNA were negative, probably because these viruses don't latently infected PBL and brain tissue, being monocytes the true site of virus latency (Gerna et al. 2004). However, serological data showed that almost 90% of samples were positive to CMV IgG antigens.

Then we divided our samples in two groups CTR-CTR and CTR-AD based on the cognitive performances at the baseline of the study and at the end of the five year follow up where CTR-CTR remain cognitively healthy, while CTR-AD represented elderly that developed clinical AD at the end of the follow up. At the end of the follow up 42 subjects remained cognitively healthy (CTR-CTR) and 24 subject suffered cognitive deterioration and developed clinical AD (CTR-AD).

IgG CMV titers were significantly increased in those subjects who developed AD (fig N)

1% of CTR and 45% of AD patients were positive for EBV DNA (p=0.05; OR 1.843), and the difference between CTR (4.4%) and AD (23.1%) for the presence of HHV-6 DNA was statistically significant (p=0.002; OR=6.5).

In brain tissue the frequency of EBV and HHV-6 positivity was as follows: 4. 6% of brain samples were positive for EBV DNA (all samples were APOE ε4 carrier) and 17% positive for HHV-6.

Moreover, we described an association between the presence of EBV and HHV-6 DNA from PBL positivity with the cognitive deterioration and progression to AD. In fact, EBV and HHV-6 DNA positivity was higher in subjects who developed AD during a five year follow up.

Moreover, EBV IgG plasma levels were higher in elderly subjects that developed clinical AD at the end of the five year follow up (Fig 5)
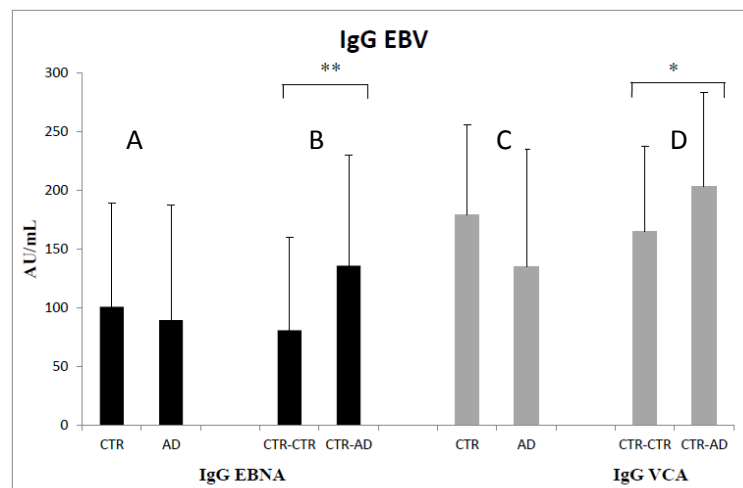

Figure 5. IgG plasma levels specific for 2 different EBV antigen from CTR and AD at the beginning of the follow up (panel A and C) and at the end of five years follow up (panel B and D). CTR-CTR remained cognitively healthy, CTR-AD represented subject that developed AD.

In the present report we showed that both EBV and HHV-6 DNA positivity along with CMV and EBV IgG plasma levels were associated with cognitive deterioration and progression to clinical AD.

Our findings supported the notion that persistent cycles of latency and reactivation phases of these viruses may contribute to impair systemic immune response and induce

altered inflammatory process that in turn may affect cognitive decline during aging. These infectious agents appear to enter the brain of elderly subjects and induce brain chronic inflammation that contributes to neurodegeneration.

Conclusions

AD is a chronic and progressive neurodegenerative disorder and one of the most frequent diseases in the industrialized world. According to the World Health Organization it is estimated that there are currently nearly 35.6 millions of people worldwide suffer AD. AD develops for an indeterminate period of time before becoming clinically apparent, and it can progress undiagnosed for years.

Late onset Alzheimer (LOAD) is the most common form of the disease and it is defined by the onset after the age 65 years. It is well established that the inheritance of the APOE ε4 allele is, after age, the single most potent and common risk factor for LOAD (Tanzi 2012).

Moreover, many other genes, involved in different pathways, have been proposed as candidates for AD susceptibility. Our previews studies confirmed the association of several SNPs on the promoter region of genes involved in the inflammatory pathway and AD (Lio et al. 2003, Licastro et al. 2000, Porcellini et al. 2007).

AD is a complex and multifactorial disease where clinical factor as inflammation, pathogens infections, environmental factors and genetics underlines the pathogenesis of the disease.

A new statistical approach, AutoCM, has been presented. This statistical analysis is innovative because it takes in consideration that AD is a multifactorial disease. The goal was to create a network of genetic, phenotypic and clinical data that allow combining different type of variables.

We tested this statistical analysis using Conselice database and we found specific four major connecting nodes that linked apparently different factors to cognitive impairment and dementia via cholesterol, cholesterol gene dependent pathway, BMI and age.

Classical statistical methods are not able to link genetic and clinical features since the heterogeneity among different variables.

The classical genetic approach of focusing on one or a few candidate genes or one or few SNPs limits our ability to identify novel factors associated with AD.

In fact, in the last few years, the most popular genetic approach is based on GWA studies, where up to one million genetic markers are simultaneously genotyped and assessed for potential correlations with disease risk and other phenotypic variables.

My studies presented in this thesis showed several genes associated with AD risk that might be involved in herpes virus infection pathways.

We just stressed the complexity of AD 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 (Miklossy 2011). Our hypothesis suggests that virus and in particular herpes virus could reactivate in the brain when an individual becomes older, perhaps because of the decline in immune responses. Brain invasion by virus triggers various mechanisms that might lead to AD. Based on GWA results published on Nature Genetic we suggest that sets of genes were strongly associated with AD. The first set of genes was located in close vicinity of the APOE locus on chromosome 19 and consisted of the poliovirus receptor-related 2 or nectin-2 (NC-2), apolipoprotein E (APOE), the translocase of outer mitochondrial membrane 40 homolog (TOMM40), the glycoprotein carcinoembryonic antigen related cell adhesion molecule-16 (CEACAM-l 6), and Bcell/lymphoma-3 (Bcl-3) genes. Genes in the second set were located in different chromosomes: APOJ or clusterin on chromosome 8; the complement receptor l (CR-l) on chromosome 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}) (Lambert et al. 2009).

Moreover, three novel AD genes, i.e., CLU (clusterin; apolipoprotein J), CR1 (complement component (3b/4b) receptor 1), and PICALM (phosphatidylinositol binding clathrin assembly protein) were published back-to-back (Harold et al. 2009). Then Hollingworth et al. and Naj et al. in 2011 published a new set of genes associated with AD risk: ABC7A (ATP-bonding cassette, sub family A, member 7), MSA4 (Membrane spanning A4), CD2AP (CD2 associated protein), CD33 (Cluster of differentiation 33), and EPHA1 (Ephrin receptor A1).

All these genes appear to be involved in the entry and/or in the replication of herpes viruses, in the cell-to cell virus spreading and also in the host immune resistance to virus infection.

Our 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 viruses of the herpes family during aging, leading to neuronal loss, inflammation and amyloid deposition.

Then, my work focused on the investigation of the presence of CMV, EBV and HHV-6 in DNA samples from peripheral blood of a large cohort of patients with clinical diagnosis of AD and aged matched CTR and DNA samples from brain tissue of patients with neuropathological diagnosis of definitive AD.

As far as we know, no report has investigated the presence of EBV DNA in association with AD. We found that a high proportion of AD patients showed EBV positive blood leukocytes. The presence of APOE ε4 allele appeared to increase the EBV positivity only in CTR. For the first time we found that almost the 6% of AD brain tissues were

positive to EBV and all subjects positive for EBV were also APOE ε4 carriers. Our findings suggest that the presence of EBV in the peripheral blood was a risk factor for AD and the APOE ε4 allele increased EBV positivity both in the blood and in the brain. HHV-6 is a neurotropic virus associated with different neurological diseases (Yao et al. 2010). No data regarding HHV-6 DNA positivity in peripheral blood of AD have been reported. We showed that a higher proportion of AD patients than CTR were positive for this virus.

Moreover, for the first time, we described an association between the presence of EBV and HHV-6 DNA from PBL positivity with the cognitive deterioration and progression to AD. In fact, EBV and HHV-6 DNA positivity was higher in subjects who developed AD during a five year follow up.

To asses systemic immune responses in the healthy elderly and AD patients to these viruses, CMV, EBV and HHV-6 IgG plasma levels were tested. CMV and EBV IgG plasma levels were higher in elderly subjects that developed clinical AD at the end of the five year follow up.

In the last article we showed, for the first time, that both EBV and HHV-6 DNA positivity along with CMV and EBV IgG plasma levels were associated with cognitive deterioration and progression to clinical AD.

Our findings support the notion that persistent cycles of latency and reactivation phases of these viruses may contribute to impair systemic immune response and induce altered inflammatory process that in turn affect cognitive decline during aging. These infections agents appear to enter the brain of elderly subjects and induce brain chronic inflammation that contribute to neurodegeneration.

This thesis presents new data regarding environmental infective agents associated with AD. This form of dementia affects an increasing number in elderly both in industrialized and developing countries and is becoming an important social and economic problem. Unfortunately no effective therapies for this disease are now available 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.

However, many questions remain un-answered; many aspects regarding viruses latency remain to be understood and more powerful methodological approach to detect latent viral forms needs to be developed.

In fact, many molecular techniques are available to quantify viral infection clearing the active virus cycle, while, up to now, no molecular approaches have been specifically developed to quantify viral load during latency. I guess that this will be the future challenge in order to better define the association of herpes viruses infection and latency with AD and to better understand viral molecular pathways involved in AD clinical history and progression

Further studies are needed to clarify the primary or secondary role of herpes virus infection in AD. Methodology improvements of methodology focused to investigate viral latency will help in better understanding the role of these pathogens in the AD.

References to discussion and conclusions

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1. Haplotype of Single Nucleotide Polymorphisms in Exon 6 of the MZF-1 Gene and Alzheimer's Disease.

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