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Genetic and epidemiological factors in cognitive impairment and dementia

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Abbreviations

5XFAD: 5X Familial Alzheimer's disease

AC: allocortex

AD: Alzheimer's disease

AD offspring: middle-aged offspring with a parental history of AD

AMPs: antimicrobial peptides

APOE: apolipoprotein E

APP: amyloid precursor protein

Aβ: amyloid‑β peptide

BBB: blood-brain barrier

BFB: basal forebrain

BIN1: bridging integrator 1

BN: brainstem nuclei

CB: cerebellum

CDHR5: cadherin-related family member 5

ChEIs: cholinesterase inhibitors

CI: confidence interval

CIND: cognitive impairment but not demented

CLU: clusterin

CMV: cytomegalovirus

CNS: central nervous system

CR1: complement component (3b/4b) receptor 1

CSF1R: colony stimulating factor 1 receptor

CTR offspring: middle-aged offspring of healthy people

CTR: controls

CTR→AD: controls who developed AD at the end of follow-up

CTR→CTR: controls who remained cognitively healthy at the end of follow-up

CTSD: cathepsin D

CX3CR1: chemokine C-X3-C motif receptor 1

DCs: plasmacytoid dendritic cells

DSM: Diagnostic and Statistical Manual of Mental Disorders

EBNA: epstein-barr nuclear antigen

EBV: epstein-barr virus

ELISAs: enzyme-linked immunosorbent assays

ENT: enthorinal cortex

EOAD: early-onset familial AD

FAD: Familial Alzheimer's Disease

FDA: Food and Drug Administration

GFAP: glial fibrillary acidic protein

GWA: genome-wide association

HBV: hepatitis B

HCV: hepatitis C

HFs: host factors

HHV-6: human herpes virus 6

HIV: human immunodeficiency virus

HSV-1: herpes simplex virus type 1

HSV-2: herpes simplex virus type 2

IFN: interferon

IFN-λR1: IFN-λ receptor chain 1

IgG: immunoglobulin G

IL-10R2: IL-10 receptor chain 2

IRF: IFN regulatory factor

ISGF3: IFN-stimulated gene factor 3

ISGs: IFN-stimulated genes

ITGAM: integrin alpha M

LC: locus coeruleus

LOAD: late-onset AD

M: molecular ladder 100 bp

MCI: Mild Cognitive Impairment

MMSE: Mini–Mental State Examination

MRI: magnetic resonance imaging

NC: neocortex

NFTs: neurofibrillary tangles

NINCDS–ADRDA: National Institute of Neurologic and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association

NMDA: N-methyl-D-aspartate

OR: odds ratio

PBLs: pheripheral blood leukocytes

PHRF1: PHD and ring finger domains 1

PICALM: phosphatidylinositol binding clathrin assembly protein

PRRs: pattern-recognition receptors

PSEN1: presenilin 1

PSEN2: presenilin 2

p-tau: hyperphosphorylated tau

qPCR: quantitative PCR

RFLP: restriction fragment length polymorphism

RT-PCR: Real Time PCR

SEM: standard error of the mean

SNPs: single nucleotide polymorphisms

SVR: sustained virological response

TBI: traumatic brain injury

TH: thalamus

TLR: Toll-like receptor

TREM2: triggering receptor expressed on myeloid cells 2

TYROBP: TYRO protein tyrosine kinase-binding protein

VCA: viral capsid antigen

WT: wild-type

Abstract

Alzheimer's disease (AD) is a multifactorial and progressive form of dementia with a senile onset that affects specific areas of the brain. This condition is characterized by a degeneration of the cerebral cortex with a progressive memory loss, cognitive function decline and personality changes. The major neuropathological lesions of AD are loss of synapses and neurons, extracellular deposits of amyloid and amyloid plaques, mainly composed by the amyloid‑β peptide (Aβ), intraneuronal accumulation of hyperphosphorylated tau protein that lead to neuro-fibrillary degeneration, reactive astrogliosis and cerebral inflammation.

Environmental risk factors, still largely unrevealed in AD, may accumulate with advancing age and play the role of disease multiple triggers in a susceptible brain inducing an activation of microglia and chronic neuro-inflammation.

Recent genome wide association (GWA) studies reported that the allele 4 of apolipoprotein E (APOE) and single nucleotide polymorphisms (SNPs) in other genes that regulate inflammatory pathways, such as the gene coding for clusterin (CLU), are associated with AD. The hypothesis is that all of these genes may be involved in different mechanisms mediated by herpes viruses and we argued that the concomitant presence of SNPs in these genes in the same individual may represent a genetic signature predisposing to AD.

The present study is focused on SNPs in CLU, interferon (IFN)-λ3/IL-28B, Med23 and the transcription factor IRF7, which are genes involved in antiviral responses and their association with AD and cognitive deterioration. Moreover, the effects of IL-28B, Med23 and IRF7 genotypes upon the presence of epstein-barr virus (EBV) and human herpes virus 6 (HHV-6) in the peripheral blood of AD and controls (CTR) have been also investigated.

Microglial cells are the innate immune system in the brain and form the first line of defense against bacterial, viral or fungal infection. Experimental, genetic and epidemiological data indicate that the activation of the innate immune system has a key role as a promoting factor for AD and in AD patients activated microglia release cytokines that induce neuro-inflammation.

In this thesis gene variants and different expression of genes involved in the innate immune response in case-control population studies and in a mouse model of AD were investigated.

Results from these experiments suggest that individuals with a particular genetic makeup in defensive mechanisms of the innate immunity may be at risk of defective immune responses. Impaired immunity against persistent viruses such as those of herpes family, might result in chronic and inappropriate activation of microglia, abnormal Aβ production and increased amyloid deposition. Cycles of virus latency and infections may therefore contribute to neurodegeneration associated with AD in genetically predisposed elderly.

Introduction

1.1 Alzheimer's disease (AD)

1.1.1 Epidemiology

Alzheimer's disease (AD) is a heterogeneous progressive neurodegenerative disorder and the most common cause of dementia.

Worldwide, nearly 46.8 million people are living with dementia in 2015 and this number is expected to double every 20 years, reaching 74.7 million in 2030 and 131.5 million in 2050. Dementia affects people in all countries, with more than half (58%) living in low- and middle-income countries and by 2050, this is likely to rise to 68% (Alzheimer's Disease International: World Alzheimer Report 2015).

The highest standardised prevalences were those in North Africa/Middle East (8.7%) and Latin America (8.4%), and the lowest in Central Europe (4.7%). The other regions occupied a fairly narrow band of prevalence, ranging between roughly 5.6% and 7.6%.

According to the revised estimates, in 2015, East Asia is the world region with the most people living with dementia (9.8 million), followed by Western Europe (7.4 million). These regions are closely followed by South Asia with 5.1 million and North America with 4.8 million. In Italy, AD patients are 1.2 million.

The incidence of dementia increases exponentially with increasing age. The regional distribution of new dementia cases is 4.9 million (49% of the total) in Asia, 2.5 million (25%) in Europe, 1.7 million (18%) in the Americas, and 0.8 million (8%) in Africa.

Moreover, women predominate amongst older people with dementia, probably because of women's greater life expectancy. However, age-specific prevalence and incidence of dementia are also higher among women, particularly at older ages. The reasons for this feature are not clearly established and more research would be justified, seeking options for prevention and treatments.

1.1.2 Symptoms, diagnosis and treatments

AD symptoms vary among individuals. However, the most common symptoms of Alzheimer's are memory loss, challenges in planning or solving problems, difficulty completing familiar tasks, confusion with time or place, problems with words in speaking or writing, changes in mood and personality, including apathy and depression. As the disease progresses, cognitive and functional abilities decline (Alzheimer's Association. 2015 Alzheimer's Disease Facts and Figures. Alzheimer's & Dementia 2015;11(3)332).

During the last stage of AD, the patient is completely dependent upon caregivers. Individual prognosis is difficult to assess and the duration of the disease varies. While most patients are diagnosed at 65 years of age and above, evidence suggests that neurodegeneration associated with AD develops for an indeterminate period of time before becoming clinically apparent, and it can progress undiagnosed for years. Aging is a major risk factor for the development of AD, but subclinical disease probably starts in younger people (Heneka et al., 2015).

No single, definitive diagnostic tests exist for AD. Although living patients can be clinically diagnosed as having possible or probable AD, a definite diagnosis requires *post-mortem* histo-pathological confirmation.

The evaluation thus depends on obtaining an individual medical and family history, thorough physical and neurologic examinations, testing the mental status, and use of diagnostic criteria for dementia and AD that have high reliability and validity.

The criteria for assessing dementia are specified in the Diagnostic and Statistical Manual of Mental Disorders (DSM), five edition and require that a patient have cognitive loss in two or more domains, such as memory, language, calculations, orientation and judgment. In addition, the loss must be of sufficient severity to cause social or occupational disability (American Psychiatric Association. Diagnostic and statistical manual of mental disorders (5th edition). Arlington, Va.: American Psychiatric Publishing; 2013). AD must also be differentiated from other causes of

dementia, such as vascular dementia, dementia with Lewy bodies, Parkinson's disease with dementia, fronto-temporal dementia and reversible dementias.

The use of neuropsychological tests and screening instruments, such as the Mini–Mental State Examination (MMSE) (Folstein et al., 1975), is recommended for screening the cognitive decline. The interpretation of scores depends on individual age and education level, but patients with cognitive losses in two or more domains typically have an MMSE below 24 (Kawas, 2003).

The National Institute of Neurologic and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA) criteria for probable Alzheimer's disease (McKhann et al., 1984), defined that cognitive loss (in two or more domains, including memory) shows an insidious onset and gradual progression.

The intermediate stage between cognitive changes associated with aging and dementia is defined as Mild Cognitive Impairment (MCI) (Geda, 2012). Individuals with MCI show cognitive impairment greater than expected for their age, but otherwise are functioning independently and do not meet the criteria for dementia. People with MCI, especially MCI involving memory problems, are more likely to develop Alzheimer's and other dementias than people without MCI. However, in some individuals, MCI reverts to normal cognition or remains stable. MCI detection is important, since it constitutes a high risk group for dementia. Ideally, prevention strategies should target individuals who are not even symptomatic.

None of the treatments available today for AD slows or stops the damage to neurons that causes Alzheimer's symptoms and eventually makes the disease fatal.

Only symptomatic therapies for AD are available. All drugs approved by the US Food and Drug Administration (FDA) for the treatment of AD modulate neurotransmitters, either acetylcholine or glutamate. The standard medical treatment for AD includes cholinesterase inhibitors (ChEIs) and a partial N-methyl-D-aspartate (NMDA) antagonist.

Secondary symptoms of AD (depression, agitation, aggression, hallucinations, delusions, sleep disorders) can be problematic. Behavioral symptoms in particular are common and can exacerbate cognitive and functional impairment. Psychotropic medications, for example, antidepressants anxiolytics, antiparkinsonian agents, beta-blockers, have been used to treat these secondary symptoms.

Many factors contribute to the difficulty of developing effective treatments for Alzheimer's. These include the high cost of drug development, the relatively long time needed to observe disease progression in Alzheimer's and the structure of the brain, which is protected by the blood-brain barrier (BBB), through which few drugs can cross. As with current pharmacologic therapies, non-pharmacologic therapies, such as music therapy and reminiscence therapy (therapy in which photos and other familiar items may be used to elicit recall) have not been shown to alter the course of AD. They are often used with the goal of maintaining or improving cognitive function and reducing behavioral symptoms (Alzheimer's Association. 2015 Alzheimer's Disease Facts and Figures. Alzheimer's & Dementia 2015;11(3)332).

1.1.3 Neuropathological hallmarks of AD

AD is a heterogeneous progressive degenerative dementia usually with a senile onset that affects specific areas of the brain.

The neuropathological hallmarks of AD are extracellular accumulation of senile plaques composed of amyloid‑β peptide (Aβ), intraneuronal accumulation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau (p-tau), synapsis loss, neuronal atrophy and cortical neurodegeneration. These pathologic markers are accompanied by neuronal degeneration, astrogliosis, microglia activation, BBB dysfunction and cognitive decline. AD pathology is also characterized by an inflammatory response, which is primarily driven by microglia and escalates with disease progression (Raz et al., 2015; Heppner et al., 2015).

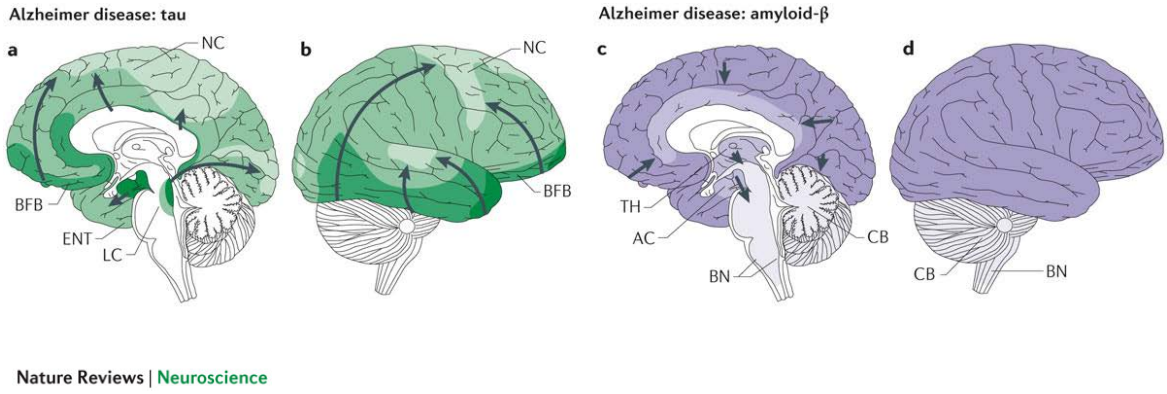
Alois Alzheimer in his original case report in 1906, described the senile plaques as the result from the abnormal extracellular accumulation and deposition of amyloid substance. Amyloid mainly consists of Aβ peptides with 40 or 42 amino acids (Aβ40 and Aβ42), two products of the metabolism of the amyloid precursor protein (APP) after its sequential cleavage by β- and γ-secretases enzymes in neurons. In particular, Aβ42 is more abundant than Aβ40 within the plaques because of its higher rate of fibrillization and insolubility (Serrano-Pozo et al., 2011). An imbalance between production, clearance and aggregation of peptides causes Aβ to accumulate and this excess may be the initiating factor in AD. This idea was called the “amyloid hypothesis” with evidence that Aβ42 was toxic to cells (Querfurth et al., 2010).

Neurofibrillary tangles, which are filamentous inclusions in pyramidal neurons, occur in AD and other neurodegenerative disorders termed tauopathies. The major component of the tangles is an abnormally hyperphosphorylated and aggregated form of tau. Normally an abundant soluble protein in axons, tau promotes assembly and stability of microtubules and vesicle transport. Hyperphosphorylated tau is insoluble, lacks affinity for microtubules and self-associates into paired helical filament structures (Querfurth et al., 2010).

In the hierarchical pattern among brain regions established for AD, tau aggregates develop in the locus coeruleus (LC), then in the transentorhinal and entorhinal regions and subsequently in the hippocampal area and in the neocortex (NC) (Figure 1, panels a and b) (Brettschneider et al., 2015).

In contrast to the dissemination of tau, patterns of Aβ plaques in AD follow essentially the opposite direction: plaques are first observed in the cortex and then detected in allocortical, diencephalic and basal ganglia structures and in the brainstem, and occasionally in the cerebellum (CB) (Figure 1, panels c and d). Why these two major disease proteins of AD show such fundamentally different patterns is incompletely understood (Brettschneider et al., 2015).

Figure 1. The hierarchical pattern among brain regions established in AD for tau aggregates (a, b) and amyloid-β (c, d). AC=allocortex; BFB=basal forebrain; BN=brainstem nuclei; ENT=entorhinal cortex; TH=thalamus (Brettschneider et al., 2015).



1.2 Genetics of AD

AD is classified into two subtypes according to the age of onset.

Early-onset familial AD (EOAD) typically develops before the age of 65 years and accounts for only a small portion (about 1-5%) of AD cases. This AD form is primarily caused by mutations in either the APP gene or genes encoding presenilin 1 (PSEN1) or presenilin 2 (PSEN2), which are essential components of the γ-secretase complexes responsible for cleavage and release of Aβ (Zou et al., 2014).

The majority of AD cases occurs late in life (>65 years) and is commonly referred to as late-onset AD (LOAD) (Liu et al., 2013). Whereas early-onset familial AD is characterized by classic Mendelian inheritance, usually in an autosomal-dominant manner, late-onset AD shows 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 (Tanzi, 2012).

The only gene variant considered to be an established late-onset AD risk factor is the ε4 allele of the apolipoprotein E gene (APOE) located on chromosome 19q13. Functionally, ApoE normally plays a role in lipid metabolism and transport. However, in AD, it is believed to play a role in the clearance of Aβ from brain (Tanzi, 2012). The human APOE gene contains several single nucleotide polymorphisms (SNPs) distributed across the gene. The most common SNPs lead to changes in the coding sequence and result in the three common isoforms of APOE: ε2 (cys112, cys158), ε3 (cys112, arg158), and ε4 (arg112, arg158). Everyone inherits one form of the APOE gene, ε2, ε3 or ε4 from each parent. Having the ε4 form increases AD risk compared with having the ε3 form, while having the ε2 form may decrease AD risk compared with the ε3 form (Corder et al., 1993; Corder et al., 1994). In fact, those who inherit one copy of the ε4 allele have a three-fold higher risk of developing AD than those without the ε4 form. Subjects inheriting two copies of the ε4 allele have 8- to 12-fold higher AD risk (Holtzman et al., 2012). Researchers estimate that between 40 and 65 percent of people diagnosed with Alzheimer's have one or two copies of the

APOE ε4 allele. ApoE binds Aβ and clears soluble Aβ preventing Aβ aggregations, while ApoE ε4 isoform is thought to be less efficient in mediating Aβ clearance. The APOE ε4 allele is neither necessary nor sufficient to cause AD and for this reason, the part of the heritability that was not yet explained has been the driving force behind decades of continue search for genetic risk factors (Van Cauwenberghe et al., 2015).

Over the past several years, the most common strategy for identify novel AD gene candidates, used the genome-wide association (GWA) approach. In a GWA study, as many as one million of SNPs are tested for genetic association with disease risk (Tanzi, 2012).

In 2009, two large case–control GWA studies (Harold et al., 2009; Lambert et al., 2009) identified, in addition to the known association with the APOE gene, loci in three genes that were potentially associated with the risk of late-onset AD: CLU (clusterin or apolipoprotein J), PICALM (phosphatidylinositol binding clathrin assembly protein) and CR1 (complement component (3b/4b) receptor 1).

Clusterin is a chaperone molecule and can bind amyloid-β peptides and prevent their fibrillization. It is also a complement inhibitor and can suppress complement activation observed in AD; CLU is also present in lipoprotein particles and regulates cholesterol and brain lipid metabolism which is disturbed in AD (Nuutinen et al., 2009). The predominant form of clusterin is a secreted glycosylated α-β-heterodimer of 75-80 kDa (de Silva et al., 1990). In humans, CLU gene maps on chromosome 8p21-p12 proximal to the lipoprotein lipase gene locus. It is expressed in all mammalian tissues and overexpression of CLU levels was observed in many pathological conditions involving injury or chronic inflammation of the brain (Calero et al., 2000). CLU expression is present in amyloid plaques and in the cerebrospinal fluid of AD cases (McGeer et al., 1992).

The second gene locus that shows evidence for association with AD is PICALM, ubiquitously expressed particularly in neurons, where it is non-selectively distributed at the pre- and post-synaptic structures. PICALM is involved in clathrin-mediated endocytosis, an essential step in the

intracellular trafficking of proteins and lipids such as nutrients, growth factors and neurotransmitters and necessary for APP processing by γ-secretase into Aβ (Harold et al., 2009).

CR1 is the receptor for complement C3b, a key inflammatory molecule that is activated as part of the brain's innate immune system in AD, and may be able to protect against Aβ-induced neurotoxicity (Tanzi, 2012).

In 2010, another GWA study suggested the existence of additional AD genetic risk factors (Seshadri et al., 2010). Among them was the gene BIN1 (bridging integrator 1), which had previously been reported to be associated with AD with subgenome-wide significance (Lambert et al., 2009). BIN1 is one of two amphiphysins, and is expressed most abundantly in the brain and muscle. Amphiphysins promote caspase-independent apoptosis and also play a critical role in neuronal membrane organization and clathrin-mediated endocytosis, which affect APP processing and Aβ production or Aβ clearance from the brain.

Others AD candidates genes emerged in subsequent GWA studies: CD2AP, MS4A6A/ MS4A4E, EPHA1, ABCA7 and CD33 (Hollingworth et al., 2011; Naj et al., 2011). Roles for these genes in AD pathogenesis can be divided into different basic categories: production, degradation and clearance of Aβ, lipid metabolism, innate immunity, and cellular signaling (Tanzi, 2012).

In particular, five of the identified AD susceptibility loci in CLU, CR1, ABCA7, CD33 and EPHA1 have putative functions in the immune system. PICALM, BIN1, CD33 and CD2AP are involved in processes at the cell membrane, including endocytosis. APOE, CLU and ABCA7 are involved in lipid processing. It is conceivable that these processes would play pivotal roles in neurodegeneration and Aβ clearance from the brain (Hollingworth et al., 2011).

In addition, a rare susceptibility variant in the triggering receptor expressed on myeloid cells 2 (TREM2) was recently identified. TREM2 encodes a transmembrane glycoprotein that forms a receptor-signaling complex with the TYRO protein tyrosine kinase-binding protein (TYROBP or DAP12) and thereby triggers the activation of immune responses in macrophages and dendritic

cells. In brain cells, TREM2 is primarily expressed on microglia, the resident histiocytes of the central nervous system (CNS). Activation of microglia may lead to phagocytosis of cell debris and amyloid, but microglia can also be activated to promote the production of proinflammatory cytokines or they may differentiate into antigen-presenting cells (Guerreiro et al., 2013; Jonsson et al., 2013).

Finally, a large, two-stage meta-analysis of GWA studies in individuals of European ancestry in addition to APOE, CR1, BIN1, CD2AP, EPHA1, CLU, MS4A6A, PICALM, ABCA7 identified 11 new Alzheimer's susceptibility loci (CASS4, CELF1, FERMT2, HLA-DRB5/HLA-DRB1, INPP5D, MEF2C, NME8, PTK2B, SLC24A4/RIN3, SORL1 and ZCWPW1) (Lambert et al., 2013). These novel loci underline the significance of specific pathways already shown to be enriched for association signal in AD GWA studies, such as immune response and inflammation (HLA-DRB5/DRB1, INPP5D and MEF2C), cell migration (PTK2B) and lipid transport and endocytosis (SORL1), and strengthen the importance of some additional previously suggested pathways including APP (SORL1 and CASS4), tau (CASS4 and FERMT2) pathology, hippocampal synaptic function (MEF2C and PTK2B), cytoskeletal function and axonal transport (CELF1, NME8 and CASS4), regulation of gene expression and post-translational modification of proteins, and microglial and myeloid cell function (INPPD5) (Reitz, 2014).

In previous works from our laboratory (Porcellini et al., 2010, Licastro et al., 2011) it was suggested that all the genes reported in GWA studies (Harold et al., 2009; Lambert et al., 2009; Hollingworth et al., 2011; Naj et al., 2011), regulating inflammation pathways, might be linked to different herpes viral infections. Moreover, we argued that the concomitant presence of these gene variations in the same individual might represent a genetic signature predisposing to AD, via complex and diverse mechanisms, each contributing to an increase of individual susceptibility to herpes virus infection. In a complex disease as AD, several of the loci with weak effects might code for proteins that could interact in common pathways. In fact, in spite of the elevated numbers of patients and controls from AD GWA studies, each single SNP showed a low odds ratio (OR)

value for the disease. Interactions among different SNPs in diverse genes might be more informative than a single SNP, considering that none of these genes alone is causative for the diseases and that all described genes are involved in different aspects of AD pathogenesis and/or clinical history. In addition, environmental factor(s) might trigger several of these genes, which could turn on or influence other genes affecting secondary pathogenetic mechanisms in the brain (such as apoptosis, immune responses, cholesterol synthesis and transportation, oxidative stress). Our hypothesis suggests that the set of genes upstream of the APOE locus or located on different chromosomes may constitute a genetic susceptibility trait for AD by affecting different mechanism involved in virus entrance or resistance to virus infection. Therefore, we suggest that infective agents of the CNS, such as viruses of the herpes family, could be the probable link for all these SNPs.

1.3 Environmental factors and AD: herpes viruses

AD is a complex multifactorial disease, whose pathogenesis may be multi-factorial and then different etiological factors may converge during aging. Environmental factors could interact with genetic risk factors via complex and diverse mechanisms leading to age-related neurodegeneration and dementia. Many non-genetic factors have been proposed as risks of AD, including metals, air pollution, pesticides, chronic psychological stress but their intracellular and/or extracellular mechanisms of inducing AD are still controversial.

Overall, investigations on environmental factors implicated in AD are scarce and the etiology of the disease remains up to now obscure.

However, there are significant associations between AD and various pathogens, including Herpesviridae, *Chlamydophila pneumoniae*, spirochetes, Helicobacter pylori and various periodontal pathogens. These pathogens are able to evade destruction by the host immune system, leading to persistent infection. Bacterial and viral infections increase the expression of pro-inflammatory molecules and activate the innate and adaptive immune systems inducing chronic inflammation in the elderly (Harris et al., 2015).

The Herpesviridae are a large family of enveloped DNA viruses that cause disease in several animal species, including humans. Herpes viruses have large double-stranded linear DNA genomes of 120-220 kb encoding 100-200 genes. They can be subdivided into α- (herpes simplex virus types 1 and 2, and varicella-zoster virus), β- (cytomegalovirus, and human herpesviruses 6 and 7) and γ-herpesviridae (epstein-barr virus and human herpesvirus 8).

The herpes virus family shows features relevant to AD, since it infects a large proportion of the human population, develops a persistent latent form impossible to eliminate by immune responses with subsequent reactivation, and is able to infect neurons.

A viral etiology, involving herpes viruses in AD, has been already proposed and most investigations have shown an association of herpes simplex virus type 1 (HSV-1) with AD (Burgos et al., 2006; Itzhaki et al., 2008; Carter, 2008; Wozniak et al., 2009).

HSV-1 is a neurotropic virus that affects more than 80% of people over 65 of age worldwide. It primarily infects epithelial cells of oral and nasal mucosa where it undergoes lytic replication; the newly produced viral particles may enter sensory neurons and, by axonal transport, reach the trigeminal ganglion where usually establish a latent infection. The virus undergoes periodic reactivation cycles in which the newly formed viral particles are transported back to the site of primary infection through the sensory neurons. However, the bipolar trigeminal ganglion neurons also project to the trigeminal nuclei located in the brainstem. From here, neurons project to the thalamus to finally reach the sensory cortex. This is the path through which the reactivated virus may reach the CNS, where it may cause acute neurological disorders like encephalitis or a mild, clinically asymptomatic, infection, or establish life-long latent infection (Piacentini et al., 2014).

A number of studies have been conducted to demonstrate the association between AD and HSV-1 infection by searching for antibodies against HSV-1 in the blood of AD patients. A reactivation of HSV-1 infection assessed by increased serum levels of specific anti-HSV-1 antibodies was found to almost double the AD risk in a longitudinal study on 3432 elderly (Lövheim et al., 2015). HSV-1 DNA and antigens were also detected in the cytoplasm of cortical neurons of patients with familial AD, suggesting a reactivation of HSV-1, and the presence of HSV-1 appeared to be coupled with Aβ42 deposition (Mori et al., 2004). These results were suggestive of reactivation of HSV-1 based on the cytoplasmic distribution of viral DNA and antigens which is compatible with the replication cycle of HSV. In fact, during the latent phase of HSV infection, virus genomes are harbored in the nucleus and not in the cytoplasm of neurons. The presence of HSV-1 may be involved in Aβ42 deposition. Additionally, glycoprotein B of HSV-1, which amino acid sequence has homology to the carboxyl-terminal region of the Aβ peptide, has been shown to promote fibril formation *in vitro*. Alternatively, Aβ deposition activates c-Jun N-terminal kinase, which may facilitate HSV

infection. Thus, a certain relationship between Aβ deposition and HSV-1 reactivation in the human brain has been suggested (Mori et al., 2004). Another study reported that elevated serum HSV-1 antibody titers correlated with decreased cortical grey matter volume as assessed by magnetic resonance imaging (MRI) (Mancuso et al., 2014). Recurrent HSV-1 infection in the brain may have a critical role in AD pathogenesis by directly activating intracellular pathways leading to typical AD molecular hallmarks (Piacentini et al., 2014). In addition, a higher frequency of HSV-1 DNA in brains from elderly than brains from young people was found (Itzhaki, 2014). It was, therefore, suggested that HSV-1 enters the brain in older age, as a consequence of the decline in the immune system with age. Subsequently it was found that the virus in the brain of the APOE ε4 allele carriers confers a strong risk for AD, accounting up to 60% of cases (Itzhaki, 2014).

The other human herpesviruses, under appropriate circumstances, are neuroinvasive and might therefore act as a potential risk factor for AD. 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 prevalent in humans with a seropositivity that ranges from 20%–100% depending on socioeconomic status and age; this virus can establish a persistent and most often asymptomatic infection in humans. CMV resides in the myeloid cell compartment, remaining latent in monocytes, but shows tropism for numerous cell types such as endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, neuronal cells, hepatocytes, trophoblasts, macrophages and dendritic cells. As other members of the Herpesviridae family, CMV may reactivate under stress conditions or other stimuli (Harris et al., 2015). The brain is the principal target organ for CMV infection in infants causing congenital infection and in immunocompromised patients (Tsutsui et al., 2008). CMV is a driver of age-associated immune changes in elderly populations which lead to a reduction in the number of naïve T cells available for fighting new infections (Simanek et al., 2011). Few studies have shown an association between CMV infection and increased risk of both cognitive impairment and development of AD. An increased rate of cognitive decline over a four year study

period in subjects with higher levels of antibody to CMV at baseline than those with lower levels has been reported (Aiello et al., 2006). A definitive direct infiltrative CNS role for CMV in AD was not indicate. Frontal and temporal brain cortex from both AD patients and elderly healthy subjects were positive for CMV with no statistically significant difference between the two groups (Lin et al., 2002a). In contrast, CMV DNA was found in brain of a greater proportion of vascular dementia patients than elderly controls, suggesting that this virus might play a role in the disease (Lin et al., 2002b). Our recent work showed that increased CMV antibody levels were present in the elderly who developed clinical AD during a five years follow-up compared to patients who remained cognitively healthy (Carbone et al., 2014). Findings from another investigation reported that baseline CMV seropositivity doubled the risk of developing clinical AD in a longitudinal follow-up of 849 participants (Barnes et al., 2015).

EBV infects 95% of humans early in life resulting in lifelong latent asymptomatic infection of B-lymphocytes. The virus causes acute infectious mononucleosis in a minority of immune competent subjects, while the majority develops a lifelong asymptomatic infection (Licastro et al., 2014). However, 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 CNS lymphoma in patients with the human immunodeficiency virus (HIV) infection and to contribute to the pathogenesis of multiple sclerosis (Kleines et al., 2011). Although data on the association of EBV with AD are limited, the virus may be a risk factor for the development of AD. Recently our findings showed an association of peripheral blood positivity for EBV genome and AD, with 45% of peripheral blood leukocytes (PBLs) positive for EBV DNA in AD patients compared to 31% of controls (Carbone et al., 2014). Moreover, serum immunoglobulin G (IgG) levels for EBV antigens were also significantly increased in a group of elderly individuals who developed AD during a five year follow-up period (Carbone et al., 2014).

HHV-6 is a neurotropic virus and exists in two forms: type A and type B. HHV-6 is widely spread in the population (seroprevalence>90%) and can establish a persistent and most often asymptomatic infection in humans. It has been implicated in multiple neurologic conditions, 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 (70%) than age-matched control brains (40%) (Lin et al., 2002a). However, these findings were not confirmed in another study (Hemling et al., 2003). In our recent work, HHV-6 showed a 23% positivity in PBLs samples from AD and 4% from controls and 17% of AD brains were HHV-6 positive (Carbone et al., 2014). In addition, at baseline HHV-6 DNA positivity in PBLs was significantly increased in those who developed clinical AD after a five year follow-up. Therefore, EBV and HHV-6 might be environmental risk factors for cognitive deterioration and progression to AD in the elderly (Carbone et al., 2014).

Recent studies have shown that while the adaptive immune system has limited access to the brain, the CNS can still mount a robust response to invading pathogens via antimicrobial peptides and the innate immune system (Soscia et al., 2010). The physiological role of Aβ is still unknown, however, Soscia and co-workers found that the Aβ peptide could be a defensive response of the innate immune system by showing that Aβ was active against at least eight common and clinically relevant microorganisms. They also observed that many of the physiochemical and biological properties of Aβ were similar to those of a group of biomolecules collectively known as "antimicrobial peptides" (AMPs), also called "host defense peptides", which are components of the innate immune system (Soscia et al., 2010). A recent report showed also that Aβ peptides displayed antiviral activities against the enveloped influenza A virus (White et al., 2014). Besides, Aβ peptide was shown to protect against *in vitro* infection by neurotropic virus such as HSV-1 (Bourgade et al., 2015). However, a sustained induction of Aβ peptide production by brain cells may become deleterious with aging due to an impaired efficiency in eliminating both viruses and Aβ peptides. Indeed, overproduction of Aβ peptide against latent herpes viruses may partially contribute to

amyloid plaque formation. Therefore, brain infections may play a pathogenic role in the progression of the sporadic form of AD (Bourgade et al., 2015).

1.3.1 Genetic variants in antiviral host defence: the IFN-λ family

Investigations regarding pathogen-host interactions have stressed the importance of host factors in the pathogenesis of infectious disease and polymorphisms in genes encoding these factors influence the host response to infection and the course of disease (Russell et al., 2014).

Interferon (IFN) family plays a pivotal role in the human anti-viral defenses. IFNs can be produced by several cell types and primarily act as antiviral cytokines, although they also exhibit cytostatic activities and help to activate and shape the adaptive immune response. In fact, IFNs provide the first line of innate immune defence against viruses and intra-cellular bacteria and are classified into families based upon sequence homology, receptor specificity and the responses they initiate (Griffiths et al., 2015).

Mammals have three IFNs classes: type I (IFN-α/β), type II (IFN-γ) and type III (IFN-λ). The direct antiviral effects of type II IFN are limited, but it has pleiotropic effects on a diverse set of immune cells promoting both adaptive and innate immune responses. Type I and III IFNs induce a strong antiviral state in responsive cells by initiating a transcriptional program that regulates the expression of hundred genes. Whereas almost all nucleated cells respond to type I IFN, responses to type III IFNs are restricted to tissues with a high risk of viral exposure and infection, such as those at mucosal surfaces. This allows type III IFNs to selectively induce a strong antiviral state in high-risk tissues with a limited inflammatory cost for the host organism (Wack et al., 2015).

IFN-λ is a recently discovered molecular group comprising several members: IFN-λ1, IFN-λ2 and IFN-λ3, also known as IL-29, IL-28A and IL-28B, respectively (Kotenko et al., 2003; Sheppard et al., 2003) and the more recently described IFN-λ4 (Bibert et al., 2013; Prokunina-Olsson et al., 2013).

The IFN-λ receptor complex is composed of the specific IFN-λ receptor chain 1 (IFN-λR1 (IL28RA)) and the shared IL-10 receptor chain 2 (IL-10R2 (IL-10Rβ)). Engagement of the IFN-λ receptor complex by any of the four ligands leads to activation of the JAK/STAT signaling system

(Kotenko et al., 2003; Sheppard et al., 2003). The phosphorylated STATs recruit IFN regulatory factor (IRF) 9, to form a trimeric transcription factor complex known as IFN-stimulated gene factor 3 (ISGF3), which enters the nucleus and drives the transcription of IFN-stimulated genes (ISGs) (Donnely et al., 2010).

IFN-λ is functionally an interferon, but it is clearly structurally related to members of the IL-10 family. In particular, it was found an interesting similarity between IFN-λ and IL-22, suggesting that IFN-λ and IL-22 possess parallel functions, protecting epithelial tissue against viral and bacterial infections, respectively (Gad et al., 2009).

Type III IFNs can be induced by a wide range of viruses in different cell types (Ank et al., 2008; Li et al., 2009) and activate a marked antiviral protection in a wide variety of cells (Doyle et al., 2006) with a cooperative action with type I IFNs (Pagliaccetti et al., 2008).

In particular, the IFN-λ family exerts anti-viral activity against a range of RNA and DNA viruses responsible for diverse infections, including hepatitis B (HBV) and C viruses (HCV) (Robek et al., 2005), herpes simplex virus types 1 and 2 (HSV-1/2) (Griffiths et al., 2013; Ank et al., 2006), CMV (Brand et al., 2005), HHV-6B (Nordström et al., 2012) and HIV (Hou et al., 2009). IFN-λ also considerably contributes to the control of viral infections of the respiratory tract (Griffiths et al., 2015).

IFN-λ expression was detected at low levels in human blood, brain, lung, ovary, pancreas, pituitary, placenta, prostate and testis (Sheppard et al., 2003). This family of IFNs is expressed predominantly in antigen-presenting cells such as macrophages and dendritic cells. Their receptor is expressed in hepatocytes more than nonhepatocytes and in epithelial cells more than nonepithelial cells (Suppiah et al., 2009). Owing to the restricted IFN-λR1 expression by immune cells, the immunomodulatory effects of type III IFNs are limited, compared to the ubiquitous activity of type I IFN during responses to infection. While the wider range of activity of type I IFN is crucial for the control of systemic viral infection, the risk of increased disease severity is always present and deleterious effects of inappropriate type I IFN responses during infection were

described in multiple reports. Therefore, type III IFN is the antiviral weapon of choice when a local mucosal response is sufficient to control the virus and when immune-mediated inflammation is a real risk (Wack et al., 2015). The induction of IFNs is mediated by pattern-recognition receptors (PRRs) that recognize the invading virus and initiate a transcriptional response through the transcription factors NF-κB, IRF3 and IRF7 (Osterlund et al., 2007).

The IFN-λ locus has been found by three independent GWA studies to be associated with the outcome of human HCV infection.

Ge et al. (2009) performed a GWA study on more than 1600 individuals chronically infected with HCV who were participating in a clinical treatment trial. A SNP 3 kb upstream of the IL-28B gene, rs12979860, was strongly associated with sustained virological response (SVR), defined as the absence of detectable virus at the end of the follow-up evaluation. The CC genotype of rs12979860 was associated with an approximately two-fold greater rate of SVR than the TT genotype, in patients of European, African American and Hispanic ancestries. Thomas et al. (2009) found that the frequency of the C allele versus the T allele was greater in the HCV clearance group in individuals of European ancestry, with frequencies of 80.3% versus 66.7%, respectively, and in individuals of African ancestry, with frequencies of 56.2% versus 37%, respectively. Patients with the CC genotype were three times more likely to clear HCV relative to patients with CT and TT genotypes. Genotyping of populations worldwide showed that the C allele was nearly fixed throughout east Asia, had an intermediate frequency in Europe, and was a minor allele in Africa. The frequencies in Central and South America were also intermediate, suggesting selective pressure since migration from Asia.

To identify genetic variants associated with HCV treatment response, in 2009 two other GWA studies were conducted, reporting an association to SVR with the SNP rs8099917, within the gene region encoding IL-28B (Suppiah et al., 2009) and with two SNPs near the gene IL-28B (rs12980275, rs8099917) (Tanaka et al., 2009).

These IFN-λ SNPs have been demonstrated to influence IFN-λ expression in humans. Langhans et al. (2011) found that serum IFN-λ levels were significantly higher in individuals with the rs12979860 CC genotype versus TT genotype.

Whilst the impact of IFN-λ and its SNPs on HCV is clear, its role in HBV infection remains controversial (Takahashi, 2014). Recent data demonstrate that IFN-λ genetic variation also contributes to the course of other infectious disease. For example, the protective rs12979860 CC genotype was associated with spontaneous HIV control (Machmach et al., 2013).

The IL-28B genotypes were explored in patients suffering recurrent orofacial herpes HSV-1 outbreaks, previously shown to be deficient in IFN-λ secretion. The minor T allele at rs12979860 was found to be associated with the severity and frequency of oral herpes labialis recurrence (Griffiths et al., 2013). In this study, Med23 was identify as an anti-viral host factor in HSV-1 infection *in vitro*. Med23 is a component of the largely pro-viral Mediator complex, which links specific transcription factors to RNA polymerase II and Med23 was found to up-regulate IFN-λ at the mRNA and protein level by interacting with IRF7 (Griffiths et al., 2013). The latter, was originally identified in the context of EBV infection, and has since emerged as the crucial regulator of type I IFNs after activation by pathogen recognition receptors (Ning et al., 2011).

1.4 Neuroinflammation as a risk factor for AD

Over the past decade, epidemiological evidence have identified several risk factors for AD, including aging, reduced physical activity, midlife obesity, systemic infection or inflammation, brain trauma and chronic peridontitis (Figure 2). Interestingly, most of these risk factors involve the activation of innate immunity and neuroinflammation, indicated as an important contributor to AD pathogenesis (Heneka et al., 2014).

In particular, a sedentary lifestyle leads to increased levels of pro-inflammatory cytokines in the blood circulation. Similarly, white adipose tissue is a constant source of pro-inflammatory cytokines that can affect the function of distant organs, including the brain. Moreover, clinical studies suggested that patients who have experienced systemic infection and sepsis show accelerated cognitive decline (Heneka et al., 2014).

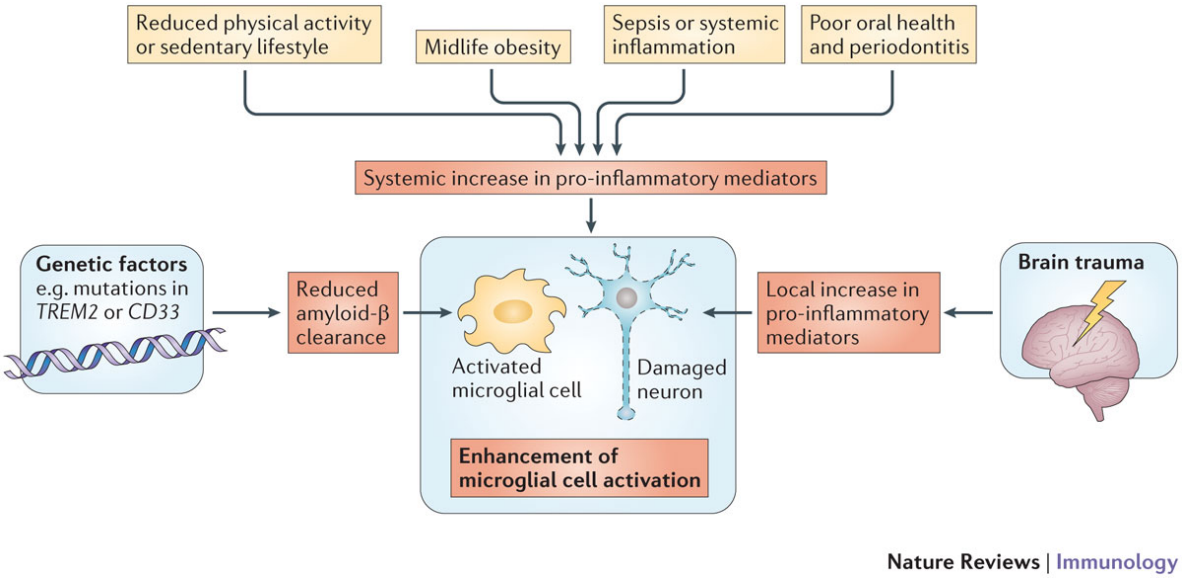
Oral microbiome and oral infections have been recently indicated as potential causes of BBB disruption and brain inflammation and these pathogens may also infect the brain via trigeminal and/or olfactory nerves (Shoemark et al., 2015). Periodontal disease is a prevalent peripheral infection induced by gram-negative anaerobic bacteria and associated with the elevation of serum inflammatory markers, including C-reactive protein (Kamer et al., 2008). Chronic inflammation in periodontitis has been suggested as a potential risk factor in AD (Sparks et al., 2012).

Traumatic Brain Injury (TBI) also increase the risk of developing AD and also leads to a local increase in the levels of neuroinflammatory mediators, which may contribute to chronic Aβ deposition and microglial activation that ultimately result in chronic neuropathology (Johnson et al., 2010; Sivanandam et al., 2012).

Genetic factors may affect the microglial cell reaction to aggregated forms of Aβ. AD-linked mutations in the microglial or myeloid genes encoding TREM2, CD33, CR1, MS4A6A and putative MS4A4E support the concept of altered microglial function in AD (Heppner et al., 2015).

Although aggregated forms of Aβ may induce the initial activation of microglial cells in the brain, the activation of these cells may be further exacerbated by systemic inflammation (Heneka et al., 2014).

Figure 2. Risk factors for AD increase innate immune activation by inducing local or systemic inflammation (Heneka et al., 2014).



Persistent infections lead to a low grade chronic inflammation which produces exacerbated activation of the microglia and dysregulation of the pro-inflammatory response. This may be especially relevant in the elderly, who show higher prevalence of systemic infections and senescent immune system prone to develop a pro-inflammatory over anti-inflammatory cell phenotype.

We now know that systemic inflammatory/immune responses transmit to the brain. Communication between peripheral immune responses and the brain can follow three different pathways: first, peripherally derived signals and even pathogen-associated molecular patterns can access the nervous system through brain sites that lack a proper BBB, or through fenestrated

capillaries; secondly, on-going peripheral reactions can be sensed and transmitted to the brain via neural afferent pathways, mainly through the vagus nerve; lastly, the BBB itself, through the role of its numerous cellular components like endothelial cells and perivascular macrophages can sense circulating signals and respond to them, affecting behavior of neurons, astrocytes, and especially resident microglia population (Solito et al., 2012).

Microglia are the innate immune cells of the central nervous system (Mosher et al., 2014). They constitute the first line of defence against invading pathogens or other types of brain tissue injury (Solito et al., 2012). In the healthy young CNS microglia have a typical ramified morphology and are distributed throughout the neural parenchyma in a "space-filling" manner, providing efficient spatial coverage of the entire CNS milieu (Wong, 2013). Microglia are equipped to sense the so called danger signals, such as AD protein aggregates, and to respond to changes in neuronal health by adopting a set of morphological and functional attributes; such cells are termed "reactive" or "primed" (Heppner et al., 2015).

With aging, the number and density of microglia appear to increase significantly in various CNS compartments, the order and regularity of their mosaic distribution appear to deteriorate and microglia cells undergo changes in their ramified morphology and decline in their dynamic motility (Wong, 2013). Besides, microglia show increased basal states of activation and increased expression of inflammatory cytokines (such as IL1β, TNF-α, IL6). It has been suggested that microglia may age, in part, as a result of cumulative activation in response to systemic infections over lifetime. Thus, systemic infections and inflammation could also drive and exacerbate neurodegeneration (Wong, 2013).

Microglia have been extensively studied in the AD field due to their dramatic responses to the pathophysiology of the disease. For instance, microglia in AD acquire what is typically considered a "reactive" morphology, with short, thick and poorly ramified processes (Mosher et al., 2014). Aβ deposits attract and activate microglia (Mosher et al., 2014).

On one hand, the ability of microglia to recognize and uptake Aβ is well established and is strongly supported by the presence of multiple receptors that bind Aβ such as Toll-like receptors (TLR) 2 and 4 (Solito et al., 2012). In addition, microglia also release Aβ-degrading enzymes and express scavenger receptors, which can mediate Aβ phagocytosis. Microglia are also able to secrete growth factors and anti-inflammatory cytokines, which are neuroprotective.

On the other hand, an inefficient clearance of Aβ by compromised microglial phagocytosis capacity might be responsible for reduced clearance of Aβ plaques *in vivo*. Impaired phagocytic activity of microglia correlated with Aβ plaque burden, indicating that Aβ plaque deposition and microglial function are closely related (Meyer-Luehmann et al., 2015). Besides, microglia can generate reactive oxygen species and secrete pro-inflammatory cytokines and additional neurotoxic factors, which contribute to the pathology of AD (Krauthausen et al., 2015).

Therefore, there is compelling evidence that microglial cells can modulate the pathological course of AD, although the exact role of microglia in AD remains to be clarified. Thus, it is critical to understand the state of activation of microglia in different AD stages to be able to determine the effect of potential anti-inflammatory therapies.

Like microglial cells, astrocytes, a CNS-resident cells of neuroectodermal origin, can respond to pathological stimuli through reactive gliosis and surround Aβ plaques. Studies using transgenic mice exhibiting cerebral amyloidosis have shown that their activation occurs early in the course of neurodegeneration and reactive astrocytes upregulate their expression of glial fibrillary acidic protein (GFAP) (Heppner et al., 2015).

Lastly, other cell types in the brain, such as neurons and endothelial cells, are also equipped with and can be activated through innate immune receptors. Therefore, these cells can also contribute to inflammatory responses in the brain.

Materials and Methods

2.1 SNPs in the CLU gene

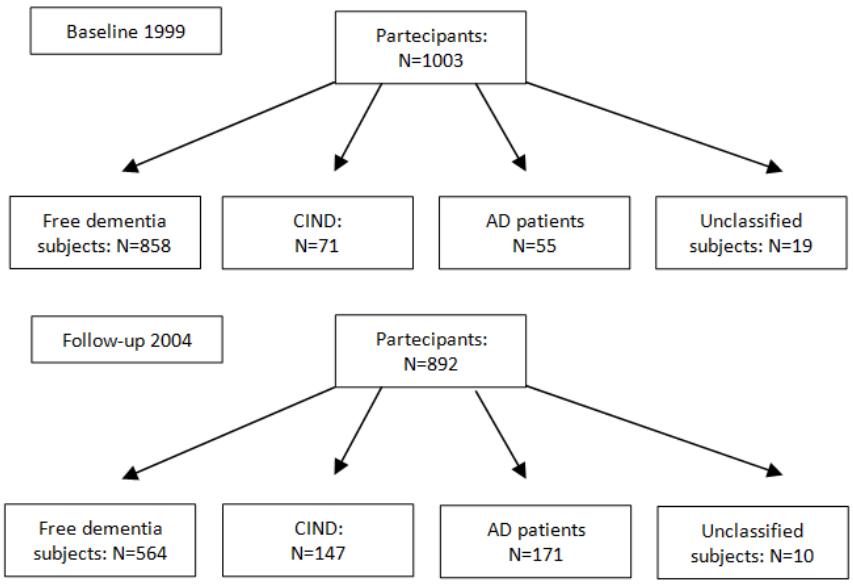
2.1.1 Patients and controls from Conselice study

Two groups of 106 AD patients and 431 controls (CTR) were included in this study.

Patients (33 male and 73 female, 77.9 ± 6.5 years of age at the beginning of the follow-up; 82.9 ± 6.5 at the end of the follow-up) with clinical diagnosis of probable AD and elderly CTR (210 male and 221 female, 72.5 ± 5.9 years of age at the beginning of the follow-up; 77.5 ± 5.9 at the end of the follow-up) were enrolled from the longitudinal "Conselice study on Brain Aging" (Ravaglia et al., 2001; Licastro et al., 2010).

A flow chart describing the enrolment of participants and their follow-up after five years has been summarized in Figure 3.

Figure 3. Flow chart of the "Conselice study on Brain Aging". Patients and controls were selected randomly from followed up subjects (CIND=cognitive impairment but not demented).



Controls and patients were randomly selected from this study.

Cognitive performance was measured according to the Mini-Mental State Examination (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 criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSMIV), the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (now called the Alzheimer's Association) (NINCS-ADRDA) (Forti et al., 2001).

2.1.2 Offspring with and without a parental history of AD

Offspring from patients with late-onset AD and offspring without such a parental history were recruited between 2006 and 2007 in a family study to investigate midlife factors that are associated with an increased risk of late-life AD. Ninety-two consecutive patients aged 70 years and older (mean age 82 years) with a diagnosis of probable AD according to National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria were recruited from the memory clinic of the Alzheimer Center of the Vrije Universiteit Medical Center of Amsterdam and affiliated nursing homes. Subjects with mixed-type dementia or vascular dementia were excluded. Ninety-seven married couples, aged 70 years and older (mean age 82.6 years), who were free from dementia, were also recruited. At least 1 spouse participated in either the Longitudinal Aging Study Amsterdam or the Leiden 85-Plus Study, 2 Dutch prospective population-based studies. Subjects were classified as free from dementia when having a Mini-Mental State Examination score greater than 27 points. When one of the spouses was deceased (n=55), a history of cognitive function from the surviving spouse was obtained. Children from the patients with AD (n=203) and the married couples without AD (n=197) were invited to participate in the study. All measurements were confined to the offspring of patients with AD and offspring of couples with good cognitive function, hereafter described as

“offspring with or without a parental history of AD”. The Medical Ethical Committee for Mental Health Care of the Netherlands approved the study and consent for participation in the study was given by all married couples or the legal guardian of eligible patients with AD (Van Vliet et al., 2009).

2.1.3 Genomic DNA samples

1.5 ml of blood diluted in a Falcon with ¾ volumes of Phosphate Buffer Saline 1X were obtained from the patients and were centrifuged for 10 minutes at a temperature of 4 °C at 3000 rpm. 900 µl of saline solution Nonidet P40/NaCl (Nonidet P40 0.1% e NaCl 0.9%) were added to the pellet and was centrifuged again for 10 minutes at 4 ºC at 3000 rpm. 2.5 ml of lyses buffer (Urea 7 M; NaCl 0.3 M; EDTA 10 mM; Tris-HCl 10 mM a pH 7.5) and then 500 µl of 10% SDS were added to the pellet and incubated for the lysis in thermostat bath at 37 ºC for 10 minutes. 4 ml of phenol / chloroform / isoamyl alcohol 25:24:1 were added and centrifuged for 10 minutes at 4 ºC to 3000 rpm. The supernatant was collected and measured. 90 µl Sodium acetate was added to the supernatant to obtain a final concentration of 0.2 M. Then 2-3 volumes of 95% ethanol were added to the solution; shaking slightly, a suspension of filamentous DNA (jellyfish) was observed. The suspension was centrifuged at 4 ºC at 3500 rpm for 10 minutes. To collect the DNA, the pellet coated into the tube was resuspended in 1 ml of 70% ethanol. The pellet collected was then centrifuged at 4 ºC at 10000 rpm for 10 minutes. After removing the ethanol, the pellet was dried and finally resuspended in water by controlling the viscosity to add an appropriate quantity of water. The concentration of DNA was read in a spectrophotometer at 260 nm. The samples obtained were maintained at -20 ºC.

2.1.4 SNPs detection

TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA) was used to genotype three intronic CLU SNPs (rs2279590, rs11136000, rs9331888) and one promoter CLU SNPs (rs9314349), according to the manufacturer's instructions. It included an unlabelled PCR primer pair to detect specific SNP targets and two different TaqMan probes that distinguished two alleles of the SNP: one probe labeled with VIC® dye and the other one labeled with FAM® dye. Allelic discrimination was based on the generated signal from each probe at the end of the Real Time PCR (RT-PCR) using a CFX96 BioRad Real Time cycler.

2.1.5 Statistical analysis

Genotype and allele frequency analysis was performed by χ^2 test and odds ratio calculation by using the Statistical Package for the Social Sciences (version 20.0; SPSS Inc, Chicago, IL).

2.2 Variants in antiviral genes as risk factors for cognitive decline and dementia

2.2.1 Patients and controls

Two groups of 158 AD patients and 228 CTR were included in this study.

Patients (47 male and 111 female, 77.7 ± 6.2 years of age at the beginning of the follow-up; 82.7 ± 6.2 at the end of the follow-up) with clinical diagnosis of probable AD and elderly CTR (113 male and 115 female, 72.9 ± 6.9 years of age at the beginning of the follow-up; 77.9 ± 6.9 at the end of the follow-up) were enrolled from the longitudinal "Conselice study on Brain Aging" (Ravaglia et al., 2001; Licastro et al., 2010).

Controls and patients were randomly selected from this study.

Cognitive performance was measured according to the Mini-Mental State Examination (MMSE) at the baseline of the study (1999) and at the end of the five-year follow-up (2004), as previously reported (Licastro et al., 2010).

2.2.2 Genomic DNA samples

Genomic DNA from PBLs was purified as previously described.

2.2.3 SNPs detection

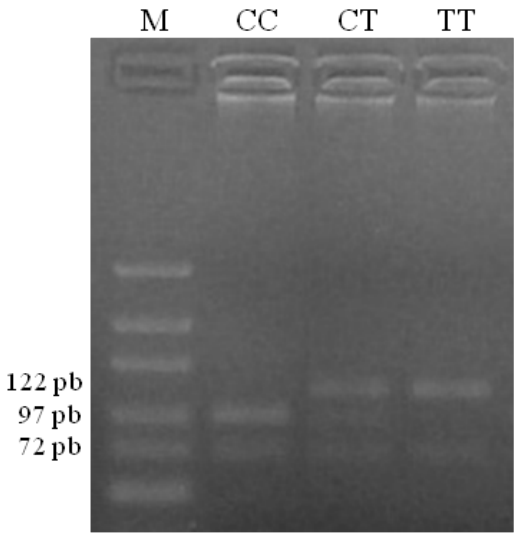
APOE genotyping for the ε alleles from DNA samples was assessed as previously described (Licastro et al., 1999; Licastro et al., 2007).

The rs12979860 SNP (substitution C/T) located ~ 3 kb upstream of IL-28B was detected by restriction fragment length polymorphism (RFLP).

Patients and CTR were genotyped by PCR DNA amplification using the following primer pairs: 5′TCAATCACAGAAGGGAGCCC3′/5′TAACCTCTGCACAGTCTGGG3′ with 5 min at 98 °C for the initial denaturation and 30 cycles of 30 s at 98 °C, 30 s at 58 °C and 45 s at 72 °C. After 10 min incubation at 72 °C the final extension was performed. The restriction enzyme *BstUI* (MBI Fermentas, Italy; 10 U/sample) resolved three different bands identifying three different genotypes on 3.5% agarose gel.

In particular, the specific primer pair amplified a 194 bp DNA fragment and then digestion of this product with the restriction enzyme *BstUI* produced 2 fragments of 122 bp and 72 bp for TT genotype; 4 fragments of 122 bp, 97 bp, 72 bp and 25 bp for CT genotype; 3 fragments of 97 bp, 72 bp and 25 bp for CC genotype (Figure 4).

Figure 4. Pattern of bands for the promoter polymorphism of the IL-28B (rs12979860) after digestion with the enzyme *BstUI* (M=molecular ladder 100 bp).



The 2 kb upstream SNP of Med23 (rs3756784 T/G) and an upstream gene variant of IRF7 (rs6598008 A/G) were analyzed by Real Time PCR using TaqMan® probes according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Hardy-Weinberg *equilibrium* was verified for all genotypes.

2.2.4 Anti-EBV and HHV-6 IgG plasma levels

Plasma samples were collected from AD and CTR enrolled in the Conselice study. Assays included enzyme-linked immunosorbent assays (ELISAs) for EBV Epstein-Barr nuclear antigen (EBNA) IgG, EBV viral capsid antigen (VCA) IgG and HHV-6 IgG. A total of 75 plasma samples were assessed, according to the manufacture's recommendations, using commercially available assays, as described by Carbone and co-workers (Carbone et al., 2014).

2.2.5 Detection of EBV and HHV-6 DNA

EBV DNA positivity from PBL was analyzed by nested PCR amplification in 2 PCR steps and the detection of HHV-6 DNA was performed by quantitative PCR (qPCR), as described by Carbone and co-workers (Carbone et al., 2014).

2.2.6 Statistical analysis

Statistical analysis for quantitative variables from AD and CTR was performed by one-way ANOVA or *t* test and frequency analysis was performed by χ^2 test and odds ratio calculation by using the Statistical Package for the Social Sciences (version 20.0; SPSS Inc, Chicago, IL).

2.3 Gene expression in a model of AD pathology

2.3.1 Mice

5X Familial Alzheimer's disease (5XFAD) mice strain Tg6799 (Oakley H. et al., 2006) were used in Marco Prinz laboratory, University Medical Center Freiburg. The 5XFAD transgenic mice overexpress both mutant human APP (695) with the Swedish (K670N and M671L), Florida (I716V) and London (V717I) Familial Alzheimer's Disease (FAD) mutations and human PS1 harboring two FAD mutations, M146L and L286V. Expression of both transgenes is regulated by neural-specific elements of the mouse Thy1 promoter to drive overexpression in the brain.

Animals were bred for heterozygosity. FAD-negative littermates were used as wild type controls. Only female mice have been used in this study. Mice were bred under pathogen-free conditions in a temperature and humidity controlled vivarium and subjected to a standard 12 h light/dark cycle with food and water were available *ad libitum*. All animal experiments were performed in accordance with the guidelines of the Regierungspräsidium Freiburg legislation for animal experiments.

2.3.2 qRT-PCR

Tissues were dissected from brain and snap frozen in liquid nitrogen. RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Samples were treated with RNase-Free DNase Set (Qiagen) and RNA was transcribed into cDNA using oligo(dT) primers and the SuperScript II RT kit (Invitrogen, Carlsbad, CA). Two µl of diluted cDNA were used for performing qRT-PCR using LightCycler 2X SYBR Green master mix (Roche) and were analyzed with a LightCycler 480 (Roche) using the following primer pairs: mouse GAPDH (forward, TCC TGC ACC ACC AAC TGC TTA GCC; reverse, GTT CAG CTC TGG GAT GAC CTT GCC),

B-ACTIN (forward, TCCTGTGGCATCCATGAAACT; reverse, GAAGCACTTGCGGTGCAC),

CCL3 (forward, TCCCAGCCAGGTGTCATTTTC; reverse, AGGCATTCAGTTCCAGGTCA),

CCL6 (forward, GGCTGGCCTCATACAAGAAATG; reverse, GGTTCCCCTCCTGCTGATAA),

CD68 (forward, ACTTCGGGCCATGTTTCTCT; reverse, GGGGCTGGTAGGTTGATTGT),

CCL12 (forward, GAGAGACACTGGTTCCTGACTC; reverse,
TCCGGACGTGAATCTTCTGC),

CSF1R (forward, CTTGGGAGCCTGTACTCACG; reverse, ACTGTCCCTGCGCACATATT),

TLR2 (forward, CCTGAGAATGATGTGGGCGT; reverse, GCTGGACCATGAGGTTCTCC),

TLR7 (forward, CTGGAGTTCAGAGGCAACCA; reverse, GGCGGCATACCCTCAAAAAC),

TGFBR1 (forward, AACCGCACTGTCATTCACCA; reverse,
AGCAGTGGTAAACCTGATCCA),

CD11B (forward, GACTCAGTGAGCCCCATCAT; reverse, AGATCGTCTTGGCAGATGCT),

CX3CR1 (forward, GGAGACTGGAGCCAACAGAG; reverse, TCTTGTCTGGCTGTGTCCTG),

GFAP (forward, AGAAAACCGCATCACCATTC; reverse, TCACATCACCACGTCCTTGT),

TYROBP (forward, GCTGGGATTGTTCTGGGTGA; reverse,
CTCTGACCCTGAAGCTCCTGA),

CTSD (forward, CTGAACAGGGACCCAGAAGG; reverse, CTCATTGCCCACCTCCAACT),

IRF8 (forward, CAGCAATTCTACGCCACCCA; reverse, CTGCTCTACCTGCACCAGAAT),

RUNX1 (forward, TGGCAGGCAACGATGAAAAC; reverse, TGAAGCTCTTGCCTCTACCG),

LY6C (forward, GGACTGCAGTGCTACGAGTG; reverse, AAGGCACTGACGGGTCTTTA).

2.3.3 Statistical Analysis

Statistical differences were evaluated using the unpaired Student's t test (GraphPad Prism) or ANOVA test. Differences were considered to be significant when $p < 0.05$, $p < 0.01$ or $p < 0.001$.

Results

3.1 SNPs in the CLU gene

3.1.1 Conselice study

Researchers have found several genes that increase the risk of AD. APOE ε4 is the first risk gene identified, and remains the gene with the strongest impact on AD risk. With this in mind, we genotyped our cohort of probable AD patients and CTR subjects for the APOE gene. This association was confirmed also in our population. In particular, APOE ε4 was over represented in AD patients when compared with controls (APOE ε4 frequency: AD 23.8% vs 15.5% CTR, p=0.035).

To establish the association of CLU, we genotyped SNPs in the CLU gene in a cohort of 106 patients with clinical diagnosis of AD and 431 control subjects matched for age and sex.

Three intronic SNPs in the CLU gene (rs2279590 (C/T), rs11136000 (C/T), rs9331888 (C/G)) and one SNP located in the promoter region (rs9314349 (G/A)) were analyzed in AD and CTR populations. CLU SNPs genotypes were in Hardy-Weinberg *equilibrium* in control group.

Results obtained are shown in Tables 1, 2, 3 and 4.

Table 1. Allele and genotype distribution of the rs2279590 (C/T) in the CLU gene from AD patients and CTR.

	CC		CT		TT		C carr		T carr	
	N	%	N	%	N	%	N	%	N	%
AD=103	30	29.1	59	57.3	14	13.6	89	86.4	73	70.9
CTR=430	147	34.2	204	47.4	79	18.4	351	81.6	283	65.8

AD vs CTR χ^2 3.359 p=0.186

Table 2. Allele and genotype distribution of the rs11136000 (C/T) in the CLU gene from AD patients and CTR.

	CC		CT		TT		C carr		T carr	
	N	%	N	%	N	%	N	%	N	%
AD=102	33	32.4	56	54.9	13	12.7	89	87.3	69	67.6
CTR=431	157	36.4	206	47.8	68	15.8	363	84.2	274	63.6
AD vs CTR χ^2 1.730 p=0.421										

Table 3. Allele and genotype distribution of the rs9331888 (C/G) in the CLU gene from AD patients and CTR.

	CC		CG		GG		C carr		G carr	
	N	%	N	%	N	%	N	%	N	%
AD=106	53	50.0	46	43.4	7	6.6	99	93.4	53	50
CTR=431	231	53.6	167	38.7	33	7.7	398	92.3	200	46.4
AD vs CTR χ^2 0.798 p=0.671										

Table 4. Allele and genotype distribution of the rs9314349 (G/A) in the CLU gene from AD patients and CTR.

	GG		AG		AA		G carr		A carr	
	N	%	N	%	N	%	N	%	N	%
AD=131	20	15.3	66	50.4	45	34.4	87	66.4	111	84.7
CTR=341	48	14.1	169	49.6	124	36.4	217	63.6	293	85.9
AD vs CTR χ^2 0.380 p=0.899										

As showed from tables 1, 2, 3 and 4, no statistically significant differences in the allele and genotype frequencies for any SNPs of the CLU (rs2279590, rs11136000, rs9331888 and rs9314349) between AD and CTR were found.

Because all these subjects belonged to the longitudinal "Conselice study on Brain Aging", they were followed up for five years and cognitive performances were detected at the beginning (1999) and at end of the study (2004). All DNA samples were obtained at the beginning of the clinical follow-up.

We performed experiments to evaluate potential difference in the genotype frequencies of CLU SNPs between subjects that after five years developed AD (CTR→AD) and subjects who remained cognitively healthy at the end of follow-up (CTR→CTR).

Results are summarized in Tables 5, 6, 7 and 8.

Table 5. Allele and genotype distribution of the rs2279590 (C/T) in the CLU gene in subjects converting to AD compared to those remaining cognitively healthy.

	CC		CT		TT		C carr		T carr	
	N	%	N	%	N	%	N	%	N	%
CTR→AD=68	18	26.5	39	57.4	11	16.2	57	83.9	50	73.6
CTR→CTR=430	147	34.2	204	47.4	79	18.4	351	81.6	283	65.8

CTR→AD vs CTR→ CTR χ^2 2.394 p=0.30

Table 6. Allele and genotype distribution of the rs11136000 (C/T) in the CLU gene in subjects converting to AD compared to those remaining cognitively healthy.

	CC		CT		TT		C carr		T carr	
	N	%	N	%	N	%	N	%	N	%
CTR→AD=68	19	27.9	39	57.4	10	14.7	58	85.3	49	72.1
CTR→CTR=431	157	36.4	206	47.8	68	15.8	363	84.2	274	63.6

CTR→AD vs CTR→ CTR χ^2 2.335 p=0.31

Table 7. Allele and genotype distribution of the rs9331888 (C/G) in the CLU gene in subjects converting to AD compared to those remaining cognitively healthy.

	CC		CG		GG		C carr		G carr	
	N	%	N	%	N	%	N	%	N	%
CTR→AD=72	39	54.2	31	43.1	2	2.8	70	97.3	33	45.9
CTR→AD=431	231	53.6	167	38.7	33	7.7	398	92.3	200	46.4

CTR→AD vs CTR→ CTR χ^2 2.405 p=0.30

Table 8. Allele and genotype distribution of the rs9314349 (G/A) in the CLU gene in subjects converting to AD compared to those remaining cognitively healthy.

	GG		AG		AA		G carr		A carr	
	N	%	N	%	N	%	N	%	N	%
CTR→AD=64	9	14.1	34	53.1	21	32.8	43	67.2	55	85.9
CTR→CTR=341	48	14.1	169	49.6	124	36.4	217	63.7	293	86

CTR→AD vs CTR→ CTR χ^2 0.326 p=0.84

As can be seen from the above tables, no statistically significant differences in the genotype frequencies of the CLU SNPs analyzed were present among subjects that developed AD and subjects that remained healthy after five years of follow-up.

3.1.2 Offspring study

A collaboration with Dr. Eric Van Exel belonging to VUMC (Vrij University, Medical Centre, Amsterdam) made possible the use of a different population model. Intronic polymorphisms rs9331888 and rs11136000, and the promoter polymorphisms rs9314349 in the CLU gene were analyzed in 198 DNA samples extracted from PBLs of middle-aged offspring with a parental history of AD (AD offspring) and 191 DNA samples taken from middle-aged offspring from

healthy parents (CTR offspring) (Tables 9, 10 and 11). AD offspring are particularly interesting because this population can be considered at high risk for AD.

Table 9. Allele and genotype distribution of the rs9314349 (G/A) in the CLU gene from AD offspring and CTR offspring.

	GG		AG		AA		G carr		A carr	
	N	%	N	%	N	%	N	%	N	%
AD offspring=195	27	13.8	97	49.7	71	36.4	124	63.5	168	86.1
CTR offspring=191	42	22.0	78	40.8	71	37.2	120	62.8	149	78

AD vs CTR χ^2 5.283 p=0.071

G carrier AD offspring vs CTR offspring χ^2 0.068 p=0.794

A carrier AD offspring vs CTR offspring χ^2 4.359 p=0.037 OR=1.754 CI=1.031-2.984

Table 10. Allele and genotype distribution of the rs11136000 (C/T) in the CLU gene from AD offspring and CTR offspring.

	CC		CT		TT		C carr		T carr	
	N	%	N	%	N	%	N	%	N	%
AD offspring =198	79	39.9	98	49.5	21	10.6	177	89.4	119	60.1
CTR offspring =182	69	37.9	90	49.5	23	12.6	159	87.4	113	62.1

AD vs CTR χ^2 0.434 p=0.805

C carrier AD offspring vs CTR offspring χ^2 0.382 p=0.536

T carrier AD offspring vs CTR offspring χ^2 0.088 p=0.767

Table 11. Allele and genotype distribution of the rs9331888 (C/G) in the CLU gene from AD offspring and CTR offspring.

	CC		CG		GG		C carr		G carr	
	N	%	N	%	N	%	N	%	N	%
AD offspring =198	98	49.5	81	40.9	19	9.6	179	90.4	100	50.5
CTR offspring =190	91	47.9	71	37.4	28	14.7	162	85.3	99	52.1

AD vs CTR χ^2 2.477 p=0.290

C carrier AD offspring vs CTR offspring χ^2 2.407 p=0.121

G carrier AD offspring vs CTR offspring χ^2 0.099 p=0.753

No difference in the frequency of rs11136000 (χ^2 0.434 p=0.805) and rs9331888 (χ^2 2.477 p=0.290) SNPs between offspring with a parental history of AD and offspring from healthy parents was found.

Only the SNP rs9314349, showed different distribution in the two offspring population (Table 9). In particular, A carrier frequency in offspring with a parental history of AD was increased, as shown in Table 9, being 86.1% vs 78%; χ^2 4.359; p=0.037; OR 1.754. The APOE ε4 allele was also over represented in the offspring with a parental history of AD (46.5% AD offspring vs 21.1% CTR offspring, χ^2 27.894, p=0.0001), as shown in Table 12.

Table 12. AD offspring and CTR offspring APOEε4 carrier or APOEε4 non carrier.

	ε4 carr		ε4 non carr	
	N	%	N	%
AD offspring =198	92	46.46	106	53.54
CTR offspring =190	40	21.05	150	78.95

χ^2 27.894 p=0.0001

To determine if the association for the A carrier of rs9314349 SNP was dependent or independent of APOE ε4 allele we restricted the analysis to offspring rs9314349 A carrier with or without the APOE ε4 allele. The results were statistically significant only for APOE ε4 non carrier (χ^2 3.844 p=0.050) (Table 13).

Table 13. Allele A distribution of the rs9314349 CLU SNP in AD offspring and CTR offspring APOEε4 carrier or APOEε4 non carrier.

ε4 carr		A carr		A non carr	
		N	%	N	%
	AD offspring	78	85.7	13	14.3
	CTR offspring	34	82.9	7	17.1
ε4 non carr		A carr		A non carr	
		N	%	N	%
	AD offspring	90	86.5	14	13.5
	CTR offspring	115	76.7	35	23.3

APOE ε4 carr χ^2 0.171 p=0.679

APOE ε4 non carr χ^2 3.844 p=0.050

3.2 Variants in antiviral genes as risk factors for cognitive decline and dementia

We genotyped AD patients and CTR subjects belonging to the longitudinal "Conselice study on Brain Aging" for the APOE gene. APOE ε4 was over represented in AD patients respect to controls (APOE ε4 frequency: AD 23.7% vs 11.2% CTR, p=0.001).

Genotype distribution of IL-28B polymorphism is shown in Table 14, panel A. TT genotype frequency between AD and CTR (15.9% in AD vs 9.2% in CTR) was statistically different (p=0.047).

All subjects were followed up for five years and cognitive performances were detected at the beginning and at end of the study; 85 converted to AD and 218 remained cognitively normal. The frequency of the IL-28B TT genotype was 16.5% in subjects converting to AD (CTR→AD) and 9.2% in those without cognitive deterioration at the end of follow-up (CTR→CTR) (p=0.071), as shown in Table 14, panel A. No difference in genotype distribution of Med23 polymorphism (rs3756784) between AD and CTR was found (Table 14, panel B). On the contrary, the GG genotype was slightly more frequent in subjects who developed AD (5.8%) than in those who remained cognitively healthy (1.8%) (p=0.055).

Table 14. A) Allele and genotype distribution of the rs12979860 in the IL-28B gene and **B)** rs3756784 in the Med23 gene from patients with AD and CTR and in subjects converting to AD compared to those remaining cognitively healthy after a five year follow-up.

A)

	IL-28B									
	TT		CT		CC		T carr		C carr	
	N	%	N	%	N	%	N	%	N	%
AD=157	25	15.9	66	42.0	66	42.0	91	58.0	132	84.1
CTR=218	20	9.2	94	43.1	104	47.7	114	52.3	198	90.8
CTR→AD=85	14	16.5	39	45.9	32	37.6	53	62.4	71	83.5
CTR→CTR=218	20	9.2	94	43.1	104	47.7	114	52.3	198	90.8

AD vs CTR TT genotype: χ^2 3.937 p=0.047 OR=1.875 CI= 1.001 - 3.513

CTR→AD vs CTR→CTR TT genotype: χ^2 3.268 p=0.071

B)

	Med23									
	TT		GT		GG		T carr		G carr	
	N	%	N	%	N	%	N	%	N	%
AD=158	104	65.8	47	29.7	7	4.4	151	95.6	54	34.2
CTR=228	158	69.3	66	28.9	4	1.8	224	98.2	71	31.1
CTR→AD=86	53	61.6	28	32.6	5	5.8	81	94.2	33	38.4
CTR→CTR=228	158	69.3	66	28.9	4	1.8	224	98.2	71	31.1

AD vs CTR GG genotype: χ^2 2.414 p=0.120

CTR→AD vs CTR→CTR GG genotype: χ^2 3.696 p=0.055 OR=3.457 CI= 0.906 - 13.190

We evaluated also the possible APOE ε4 effect, but the ε4 allele presence did not affect IL-28B genotype distribution in these AD groups (Table 15, panel A).

Conversely, the GG genotype was slightly higher in the APOE ε4 non carrier AD patients than CTR (p=0.059) and significantly increased in the APOE ε4 non carrier elderly who developed AD (p=0.018; Table 15, panel B) during the five years follow-up.

Table 15. Genotype distribution of the **A)** rs12979860 in the IL-28B SNP and **B)** rs3756784 Med23 in AD patients and CTR APOE ε4 carrier/or ε4 non carrier and in subjects converting to AD compared to those remaining cognitively healthy.

A)

	IL-28B									
	ε4 carr						ε4 non carr			
	TT		CT		CC		TT		CT		CC	
	N	%	N	%	N	%		N	%	N	%	N	%
AD=37	7	18.9	14	37.8	16	43.2	AD=119	18	15.1	52	43.7	49	41.2
CTR=24	3	12.5	7	29.2	14	58.3	CTR=191	17	8.9	87	45.5	87	45.5
CTR→AD=18	3	16.7	6	33.3	9	50.0	CTR→AD=67	11	16.4	33	49.3	23	34.3
CTR→CTR=24	3	12.5	7	29.2	14	58.3	CTR→CTR=191	17	8.9	87	45.5	87	45.5

AD vs CTR APOE Ɛ4 carr χ^2 1.358 p=0.507

AD vs CTR APOE Ɛ4 non carr χ^2 2.893 p=0.235

AD vs CTR APOE Ɛ4 non carr TT genotype χ^2 2.837 p=0.092

CTR→AD vs CTR→CTR APOE Ɛ4 carr χ^2 0.313 p=0.855

CTR→AD vs CTR→CTR APOE Ɛ4 non carr χ^2 4.194 p=0.123

CTR→AD vs CTR→CTR APOE Ɛ4 non carr TT genotype χ^2 2.897 p=0.089

B)

Med23												
ε4 carr							ε4 non carr					
	TT		GT		GG			TT		GT		GG	
	N	%	N	%	N	%		N	%	N	%	N	%
AD=38	25	65.8	11	28.9	2	5.3	AD=119	79	66.4	35	29.4	5	4.2
CTR=25	16	64.0	7	28.0	2	8.0	CTR=200	140	70.0	58	29.0	2	1.0
CTR→AD=19	12	63.2	6	31.6	1	5.3	CTR→AD=67	41	61.2	22	32.8	4	6.0
CTR→CTR=25	16	64.0	7	28.0	2	8.0	CTR→CTR =200	140	70.0	58	29.0	2	1.0

AD vs CTR APOE Ɛ4 carr χ^2 0.190 p=0.909

AD vs CTR APOE Ɛ4 non carr χ^2 3.631 p=0.163

AD vs CTR APOE Ɛ4 non carr GG genotype χ^2 3.563 p=0.059

CTR→AD vs CTR→CTR APOE Ɛ4 carr χ^2 0.167 p=0.920

CTR→AD vs CTR→CTR APOE Ɛ4 non carr χ^2 6.337 p=0.42

CTR→AD vs CTR→CTR APOE Ɛ4 non carr GG genotype χ^2 5.644 p=0.018 OR=6.286 CI=1.125 - 35.134

We analyzed also the rs6598008 polymorphism in IRF7 gene. However, no statistically significant difference was found neither between AD and CTR nor between subjects who developed AD and subjects who remained cognitively healthy (Table 16). The presence or the absence of the APOE ε4 allele did not influence IRF7 SNP distribution, ad shown in Table 17.

Table 16. Allele and genotype distribution of the rs6598008 IRF7 SNP in patients with AD and CTR and in subjects converting to AD compared to those remaining cognitively healthy.

	IRF7									
	GG		GA		AA		G carr		A carr	
	N	%	N	%	N	%	N	%	N	%
AD= 129	43	33.3	59	45.7	27	20.9	102	79.1	86	66.7
CTR=172	55	32.0	73	42.4	44	25.6	128	74.4	117	68.0
CTR→AD=79	27	34.2	33	41.8	19	24.1	60	75.9	52	65.8
CTR→CTR=172	55	32.0	73	42.4	44	25.6	128	74.4	117	68.0

AD vs CTR χ^2 0.900 p=0.638

CTR→AD vs CTR→CTR χ^2 0.137 p=0.934

Table 17. Genotype distribution of the rs6598008 IRF7 SNP in AD patients and CTR APOE ε4 carrier/or ε4 non carrier and in subjects converting to AD compared to those remaining cognitively healthy.

	ε4 carr						ε4 non carr					
	GG		GA		AA			GG		GA		AA	
	N	%	N	%	N	%		N	%	N	%	N	%
AD=30	8	26.7	13	43.3	9	30.0	AD=98	35	35.7	45	45.9	18	18.4
CTR=20	3	15.0	12	60.0	5	25.0	CTR=149	52	34.9	60	40.3	37	24.9
CTR→AD=17	6	35.3	7	41.2	4	23.5	CTR→AD=62	21	33.9	26	41.9	15	24.2
CTR→CTR=20	3	15.0	12	60.0	5	25.0	CTR→CTR=149	52	34.9	60	40.3	37	24.8

AD vs CTR APOE Ɛ4 carr χ^2 1.516 p=0.469

AD vs CTR APOE Ɛ4 non carr χ^2 1.565 p=0.457

CTR→AD vs CTR→CTR APOE Ɛ4 carr χ^2 2.198 p=0.333

CTR→AD vs CTR→CTR APOE Ɛ4 non carr χ^2 0.5 p=0.975

Leukocyte DNA from a subgroup of AD and CTR belonging to the Conselice study was previously analyzed for the presence of HHV-6 and EBV nucleic acids (Carbone et al., 2014). The difference for the presence of HHV-6 DNA between AD (87.5%) and CTR (12.5%) was statistically significant (p=0.019) (Table 18). Frequency of EBV DNA positivity in AD was also increased but at the limit of statistical significance (p=0.065) (Table 19).

Table 18. Presence or absence of human herpes virus 6 (HHV-6) DNA in peripheral blood leukocytes (PBL) from AD patients and CTR.

	AD		CTR	
HHV-6	N	%	N	%
Positive= 8	7	87.5	1	12.5
Negative=73	32	43.8	41	56.2

χ^2 5.506 p=0.019 OR=8.969 CI= 1.049 - 76.664

Table 19. Presence or absence of Epstein-Barr virus (EBV) DNA in peripheral blood leukocytes (PBL) from AD patients and CTR.

	AD		CTR	
EBV	N	%	N	%
Positive= 44	25	56.8	19	43.2
Negative= 36	13	36.1	23	63.9

χ^2 3.404 p=0.065

In order to evaluate whether polymorphisms in antiviral gene such as IL-28B, Med23 and IRF7 might influence anti-HHV-6 and EBV immune responses in AD, we stratified results from DNA virus positivity from AD patients and CTR according to genotype distributions.

Results are presented in Table 20, panel A, B, C. TT carrier for IL-28B SNP showed an increased positivity for the presence of EBV DNA (16.3%, panel A). There were only three subjects with GG genotype for Med23 SNP and they were all positive for EBV DNA.

Table 20. Presence or absence of Epstein-Barr virus (EBV) DNA in peripheral blood leukocytes (PBL) from all subjects (AD+CTR) in relation to different genotype of **A)** IL-28B, **B)** Med23 and **C)** IRF7 SNP.

A)

IL-28B	TT		CT		CC	
	N	%	N	%	N	%
Positive=43	7	16.3	17	39.5	19	44.2
Negative=31	2	6.5	11	35.5	18	58.1

χ^2 2.202 p=0.332

B)

Med23	TT		GT		GG	
	N	%	N	%	N	%
Positive=43	33	76.7	7	16.3	3	7.0
Negative=34	25	73.5	9	26.5	-	-

χ^2 3.347 p=0.188

C)

IRF7	GG		GA		AA	
	N	%	N	%	N	%
Positive=43	8	18.6	22	51.2	13	30.2
Negative=34	7	20.6	20	58.8	7	20.6

χ^2 0.923 p=0.630

IL-28B genotypes did not affect HHV-6 DNA presence or absence (Table 21, panel A). In particular, only one subject TT carrier for IL-28B SNP was positive for HHV-6 DNA.

On the other hand, Med23 genotypes were associated with differential degrees of HHV-6 DNA positivity (p=0.049), the percentage of positivity for the virus being elevated among carriers of the GG genotype (p=0.041), as shown in Table 21, panel B.

Table 21 panel C also shows that IRF7 genotypes did not affect HHV-6 DNA presence or absence.

Table 21. Presence or absence of human herpes virus 6 (HHV-6) DNA in peripheral blood leukocytes (PBL) from all subjects (AD+CTR) in relation to different genotype of **A)** IL-28B, **B)** Med23 and **C**) IRF7 SNP.

A)

IL-28B	TT		CT		CC	
	N	%	N	%	N	%
Positive=7	1	14.3	3	42.9	3	42.9
Negative=68	7	10.3	28	41.2	33	48.5

χ^2 0.142 p=0.932

B)

Med23	TT		GT		GG		G carr		non G carr	
	N	%	N	%	N	%	N	%	N	%
Positive=7	3	42.9	3	42.9	1	14.3	4	57.1	3	42.9
Negative=70	53	75.7	16	22.9	1	1.4	17	24.3	53	75.7

χ^2 6.026 p=0.049

GG genotype χ^2 =4.158 p=0.041 OR=11.5 CI= 0.636 - 207.882

G carriers χ^2 =3.464 p=0.063 OR=4.157 CI= 0.845 - 20.456

C)

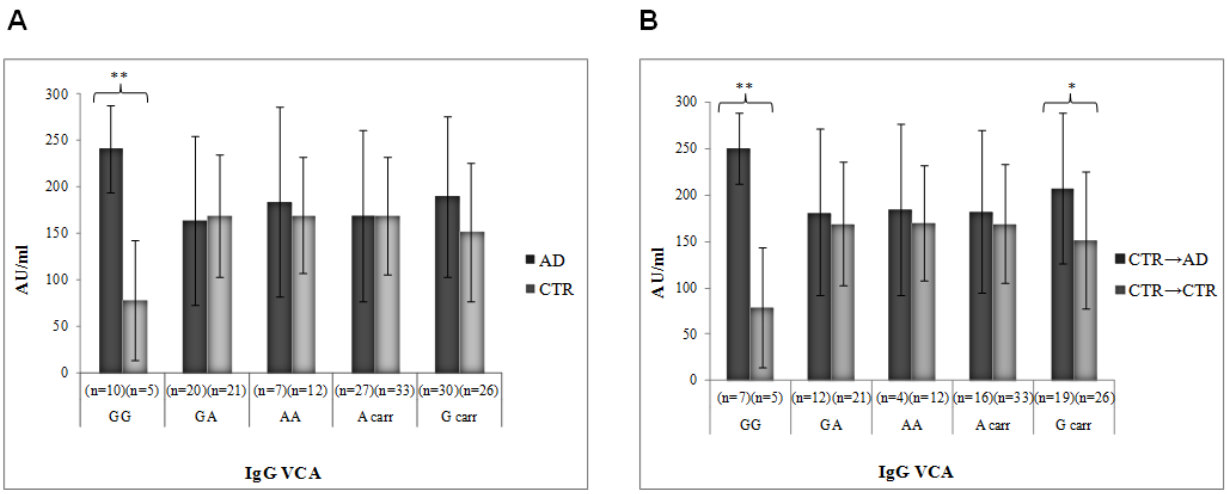
IRF7	GG		GA		AA	
	N	%	N	%	N	%
Positive=7	2	28.6	3	42.9	2	28.6
Negative=70	21	30.0	29	41.4	20	28.6

χ^2 0.007 p=0.996

In order to investigate EBV and HHV-6 serum positivity in AD and CTR samples, virus antigen specific IgG plasma levels, such as HHV-6 IgG plasma levels and EBNA and VCA for EBV, were also previously measured (Carbone et al., 2014). HHV-6 specific IgG levels and EBNA IgG-specific levels were not affected by IL-28B, Med23 or IRF7 genotypes in AD, CTR and in subjects that developed AD at the end of follow-up.

On the other hand, IRF7 genotypes influenced VCA IgG levels in both AD and CTR, as shown in Figure 3 (panel A) and the GG carriers with AD showed the highest serum levels of these antibodies ($p<0.001$; Figure 2, panel A). In addition, VCA IgG titers were higher in IRF7 GG ($p=0.0001$) and G carriers ($p=0.022$) who developed AD than those from elderly who remained cognitively healthy during the five years follow-up (Figure 3, panel B).

Figure 5. Immunoglobulin G (IgG) plasma levels specific for VCA antigens for Epstein-Barr virus (EBV) (AU/ml) according to the IRF7 genotypes and alleles in **A)** AD and CTR and **B)** subjects converting to AD compared to those remaining cognitively healthy after a five year follow-up.



A) AD and CTR t-test: AD p=0.074; CTR p=0.023. GG carr AD vs CTR p=0.000

B) Subjects converting to AD compared to those remaining cognitively healthy after a five year follow-up t-test: CTR→AD p=0.182; CTR→CTR p=0.023. GG carr p=0.000. G carr p=0.022

*p<0.05, **p<0.001.

3.3 Gene expression in a model of AD pathology

5XFAD mice rapidly recapitulate major features of AD amyloid pathology and are useful models of intraneuronal Abeta42-induced neurodegeneration and amyloid plaque formation (Oakley et al., 2006).

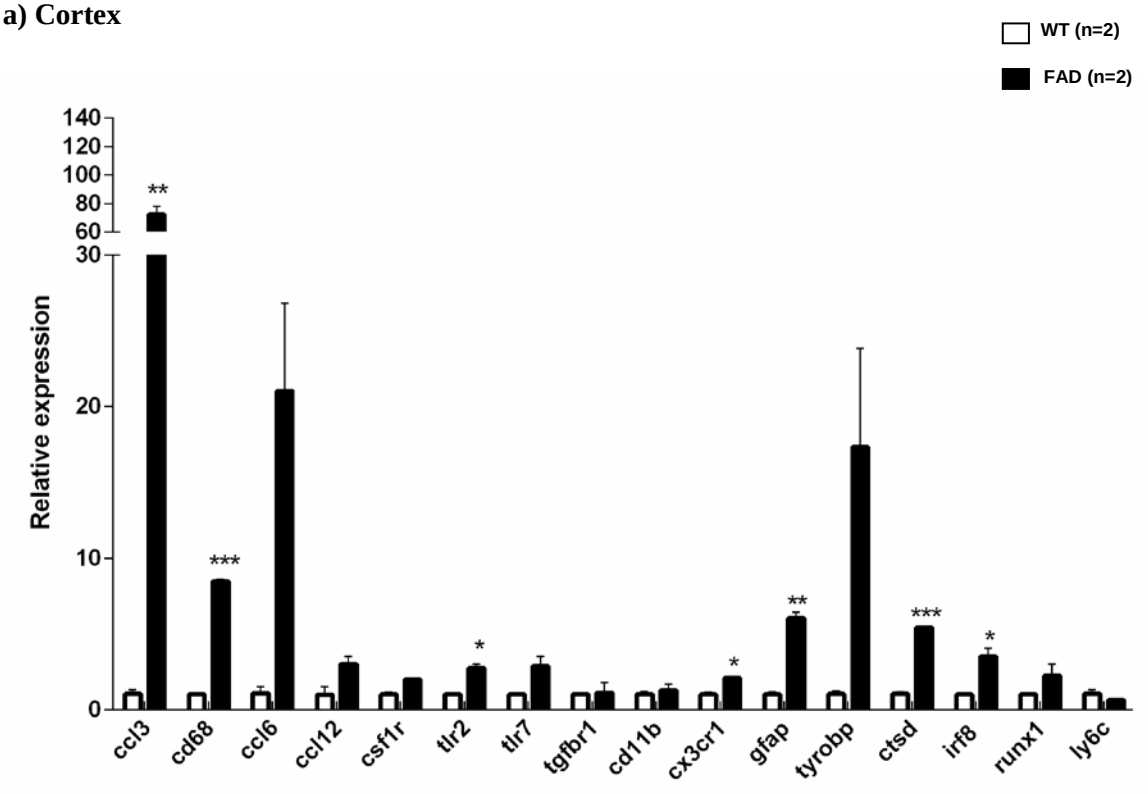
Here we demonstrated that several genes encoding chemokines and other myeloid cell markers were up-regulated in aged 5XFAD female mice at 40 weeks (Figure 6 a, b and c) and 48 weeks of age (Figure 7 a, b and c) compared with wild-type (WT) mice particularly in the cortex and hippocampus, the two major brain regions affected in AD, but much less in the cerebellum. Our results showed an increase in the number of differently expressed genes between 40 weeks and 48 weeks old mice in each tissue and most of these genes were shared by cortex and hippocampus.

In particular, upregulated genes mostly belong to the innate immune response and inflammatory response category, as illustrated by changes in expression of genes encoding for chemokines (CCL3, CCL6, CCL12) and the glial fibrillary acidic protein (GFAP) both in the cortex and hippocampus of aged mice. We also found an up-regulation of TLRs (TLR2, TLR7) in 40 and 48 weeks mice and to a lesser extent of integrin alpha M (ITGAM or CD11b) gene. Genes encoding for microglial activation markers such as CD68 and the chemokine C-X3-C motif receptor 1 (CX3CR1) were overexpressed in 5XFAD mouse model, instead, LY6C, a myeloid cells marker, did not change or was down-regulated in 5XFAD mice. Transcription factors IRF8 and RUNX1 genes were highly up-regulated in the cortex and hippocampus whereas TGFß signalling molecule (TGFBR1) was not differently expressed. Colony stimulating factor 1 receptor (CSF1R) and TYROBP genes, which belong to the same signalling pathway, were also up-regulated.

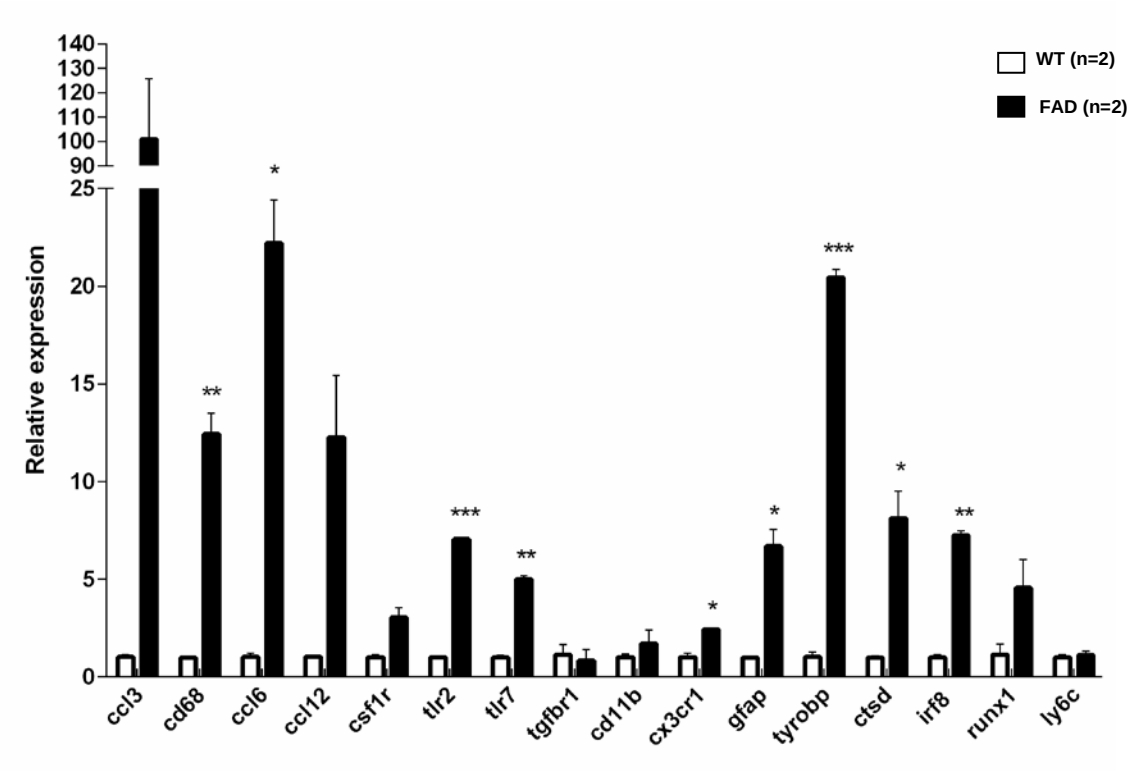
An increased expression of cathepsin D (CTSD), one of the lysosomal proteinases, was also present in 5XFAD mouse model.

Figure 6. Gene expression in 5XFAD mice at 40 weeks of age. Each panel represents gene expression in the indicated brain regions in wild type and in 5XFAD mice. Each bar represents the mean ± SEM gene expression level relative to the average of two animals per group, normalized over GAPDH and β ACTIN expression as reference genes (*p ≤ 0.05, ** p≤ 0.01,*** p ≤ 0.001).

a) Cortex



b) Hippocampus



c) Cerebellum

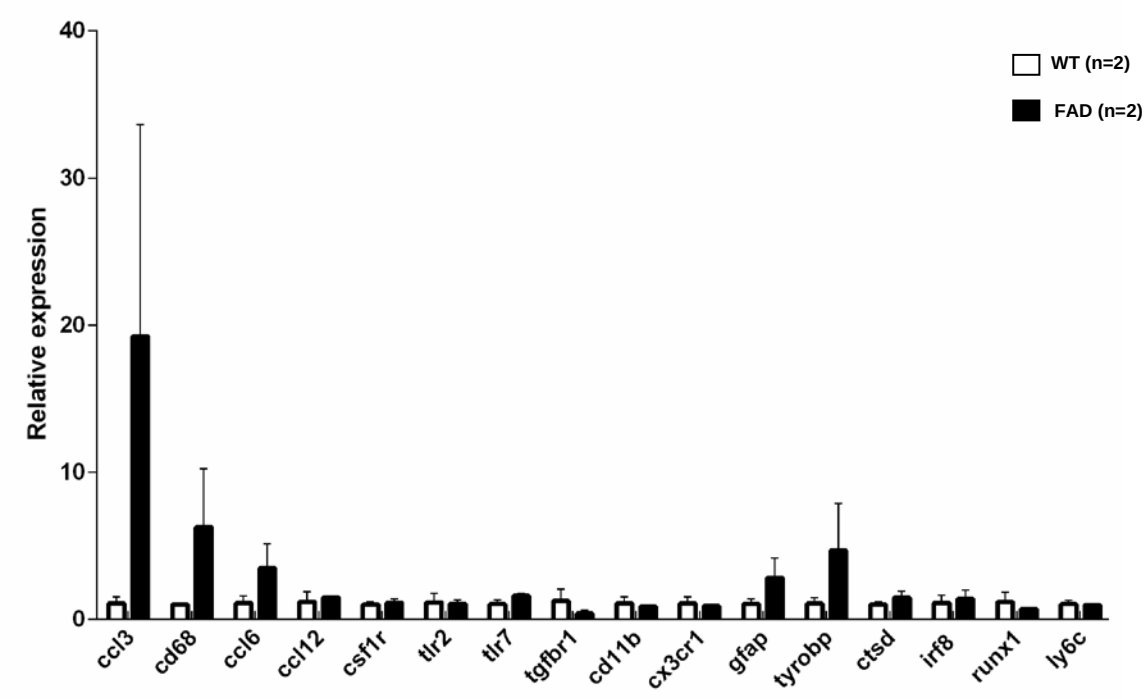
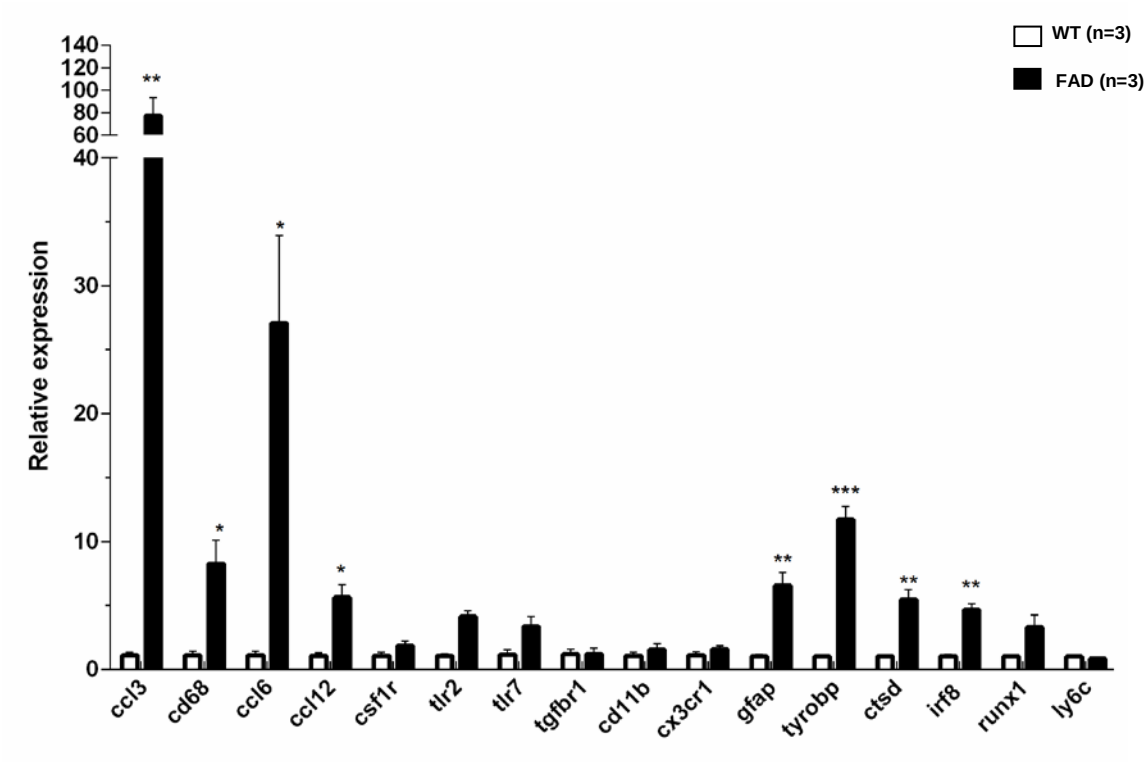
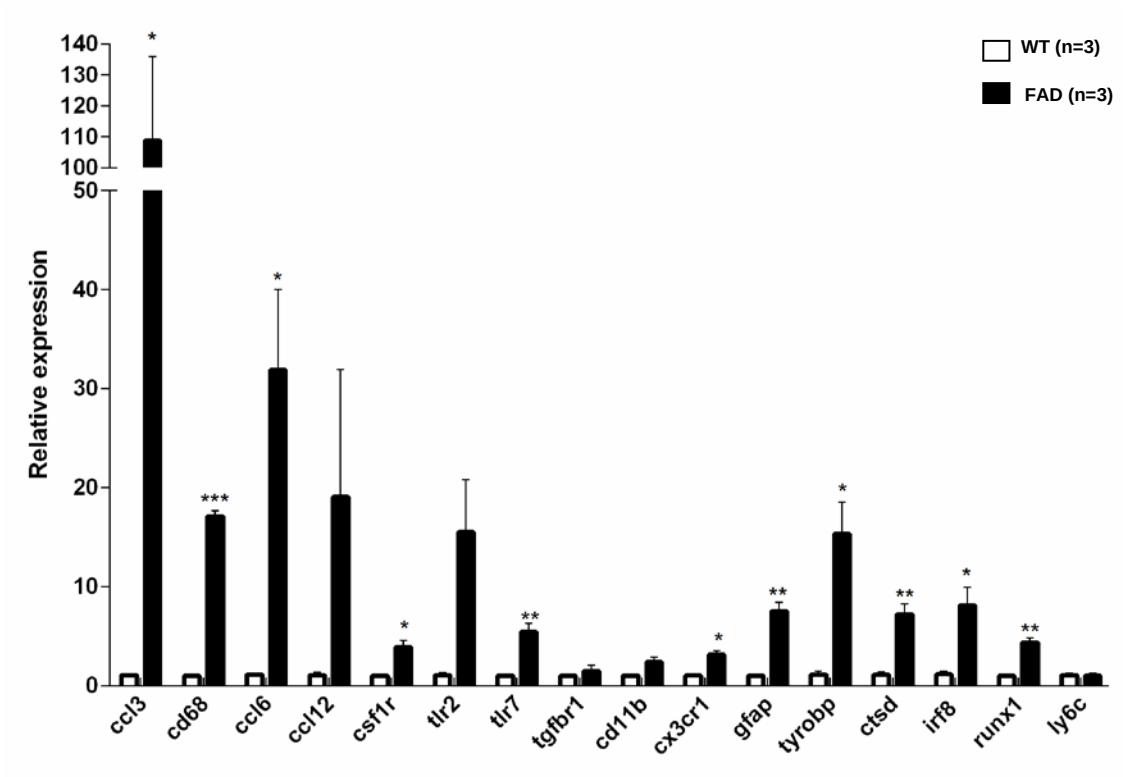


Figure 7. Gene expression in 5XFAD mice at 48 weeks of age. Each panel represents gene expression in the indicated brain regions in wild type and in 5XFAD mice. Each bar represents the mean ± SEM gene expression level relative to the average of three animals per group, normalized over GAPDH and β ACTIN expression as reference genes (*p ≤ 0.05, ** p≤ 0.01,*** p ≤ 0.001).

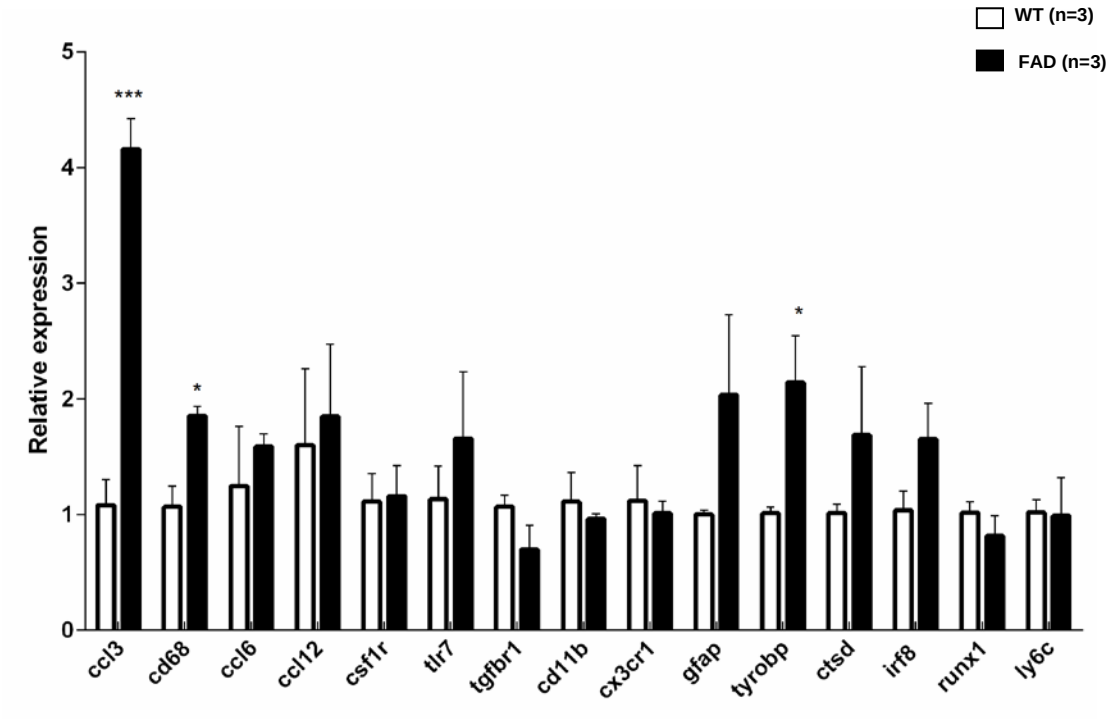
a) Cortex



b) Hippocampus



c) Cerebellum



Discussion

4.1 SNPs in the CLU gene

4.1.1 Conselice study

Late-onset AD is the most common form of dementia with age onset after 65 years. AD shows a genetically complex pattern of inheritance in which genetic risk factors together with environmental factors and life exposure events could contribute to increase the risk for AD. Consequently, it is difficult to identify novel AD loci, also because studies are often characterized by replications and refutations.

Genetic studies identified ε4 allele of the APOE gene as the major susceptibility locus for late-onset AD (Corder et al., 1993) and several recent GWA studies have, in addition to APOE ε4, identified the CLU gene, which encodes for Clusterin protein, as an indipendent genetic locus involved in AD risk.

One of the SNPs located in the CLU gene, rs11136000, was reported to be significantly associated with AD by two independent and large-scale GWASs of Caucasian ancestry by Harold et al. (2009) and Lambert et al. (2009).

Several association studies between the CLU SNPs and AD were conducted after the GWA studies with controversial results. Among them, subsequent two case-controlled studies (Carrasquillo et al., 2010; Corneveaux et al., 2010) and three meta-analysis (Jun et al., 2010; Seshandri et al., 2010; Kamboh et al., 2012) performed on Caucasian populations confirmed this finding. However, despite of these studies, Chen at al. showed evidence of a weak association of the CLU SNP with AD (Chen et al., 2012) whereas Lu et al. conducted another association study with no positive results (Lu et al., 2014) in two independent southern Chinese population. Finally, a meta-analysis study demonstrated that the rs11136000 polymorphism contributed to AD with a similar genetic risk in both Asian and Caucasian populations (Liu et al., 2014).

In the CLU gene, other two SNPs (rs2279590, rs9331888) showed statistically significant association with AD in samples from European countries (Lambert et al., 2009).

The rs2279590 polymorphism was also investigated in Asian populations, which included three studies in Chinese population (Yu et al. 2010; Chen et al. 2012; Lu et al., 2014) and one study in Japanese population (Komatsu et al. 2011), resulting in no or only weak association with AD. On the contrary, this polymorphism has been shown to contribute to AD susceptibility both in Caucasian and Asian populations in a recent meta-analysis (Zhang et al., 2015).

Inconsistent results regarding the rs9331888 SNP have also been reported in Asian population (Yu et al. 2010; Chen et al. 2012; Lu et al., 2014; Komatsu et al. 2011). This finding was reinforced by two recent analysis that confirmed that this SNP was associated with an increased AD risk in Caucasian population but not in Asian population (Shuai et al., 2015; Zhang et al., 2015).

This discrepancy might be attributable to the heterogeneity in the genetic background in different populations. On the other hand, studies conducted in Asian population generally had small sample size, compared with large-scale GWASs in populations of European ancestry (Harold et al. 2009; Lambert et al. 2009).

To help clarify the relevance of CLU as genetic determinant of AD, we analyzed its association in our Conselice population. This study comprised 106 probable sporadic AD patients and 431 age- and sex-matched healthy controls. Four SNPs within CLU gene were selected for genotyping: the previously mentioned rs2279590, rs9331888 and rs11136000 from GWA studies (Harold et al., 2009; Lambert et al., 2009) and meta-analysis studies (Jun et al., 2010; Seshadri et al., 2010) and the rs9314349. The last one is in the CLU promoter region and might affect CLU expression.

Nevertheless, no positive association was found between these CLU variants and AD and we were unable to replicate CLU SNPs associations in our population. CLU SNPs association with AD risk reported by GWA studies and meta-analysis was small according to the relatively small OR values obtained from the large population of AD and controls investigated.

We could not exclude that the number of AD and controls was too small to reproduce data from much larger populations from GWA investigations.

Because all these subjects belonged to the longitudinal "Conselice study on Brain Aging", they were followed up for five years and cognitive performances were detected at the beginning and at end of the study.

This allowed us to divide our samples into two groups: CTR→CTR and CTR→AD, in which CTR→CTR were elderly persons who stayed cognitively healthy, whereas CTR→AD represented elderly persons who developed clinical AD at the end of the follow-up after five years.

In order to verify whether these polymorphisms of CLU could be associated with progression of AD, we evaluated the genotype frequencies of CLU SNPs between CTR→AD and CTR→CTR.

The negative result suggests that CLU variants may not influence AD progression in our cohort and this can be explained by our relatively small sample size suggesting that CLU may only exert a modest effect to the risk.

4.1.2 Offspring study

In light of the increasing number of AD patients, the research for factors that might increase or lower the risk of manifesting the disease takes on crucial importance. We yet still cannot predict with a satisfactory degree of certainty the risk for the population of adult children of persons with AD (AD offspring) to develop the same disease of their parents. Moreover, risk and protective factors still lack robust data required for devising effective preventive and treatment interventions. Therefore, further research may be of particular urgency because there are few data available on the genetic background affecting the risk faced specifically by AD offspring (Jarvik et al., 2008).

AD is a complex neurodegenerative disorder and heritable factors make an important contribution to late-onset AD. Twin studies estimated an high hereditability, $\geq 60\%$, for AD (Gatz et al., 2006), however, it remains unclear which genes contribute to AD owing to its heterogeneity.

Although late-onset AD is traditionally referred to as sporadic form of AD, familial clusters have been frequently observed and first degree relatives of AD patients are at increased risk for developing dementia (Wang et al., 2012). It remains uncertain whether the contribution of family history of AD is independent of the APOE ε4 allele as both strongly co-occur (Wang et al., 2012).

We used samples from a family study in which middle-aged offspring with and without a parental history of AD were compared (Van Exel et al., 2009).

In this study, offspring with a parental history of AD might have an increased number of risk factors but did not yet present the disease.

The over representation of the APOE ε4 allele among offspring with a parental history of AD confirm the notion that the APOE ε4 allele was associated with increased risk for the disease also in this population.

However, we were unable to find differences in genotype frequency for both rs11136000 and rs9331888 SNPs in offspring populations. This result maybe be ascribed to the population size of the group at risk for AD, since among offspring with a positive familiarity only a small percentage would develop AD in advanced age.

However, an increased representation of A allele of the rs9314349 SNP located in the CLU promoter region from offspring with positive history of AD was observed. Thus, rs9314349 SNP could represent a new genetic risk factor predisposing to cognitive decline and AD. Further studies in larger cohorts of offsprings are needed to confirm the above finding.

4.2 Variants in antiviral genes as risk factors for cognitive decline and dementia

In recent years, the involvement of infection in the etiology of AD has gained attention suggesting a role for viral and bacterial chronic infections as causative inflammatory pathway in AD. A viral role in AD, especially involving herpes simplex virus type 1 (HSV-1), was proposed several decades ago (Mori et al., 2004; Burgos et al., 2006; Itzhaki et al., 2008; Carter CJ, 2008; Wozniak et al., 2009).

HSV-1 is a neurotropic double-stranded DNA virus that primarily infects epithelial cells of oral and nasal mucosa, where it undergoes lytic replication. The newly produced viral particles may enter sensory neurons and, by axonal transport, reach the trigeminal ganglion where the virus usually establish a latent infection. The trigeminal ganglion neurons also project to the trigeminal nuclei located in the brainstem. From here, neurons project to the thalamus to finally reach the sensory cortex. This may be the path through which the reactivated virus may reach the CNS and cause acute neurological disorders like encephalitis or a mild, clinically asymptomatic, infection, or establish lifelong latent infection (Monastero et al., 2014).

In contrast to the high frequency of HSV-1 DNA in elderly brains, the viral DNA was found to be present in only a very small proportion of brains of young people suggesting that HSV-1 enters the brain in older age, as a consequence of the decline in the immune system with age (Itzhaki, 2014). Subsequently the presence of this virus was found increased in the brain of carriers of the APOE ε4 allele with AD (Itzhaki et al., 1997).

Other herpes viruses have the ability to become latent in the infected host and eventually latently infect neurons (Monastero et al., 2014). However, investigations focused on these viruses, such as, EBV or HHV-6 in AD are limited.

The sero-positivity to these viruses is very high worldwide and both EBV and HHV-6 seem to be involved in the pathogenesis of various neurological diseases (Licastro et al., 2014).

Our previous findings showed an association of peripheral blood positivity for EBV DNA with AD and elevated levels of EBV specific antibodies were associated with an increased AD risk (Carbone et al., 2014).

HHV-6 has been found in a higher proportion of AD brains than age-matched control brains (Lin et al., 2002a). However, this result was not confirmed by another investigation (Hemling et al., 2003).

An elevated positivity for HHV-6 DNA in peripheral blood and brains of AD has been demonstrated beforehand and an increased sero-positivity for this virus was also associated with clinical diagnosis of AD (Carbone et al., 2014).

Moreover, it is also relevant how the host responds to viral infections. Several studies have suggested that factors such as number or expression of viral genes and host susceptibility might be related to incidence of AD (Monastero et al., 2014). These findings suggested that a differential genetic background in genes regulating immune defences against herpes viruses might be associated with age-related cognitive deterioration and AD. Cycles of virus latency and infections may therefore contribute to neurodegeneration associated with AD in genetically predisposed elderly, leading to neuronal loss, inflammation and amyloid deposition (Porcellini et al., 2010; Licastro et al., 2011).

Molecules belonging to the IFNs family are produced by both by innate and adaptive immunity and are the major factors with antiviral activity.

In particular, type III IFNs (IFN-λ) exhibit antiviral activity against several viruses. GWA studies have revealed multiple IFN-λ polymorphisms that are linked to clearance of HCV infection and possibly improved outcomes with other viral infections, including HBV, CMV, HSV-1, HSV-2 (Lazear et al., 2015; Griffiths et al., 2015) and HHV-6B (Nordström et al., 2012).

The type III IFNs genes (IFN-λ1 or IL-29, IFN-λ2 or IL-28A, IFN-λ3 or IL-28B and the most recently discovered IFN-λ4) map on chromosome 19q13. Like type I, type III IFNs trigger a signal transduction pathway that induces the activation of JAK-family kinases, phosphorylation of STAT1 and STAT2, and association between activated STAT complexes and IRF-9 to form ISGF3, which translocates to the nucleus and induces expression of hundreds of ISGs (Kotenko et al, 2003; Sheppard et al, 2003).

Among the IFN-λ subtypes, IFN-λ3 shows the most potent bioactivity compared with IFN-λ1 and IFN-λ2 (Dellgren et al., 2009) although IFN-λ2 and IFN-λ3 are nearly identical (96% amino acid identity) (Sheppard et al., 2003). IFN expression occurs after host detection of pathogen-associated molecular patterns by specific PRRs. Transcription factors activated downstream of PRR signaling include interferon regulatory factors (IRFs) and NF-kB (Lazear et al., 2015). The IFN-λ1 (IL-29) gene expression is regulated by IRF3 and IRF7, thus resembling the regulation of the IFN-β gene, whereas IFN-λ2/3 (IL-28A and B) gene expression is mainly controlled by IRF7, thus resembling IFN-α genes (Li et al., 2009).

Most cell types express both types IFN-α/β and IFN-λ after TLRs stimulation or virus infection, whereas the ability of cells to respond to IFN-λ is restricted to a narrow subset of cells, including plasmacytoid dendritic cells (DCs) and epithelial cells (Ank et al., 2008).

Almost any cell type is able to express IFN-λ1-3 mRNA in response to diverse viral infections. High levels of IFN-λs, but not IFN-α, were observed during viral infection of lung and liver tissues and IFN-λs seem to be the major IFNs induced in airway epithelial cells during infection with respiratory viruses (Egli et al., 2014a). However, the most potent producers of IFN-λs seem to be myeloid and DCs (Egli et al., 2014a).

Recently, a variant in the upstream region of IFN-λ3, designated as IFN-λ4, has been discovered (Prokunina-Olsson et al., 2013). This region harbors a dinucleotide variant (ss469415590) that is found in two alternative forms (ΔG or TT alleles). The one-base deletion in the ΔG variant results

in a frameshift that in turn creates IFN-λ*4* gene encoding the interferon-λ4 protein, which is moderately similar to IFN-λ3 (Prokunina-Olsson et al., 2013).

Recent studies showed that IFN-λ exerts its antiviral activity *in vivo* by the stimulation of the immune system rather than through induction of antiviral state mediators. These activities include the increase of the NK and T cell dependent cytotoxicity (Li et al., 2006) and the induction of T helper 1 cell responses (Kotenko et al., 2003; Sheppard et al., 2003).

In a GWA study from a clinical trial on more than 1600 individuals chronically infected with HCV Ge et al. found that the rs12979860 SNP, which is located 3kb upstream of the IFN-λ3/IL-28B gene, was associated with SVR to antiviral therapy, defined as the absence of detectable virus at the end of follow-up evaluation (Ge et al., 2009). The CC genotype showed a two-fold greater rate of SVR in patients of European ancestry and Hispanics and a three-fold higher rate in African-American patients group in comparison to the TT genotype.

Moreover, genotypic analysis of patients suffering recurrent orofacial HSV-1 outbreaks, shown to be deficient in IFN-λ secretion, found a significant correlation with the rs12979860 SNP in the IL-28B promoter; the homozygous TT genotype being associated with the disease severity progression (Griffiths et al., 2013). Besides, IL-28B SNP influenced the replication and viremia level of CMV (Egli et al., 2014b) and EBV (Akay et al., 2014).

Our findings showed that IL-28B TT carriers were significantly over represented in AD patients compared to CTR ($p=0.047$). An over representation of IL-28B TT genotype in the elderly developing AD during a five years follow-up compared to subjects that remained cognitively healthy was also present. These results suggest that this genotype may favor cognitive deterioration by defective anti viral immune responses.

Using a combined two genome-scale screens for host factors (HFs) involved in virus replication to investigate the complex interaction between HSV-1 and its host, Griffiths et al. showed that IL-28B was up-regulated by Med23, an anti-viral component of the largely pro-viral multi-protein

Mediator complex, at the mRNA and protein level by directly interacting with the transcription factor IRF7 (Griffiths et al., 2013).

It is of interest that a missense mutation (R617Q) in Med23 gene failed to enhance IRF7-induced IFN-λ expression (Griffiths et al., 2013) and this mutation was associated with hereditary dementia (Hashimoto et al., 2011). The failure to induce IFN-λ and thereby control HSV-1 in the brain may be a potential cofactor for the development of dementia, similar to AD.

Since the synergistic effect of Med23 and IRF7 on IFN-λ induction suggested that IRF7 could be the major transcription factor for IFN-λ expression, we decided to investigate the role of SNPs in Med23 and IRF7 genes in AD. Med23 and IRF7 SNPs were not investigated in recent GWA studies; the IL-28B (rs12979860) was included in the first European GWA investigation, but its association with the disease was below 10^{-5} threshold (Lambert et al., 2009).

Our data showed that the GG genotype in the rs3756784 SNP in the promoter region of the Med23 gene was over represented in the elderly converting to AD when compared to the frequency from those subjects that remained cognitively healthy at the end of the follow-up ($p=0.055$) and significantly increased in subjects converting to AD without the APOE ε4 allele ($p=0.018$). This could suggest that the SNP in Med23 gene could be associated with the progression of AD independently of APOE ε4.

We also analyzed the rs6598008 SNP in IRF7 gene, which belong to PHRF1 (PHD and ring finger domains 1 (PHRF1, also known as KIAA1542) - IRF7 - CDHR5 (cadherin-related family member 5) locus (Carmona et al., 2012). This SNP was not associated with the AD risk or the progression to AD.

HHV-6 and EBV have been implicated in the development of different neurological diseases (Yao et al., 2010; Kleines et al., 2011), but studies regarding these viruses in AD are scarce. As previously reported by our laboratory (Carbone et al., 2014), we have shown also in our cohort of

subjects that a higher proportion of AD patients than CTR were positive for HHV-6 virus and, though to a lesser extent, for EBV virus.

Therefore, the viral DNA presence were stratified according to IL-28B, Med23 and IRF7 genotypes. Of the above genes, only Med23 GG genotype was associated with increased positivity for HHV-6 DNA presence in PBLs from AD.

Age-associated immune alterations induced by chronic sub-clinical infections might substantially contribute to the appearance of neuroinflammation in the elderly (Licastro et al., 2014).

Therefore, to assess systemic immune responses to EBV and HHV-6 in AD patients and CTR with a different genetic background, serological data about IgG plasma levels (Carbone et al., 2014) were stratified for the genotype distribution of SNPs in IL-28B, Med23 and IRF7 genes. AD patients with the IRF7 GG genotype showed elevated levels of VCA IgG for EBV. Moreover, the elderly converting to AD had increased serum titers of these antibodies. These findings suggested that a specific polymorphism in antiviral genes might influence immune responses and progression to AD.

However, these preliminary results should be interpreted with caution and they should be replicated in a larger case-control study. We included in this study 158 AD patients and 228 controls subjects, genotyped for different SNPs in APOE, IL-28B, Med23 and IRF7 genes. The discrepancy in subject number in the different genotyping investigations was due to the fact that DNA and plasma samples were not available for all genotyping and for all plasma Ig viral detection. Besides, we have differentially genotyped subjects, with inherent implications of selection. Furthermore, plasma analyses were run in only 75 subjects.

4.3 Gene expression in a model of AD pathology

5XFAD transgenic mice that overexpress mutant human APP (695) with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) Familial Alzheimer's Disease (FAD) mutations and human PS1 harboring two FAD mutations, M146L and L286V were used. Expression of both transgenes is under the transcriptional control of the neuron-specific Thy-1 promoter to drive overexpression in the brain.

Mutations in genes encoding for APP and presenilins (PS1 and PS2) cause an enhanced production of Aβ42 and familial Alzheimer's disease (FAD). Mice expressing FAD mutations (such as 5XFAD line) overproduce Aβ42 and present amyloid plaque pathology similar to that found in AD. In 5XFAD mouse model extracellular amyloid deposition begins at 2 months reaching a very large burden while intraneuronal Aβ42 accumulates in brain starting at 1.5 months of age. These transgenic mice have reduced synaptic markers, increased p25 (activation subunit of cyclin-dependent kinase 5) levels, increased neuron loss and memory impairment in the Y-maze (Oakley et al., 2006).

Thus, 5XFAD mice rapidly summarize the major features of AD amyloid pathology and are useful models of amyloid plaque formation and intraneuronal Aβ42-induced neurodegeneration and amyloid plaque formation (Oakley et al., 2006).

Little is known about gene expression changes during neurodegeneration in this AD mouse model. Recently, Landel and co-workers carried out a transcriptomic analysis on RNAs from the neocortex and the hippocampus of 5XFAD female mice at the ages of one, four, six and nine months (Landel et al., 2014).

Here, we showed an increased expression of chemokines and myeloid cell markers in the two major brain regions affected in AD, cortex and hippocampus, obtained from 5XFAD female mice at 40 and 48 weeks of age.

Upregulated genes both in the cortex and hippocampus mostly belong to the innate immune response and inflammatory response as demonstrated by an over-expression of genes coding for chemokines CCL3, CCL6, CCL12 and GFAP, the main astrocytic intermediate filament protein.

Neuroinflammation is a well-known hallmark of AD characterized by the activation of astrocytes and microglia. Chemokines are considered pro-inflammatory factors because they can be induced during the immune response to recruit cells of the immune system to the site of infection (Chang et al., 2014). Amyloid plaques in human AD and AD mouse models are surrounded by reactive astrocytes which express increased GFAP levels; the latter is considered a marker for activated astrocytes (Kamphuis et al., 2012). Moreover, recently transcript levels of most known GFAP isoforms have been shown to increase with AD progression in human hippocampal tissue at different stages of the disease (Kamphuis et al., 2014).

We found that Toll-like receptors (TLR2 and TLR7) expression was also increased in 5XFAD mice, particularly in the hippocampus. On the other hand, the expression of the microglial integrin marker CD11b was not statistically different from wild-type mice.

It was already observed that 6-month-old 5XFAD mice showed an upregulation of TLR2, TLR7 and TLR9 and GFAP (Hillmann et al., 2012). TLR2 and TLR7 were higher also in the cortex of another transgenic mice (APP TgCRND8) (Letiembre et al., 2009) and a strong upregulation of TLR2 and TLR7 mRNAs was detected in plaque material, compared to plaque-free tissue, in the APP23 transgenic mouse model (Frank et al., 2009).

Fibrillar Aβ1–42 peptides increased the expression of different TLRs, proinflammatory molecules and microglial integrin markers (CD11a, CD11b, CD11c, and CD68) in mouse primary microglia and BV-2 microglial cells (Jana et al., 2008).

In our study from 5XFAD mice microglial activation markers CD68 and CX3CR1 were strongly overexpressed in the cortex and hippocampus. Given the importance of microglia in physiological brain function, it is not surprising that an increasing number of microglia-related genes have now

been associated with neuropsychiatric or neurologic disorders. “Activated microglia” were already found to exhibit greater expression of CD68, a low-density lipoprotein associated with microglial phagocytosis (Walker et al., 2013). Fractalkine receptor CX3CR1, which normally promotes phagocytosis of apoptotic cells, shifted microglia towards a phenotype with less capacity to phagocytose fibrillary congophilic Abeta in diseased condition (Meyer-Luehmann et al., 2015).

The transition from the “resting” but surveying microglial phenotype to an activated stage is tightly regulated by several extrinsic (e.g., interaction between CX3CR1 and neuronal CX3CL1) and intrinsic factors, such as RUNX1 and IRF8, a downstream effector of IFN-γ (Kierdorf et al., 2013), which were up-regulated in cortex and hippocampus of our 5XFAD model. IRF8 expression can also be induced through TGF-β (Ju et al., 2007). TGF-β receptor is encoded by the TGFBR1 gene that was overexpressed in 5XFAD mice of our study.

We find also an overexpression of the macrophage colony stimulating factor 1 receptor gene (CSF1R), that encodes for a myeloid cell markers, which, together with its ligand (CSF1), co-signals through TYROBP (Neumann et al., 2013), another upregulated gene. TYROBP (also called DAP-12) is the trans-membrane binding partner of TREM2, a microglial/macrophage cell surface receptor, which activates a signal transduction leading to brain myelination and inflammation (Villegas-Llerena et al., 2015). In mice, TREM2 is expressed in myeloid cells in the brain and appears increased in microglia in the vicinity of plaques in APP mice (Melchior et al., 2010; Frank et al., 2008). Recently, rare variants in TREM2 have been associated with susceptibility to late-onset AD, with an odds ratio similar to that of the APOE ε4 allele (Guerreiro et al., 2013; Jonsson et al., 2013). Landel proposed that the signalling involving CSF1/CSF1R/TREM2/TYROBP might play a role in the 5XFAD physiopathology since all the corresponding genes were strongly upregulated in cortex and hippocampus of these aged mice (Landel et al., 2014).

Another gene greatly expressed in our 5XFAD mice is the Cathepsin D (CTSD). This finding was confirmed also in another aged 5XFAD mouse model (Bouter et al., 2014). It is of interest that an up-regulation of CTSD mRNA in neurons of AD patients was also reported (Cataldo et al., 1995).

CTSD is a lysosomal enzyme found in neuritic plaques and is considered to be involved in amyloid precursor protein processing (Schuur et al., 2011).

In conclusion, the upregulation of all these immune markers makes 5XFAD mice a good model for AD because it recapitulates the dysfunction of immune system seen in AD.

Conclusions

AD is a multifactorial and progressive neurodegenerative disease and the most common form of dementia in the elderly. AD is clinically characterized by a degeneration of the cerebral cortex with progressive impairment of memory and cognitive functions and personality changes. Since current treatments for AD and strategies for preventing or significantly slowing the disease progression are limited, AD is a serious health problem and the number of patients is continuously increasing. It is estimated that over 46 million people worldwide are currently living with dementia and that this number is estimated to increase to 131.5 million by 2050 (Alzheimer's Disease International: World Alzheimer Report 2015).

Neuritic senile plaques, neurofibrillary tangles, synapsis loss, neuronal atrophy, microglia activation, reactive astrogliosis and cerebral inflammation are neuropathological hallmarks of the disease. Overall, 90-95% of AD, also called late-onset AD (LOAD), belongs to the sporadic form and affects people over 65 years of age. AD is a complex disorder characterized by interactions among multiple genetic, epigenetic and environmental factors.

Recent GWA studies found that the APOE ε4 allele and SNPs of other genes that regulate inflammatory pathways are associated with AD (Harold et al., 2009; Lambert et al., 2009; Hollingworth et al., 2011; Naj et al., 2011).

All these genes can be involved in different mechanisms mediated by viral herpes virus and has been proposed that the concomitant presence of these SNPs might result in a genetic signature predisposing to AD (Porcellini et al., 2010; Licastro et al., 2011; Licastro et al., 2015).

A strong association of the CLU gene, also known as APOJ, with the disease was found. CLU is a modulator of complement and its cytolytic activity is important for virus neutralization. Polymorphisms in this gene might influence virus lytic defences by regulating complement activation (Porcellini et al., 2010).

The family of herpes virus shows characteristics relevant to AD, as the virus infects a large proportion of the population, develops a latent persistent infection impossible to eliminate by the

immune responses and is able to infect neurons. Brain infections by reactivated latent viruses may induce neuronal loss and astroglial activation leading to AD.

In a case-control study, EBV and HHV-6 were considered as possible environmental risk factors for cognitive impairment and progression of AD in the elderly (Carbone et al., 2014). Studies on host-pathogen interactions have shown the importance of host factors in the pathogenesis of infectious diseases and polymorphisms in genes encoding these factors influenced the response of the host and the course of the disease (Russell et al., 2014).

As already known, one of the most important groups of cytokines with antiviral function are the interferons (IFN).

IFN-λ has direct antiviral functions, common to other IFNs; however, complex regulatory mechanisms exist which have implications on viral infection (Egli et al., 2014a).

Med23, a subunit of the Mediator complex, is a key regulator of IFN-λ induction. It is a coactivator involved in transcriptional regulation of almost all RNA polymerase II-dependent genes. Med23 up-regulates, both at the mRNA and protein level, IFN-λ by interacting with IRF7, an important transcription factor involved in innate immunity against HSV-1 infection (Griffiths et al., 2013).

Results presented in this thesis investigated several SNPs in genes potentially associated with AD risk and disease's progression and involved in herpes virus infection pathways.

Polymorphisms in the CLU gene were investigated in a population of patients with clinical diagnosis of AD and age- and sex-comparable controls.

AD patients and controls ("Conselice study on Brain Aging") were followed up during a five year period from 1999 and ended in 2004. All subjects belonging to the study were assessed cognitively at the beginning and end of the study.

Three intronic polymorphisms of the CLU gene (rs2279590, rs11136000, rs9331888) and a SNP located in the promoter of the CLU (rs9314349) were investigated. However, no association of these SNPs in AD patients was found.

Studies of offspring whose parents were affected by late-onset AD may be informative for genetic factors relevant to AD. For this purpose, rs9331888 and rs11136000 SNPs, and the promoter polymorphisms rs9314349 in the CLU gene were analyzed in middle-aged offspring with a positive parental history of AD (AD offspring) and negative parental history of AD (CTR offspring). Despite no difference was found in the frequency of rs11136000 and rs9331888 SNPs between offspring with a parental history of AD and offspring of healthy subjects, the difference in the A carrier frequency of rs9314349 SNP between the two gropus was statistically significant.

Our results suggest that CLU genotypes may have a limited effect on AD risk and this small effect emerges only by investigating several thousand cases and controls as it is the case of large GWA population studies.

A gene association study of factors regulating antiviral response, such as interferon IFN-λ3, also known as IL-28B, Med23 and IRF7 with cognitive deterioration and AD was also the object of this thesis.

SNPs of IL-28B (rs12979860), Med23 (rs3756784) and IRF7 (rs6598008) genes were analyzed in elderly belonging to the above mentioned “Conselice study on Brain Aging”.

Differences in the TT genotype distribution of IL-28B SNP between AD and CTR were found. The GG genotype of Med23 gene appeared to influence the progression of the disease, being more frequent in the APOE ε4 negative elderly that developed AD during the five year follow-up.

Moreover, we were also interested in the immune response of the host to infections, since herpes viral replication occurs intermittently throughout life. In fact, viruses of the herpes familiy by frequent cycle of reactivation and latency constantly challenge the host immune system.

In the present study, both Med23 and IRF7 gene polymorphisms appeared to affect anti-HHV-6 and EBV immune responses in AD. These findings suggest that individual genetic makeup may influence sub-clinical infections by persistent virus and chronic infections may contribute to cognitive deterioration. Therefore, these data support the notion that peripheral infections may also modulate inflammatory responses in CNS and neurodegenerative processes associated with AD. In fact, persistent cycles of virus latency and reactivations by stressing the systemic immune responses may contribute to neurodegeneration and progression of cognitive decline in genetically predisposed elderly leading to AD.

However, our data are preliminary and further studies with larger sample sizes is needed to confirm and extend these findings.

AD mouse models can contribute to our understanding of the pathophysiology of the disease. Furthermore, they are valuable tools in the preclinical testing of potential drugs for AD therapy.

The 5XFAD mouse model carrying five mutations associated with familial Alzheimer's disease (FAD) was used. 5XFAD mice rapidly recapitulate major features of AD amyloid pathology and may be useful models of neurodegeneration and amyloid plaque formation (Oakley et al., 2006). In these mice gene expression studies were performed by using different brain samples.

Gene expression changes can be involved in the progression of AD in the brain. Like other neurodegenerative diseases, AD is the result of complex interactions between many different factors over an extended time period.

Here we showed that several genes belonging to the innate immune response and inflammatory response, such as genes coding for chemokines (CCL12,CCL3,CCL6), TLRs (TLR2, TLR7), microglial activation markers (CD68, CX3CR1), were up-regulated in female mice of 40 weeks and 48 weeks of age, particularly in the cortex and hippocampus.

Our data from this mouse model of AD suggest that, inflammation-associated pathways and sustained microglial activation represent predominant features of AD associated

neurodegeneration. Moreover, this study identifies a number of genes already known to be altered in human AD, thus confirming that the 5XFAD model can be valid and useful for understanding AD pathogenesis and for screening potential therapeutic molecules.

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- **The 21st century epidemic: infections as inductors of neuro-degeneration associated with Alzheimer's Disease.**

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- **Variants in Antiviral Genes are Risk Factors for Cognitive Decline and Dementia.**

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