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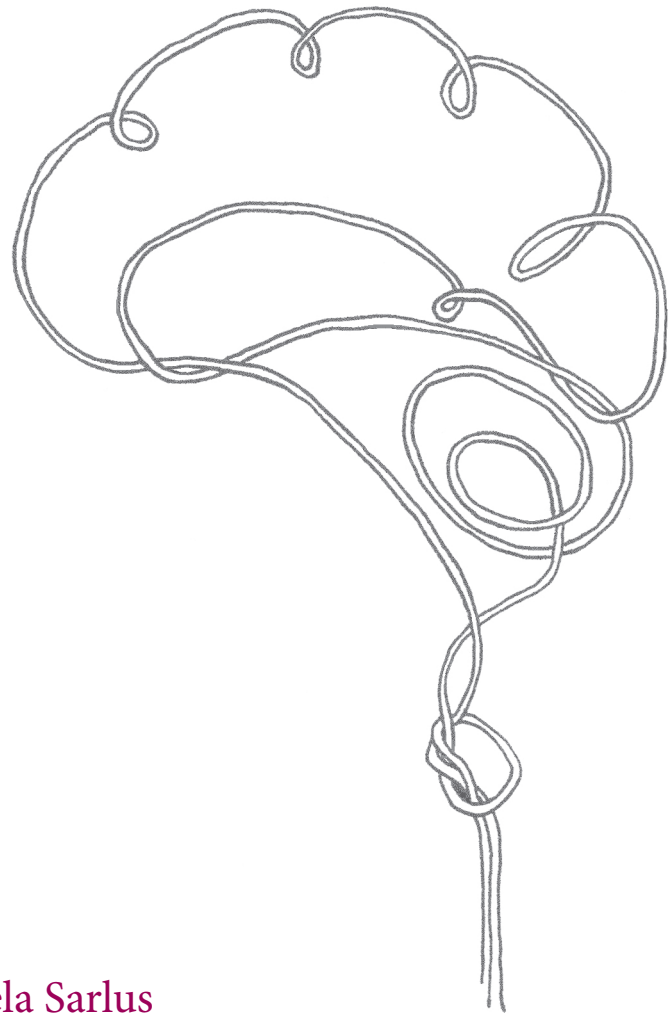
Allergy and Alzheimer Disease



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Unexamined life is not worth living.
Socrates

Qaid-e-hayaat-o-band-e-gham, asl mein dono ek hai
Maut se pehle aadmi gham se nijaat paaye kiyun

Mirza Ghalib

ABSTRACT

Alzheimer disease (AD) is a neurodegenerative disorder characterized by progressive dementia with devastating effects for the patients and their families. The treatments available are purely symptomatic and there is need for treatment strategies aiming at the etiopathogenesis of AD. The effects of systemic inflammation on the development and/or progress of AD are not clarified. Present knowledge points towards both beneficial and detrimental effects of inflammation on AD, depending on both its timing and its nature. Allergy is associated with chronic systemic inflammatory changes, and its effects on the brain are largely unknown. Epidemiological studies have shown that allergic diseases were associated with increased risk for AD. The aim of this thesis was to investigate the effects of allergy on the normal brain and in association with AD-like pathology.

In **Paper I**, we aimed to study whether chronic airway allergy affects the AD-related proteins amyloid precursor protein (APP) and hyperphosphorylated tau (p-tau), and the inflammatory status in the brain of naïve mice. We found that allergy increased p-tau levels in the brain, whereas levels of APP were not modified. Furthermore, the levels of immunoglobulin (Ig) G and E were significantly increased in the brain of allergic mice. The increase was not only confined to blood vessels but broadly in the brain parenchyma. We then aimed to study in **Paper II** the changes in gene expression induced by chronic airway allergy in the brain using microarray technology. Allergy induced changes in several inflammation-related signalling pathways. We found that the levels of insulin-degrading enzyme (IDE) and phosphorylated insulin receptor (p-IR) were decreased in the brain in response to allergy. In **Paper III**, we investigated the effects of chronic airway allergy on the brain in the 3xTgAD (Tg) mouse model for AD, and their background strain (Bg). The levels of IgG and IgE were also increased in the brain of Tg mice in response to allergy. Allergy increased the levels of C1q component C and interleukin-1β, decreased p-IR, and impaired the burrowing activity in Bg animals. The Tg mice showed increased levels of brain-derived neurotrophic factor and decay-accelerating factor (complement inhibitor), and decreased levels of phosphorylated p38. In **paper IV**, we analysed the levels of Igs and cytokines in cerebrospinal fluid (CSF) and serum obtained from patients with subjective cognitive impairment (SCI), mild cognitive impairment (MCI) and AD, with or without allergy. The relation of allergy to CSF biomarkers (p-tau, total (t)-tau, and β-amyloid (Aβ)) and mini-mental statement examination (MMSE) was investigated. We found that the CSF levels of IgG1 ratio, IgA and t-tau were lower in AD cases with allergy compared to those without allergy. The serum interferon γ levels were lower while MMSE scores were higher in MCI cases with allergy.

In conclusion, our studies suggest that allergy may have negative effects on the normal brain but seemingly beneficial effects in the presence of AD-like pathology. It is possible that stimulation of immune responses induced by allergy may lead to beneficial effects on AD. So far, little is known regarding the association between AD and allergies and further studies are needed to clarify the impact of allergy on AD pathogenesis and progression.

LIST OF SCIENTIFIC PAPERS

I. **Heela Sarlus**, Caroline Olgart Höglund, Bianka Karshikoff, Xiuzhe Wang, Mats Lekander, Marianne Schultzberg, Mircea Oprica.
Allergy influences the inflammatory status of the brain and enhances tau-phosphorylation. J. Cell. Mol. Med. 2012, 16:2401-2412

II. **Heela Sarlus**, Xiuzhe Wang, Angel Cedazo-Minguez, Marianne Schultzberg, Mircea Oprica.
Chronic airway-induced allergy in mice modifies gene expression in the brain toward insulin resistance and inflammatory responses. J Neuroinflammation. 2013, 10:99

III. **Heela Sarlus**, Alina Codita, Xiuzhe Wang, Angel Cedazo-Minguez, Marianne Schultzberg, Mircea Oprica.
Chronic airway allergy induces anti-inflammatory responses in the brain of 3xTgAD mice.

IV. **Heela Sarlus**, Helga Eyjolfsdottir, Maria Eriksdottir, Mircea Oprica Marianne Schultzberg.
Influence of allergy on immunoglobulins and tau in the cerebrospinal fluid of patients with Alzheimer's disease.

CONTENTS

List of abbreviations

6-OHDA	6-hydroxydopamine
ABCA7	Binding cassette subtype family member 7
AChR	Acetylcholine receptor
AD	Alzheimer disease
AIC	Anterior insular cortex
$Al(OH)_3$	Aluminum hydroxide
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid β
BAL	Bronchoalveolar lavage
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
Bg	Background strain for 3xTgAD mice (B6129SF1)
C	Complement component
CD16	IgG receptor III
CD32	IgG receptor II
CD64	IgG receptor I
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disorder
COX	Cyclooxygenase
CR1	Complement receptor 1
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CV	Cross validation
CVO	Circumventricular organ
DAB	Diaminobenzidine
DAF	Decay-accelerating factor
DC	Dendritic cell
DEG	Differentially expressed gene

DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
DT2	Diabetes type 2
EAA	Extrinsic allergic alveolitis
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze
FAD	Familial Alzheimer disease
FcγRI	IgG receptor I
FcγRII	IgG receptor II
FcγRIII	IgG receptor II
FcεR	IgE receptor
GFAP	Glial fibrillary acidic protein
Iba-1	Ionized calcium binding adaptor molecule 1
IC	Immune complex
IDE	Insulin-degrading enzyme
IFNγ	Interferon γ
Ig	Immunoglobulin
IgLC	Immunoglobulin light chain
IL	Interleukin
I.p.	Intraperitoneally
IR	Insulin receptor
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MCI	Mild cognitive impairment
MMSE	Mini-mental status examination
mRNA	Messenger ribonucleic acid
MSD	Meso Scale Discovery
MVA	Multivariate analysis

MW	Molecular weight
MWM	Morris water maze
NFT	Neurofibrillary tangle
NO	Nitric oxide
NSAID	Non-steroid anti-inflammatory drug
OF	Open field
OPLS-DA	Orthogonal to latent structure-discriminant analysis
OVA	Ovalbumin
p-tau	Phosphorylated tau
PA	Passive avoidance
PBS	Phosphate-buffered saline
PC	Principle component
PCA	Principle component analysis
PCR	Polymerase chain reaction
PIE	Pro-inflammatory endotype
PS	Presenilin
ROS	Reactive oxygen species
RT	Room temperature
SCI	Subjective cognitive impairment
SNS	Sympathetic nervous system
t-tau	Total tau
Tg	Transgenic
TGFβ	Transforming growth factor β
Th	T helper cell
TLR	Toll-like receptor
TNFR	Tumour necrosis factor receptor
TNFα	Tumour necrosis factor α
TREM2	Triggering receptor expressed on myeloid 2
UV	Unit variance

PREWORDS

Initial definition of inflammation dates back to the Roman Cornelius Celsus in the 1st century AD, who coined the first four cardinal signs of inflammation: *Rubor et tumor cum calore et dolore* (redness and swelling with heat and pain) (Scott *et al*., 2004). Two centuries later Galen promoted a humoral view, which persisted until the 19th century when the idea came that inflammation, especially pus cells, is a beneficial response to an injury. Although Celsus inflammation indicates an adequate acute inflammatory response following a traumatic injury, it falls short in describing the underlying cellular and molecular processes of the cardinal signs which may occur to induce inflammation at a sub-clinical level, but not be manifested as redness, swelling, heat or pain (Scott *et al*., 2004). This limitation was not overlooked by Rudolph Virchow – one of the greatest 19th century pioneers – who found that symptoms occurring due to increased blood flow (rubor and congestio) were absent in non-vascularized tissues such as cornea or connective tissue. In contrast to Galen, Virchow's inflammation was pathological and consisted of "inflammatory processes" rather than a single uniform process. He added an additional sign – functio laesa (loss of function), underlining the restriction in the function of inflamed tissue (Heidland *et al*., 2006). Virchow had observed an increased number of cells in inflammatory areas and believed that connective tissue was the breeding place for their formation. While Recklinghausen and Cohnheim disproved this concept by demonstrating the migration of inflammatory (pus) cells from their place of origin and the transmigration of leukocytes from the blood vessels into the local inflammatory area (diapedes) (Heidland *et al*., 2009), their contemporary Metchnikoff discovered a very important asset of inflammation – the phagocytosis (Gordon, 2008). The advances in microscopy during the 19th century encouraged cell-based understanding of inflammation, and the discovery in the 20th century of the molecular mediators of inflammation, namely histamine, kinins, anaphylatoxins, added another layer to the understanding of inflammation. Thus, a new definition of inflammation taking into account both the cellular and molecular events was proposed by Rocha e Silva as follows: "*multi-mediated phenomenon, of a pattern type in which all mediators would come and go at the appropriate moment… increasing vascular permeability, attracting leukocytes, producing pain, local edema and necrosis*" (Rocha e Silva, 1978). It is obvious that Rocha e Silva's

definition adds a biochemical perspective to the four cardinal signs but Celsus inflammation survives in the background and is still relevant.

In last decades, inflammation has become one of the hottest topics in the medical research. The discovery of cellular and molecular mediators involved in inflammatory processes have changed the understanding of inflammation and therefore very divergent diseases – such as Alzheimer disease and asthma – have inflammation as a common denominator.

1 INTRODUCTION

1.1 Alzheimer disease

1.1.1 Overview

Alzheimer disease (AD) is a neurodegenerative disorder, which begins with a subtle impairment in memory formation but gradually affects other cognitive domains such as language, orientation, behaviour and executive functions such as planning, problem solving and judgment. The patients' ability to function in daily life declines as the disease progresses and the patients become eventually completely dependent. AD is the most common type of dementia accounting for 50 - 70 % of dementia cases. The prevalence of dementia increases with age and the number of people with the age of 60 years or above is estimated to increase to 1.25 billion by 2050. Today, more than 35 million people live with dementia in the world and the number is expected to double every 20 years, reaching more than 100 million by 2050 (Prince *et al*., 2013). The estimated cost for dementia was approximately 604 billion USD in 2010, which corresponds to 1% of world's gross domestic product (Wimo *et al*., 2013). Thus the increasing dementia cases pose an enormous socioeconomic burden on the society and psychological burden on the family members and caregivers, not to mention the suffering of the patients.

The first description of AD dates back to 1906 when the German physician Alois Alzheimer characterized the main pathological features of AD, amyloid plaque and neurofibrillary tangles (NFTs), in the brain of a demented patient (Maurer *et al*., 1997). In addition, Alzheimer described in detail the symptoms of the disease that are in accordance with the

current diagnostic criteria for AD (McKhann *et al*., 2011) and pointed out glial changes (Alzheimer *et al*., 1995) that were not considered as signs of inflammation until in eighties. Extensive research since last three decades has imparted the field with better understanding of the underlying disease mechanisms. The discovery of causative genetic mutations leading to familial AD (FAD) has played an important role in subsequent modeling of the disease both *in vitro* and *in vivo* for studying pathological processes associated with AD. Advances in the field of genetics have identified several risk genes among which Apolipoprotein E (APOE) 4 has shown the strongest link to AD (Corder *et al*., 1993). Epidemiological studies have highlighted the importance of environmental and lifestyle factors for the risk of developing AD. Imaging techniques, by investigating pathological processes in living patients, have shed light on the time course of pathological changes beginning already at pre-symptomatic stages. Therefore, detecting early pathological changes before the development of symptoms such as memory loss was incorporated into the new diagnostic research guidelines termed as prodromal AD stage. In this front, identification of AD biomarkers, especially in body fluids such as cerebrospinal fluid (CSF) (Brinkmalm *et al*., 2014) and blood (Veitinger *et al*., 2014) are under investigation. The current criteria for diagnosing AD are based on clinical symptoms in combination with, if available, the cerebrospinal fluid (CSF) levels of AD biomarkers, amyloid β (Aβ), phosphorylated tau (p-tau), and total tau (t-tau), and imaging techniques to investigate Aβ burden, brain glucose metabolism and brain volume (Alzheimer's, 2013).

The available pharmacological treatments for AD improve the symptoms temporarily. Disease-modifying treatment strategies that were supposed to delay or halt the progression of AD have failed, despite numerous approaches, possibly because the beneficial therapeutic effect in already established AD with massive neuronal death is difficult to achieve. Furthermore, the potential beneficial effect of a therapy, if any, may have been obscured due to the disease heterogeneity in AD patients. Thus, combination of biomarkers, genetic information such as polymorphisms, epidemiological data such as risk factors and imaging techniques with cognitive assessment of AD patients, allow for better stratification of AD

patients, both at pre- and post-symptomatic stages, and will hopefully result in better outcomes in clinical trials.

1.1.2 Risk and protective factors

Risk factors for AD play an important role in the pathogenesis of the disease (Reitz *et al*., 2011). In addition to aging, which is the most important risk factor, several gene variants that increase the risk for AD including, *complement receptor type I (CR1)*, *sortilin-related receptor 1*, *clusterin* have been identified (Reitz *et al*., 2011). The most common risk gene associated with AD is *apoE,* a lipid-binding protein involved in cholesterol transport, which is present in three allelic variants: *apoE2, apoE3 and apoE4*. The heritage of a single *apoE4* allele increases the risk for AD 2-fold, and double copies of this gene are associated with a 7-fold increase in the risk for AD (Raber *et al*., 2004).

The heritability of late-onset AD is between 58 - 79 % (Humphries & Kohli, 2014) meaning that the remaining risk can be attributed to environmental risk factors. Cardiovascular and metabolic dysfunction such as hypercholesterolemia, hypertension, obesity and diabetes type 2 (DT2) are associated with increased risk for developing AD (Meng *et al*., 2014). Other factors including smoking, depression, psychological stress, and traumatic brain injuries have been linked with increased risk for developing AD (Reitz *et al*., 2011). Infections caused by viruses, especially herpes simplex virus type 1, and bacterial infections such as by *Chlamydophila pneumoniae,* the gram-negative bacteria spirochetes (Maheshwari & Eslick, 2014), and *helicobacter pylori* (Adriani *et al*., 2014), have also been associated with increased risk for AD.

Although the disease-modifying pharmacological treatment for AD have yet not been successful, preventive strategies have been proposed dependent on factors that influence life-style. Diet rich in anti-oxidants and polyunsaturated fatty acids, physica and intellectual activity were associated with improved cognitive performance and decreased risk for developing AD (Reitz *et al*., 2011). In addition, genetic factors may also protect against AD. For example, a mutation in the *amyloid precursor protein (APP)* (A673T) gene in the

Islandic population above the age of 85, was associated with improved cognition and reduced risk for AD (Jonsson *et al*., 2012). The *apoE2* gene protects against AD (Corder *et al*., 1994) probably by being associated with reduced hippocampal atrophy as observed in humans (Chiang *et al*., 2010), and increased Aβ clearance in AD mouse models (Hudry *et al*., 2013).

1.1.3 Pathogenesis

Amyloid plaques in AD brain are mainly composed of aggregated Aβ peptides that are derived from a sequential cleavage of APP by the β- and γ-secretase in the so-called amyloidogenic pathway. The non-amyloidogenic cleavage of APP by α-secretase instead of β-secretase, leads to formation of p3 fragments. In contrast to p3 fragments, Aβ peptides have the propensity to form aggregates and give rise to a wide range of higher molecular species ranging from small oligomers to protofibrils that later develop into amyloid plaques (Goto *et al*., 2008). Aβ is removed enzymatically from the brain, for example by insulin degrading enzyme (IDE) and neprilysin, as well as non-enzymatically by other clearance mechanisms such as phagocytosis, autophagy, drainage along perivascular basement membrane, and transport across blood brain barrier (BBB) through several mechanisms (Miners *et al*., 2011). The amyloid burden in the AD brain seems to be determined by the balance between the production and removal of Aβ (Hyman *et al*., 1993).

NFTs are composed of hyperphosphorylated tau protein. Amyloid plaques and NFTs develop in distinct manner spatially and temporally, and are classified in stages A-C and stages I-VI respectively (Braak *et al*., 1993). Initially, amyloid deposits occur in neocortical regions (stage A), spread into isocortical regions including hippocampus and entorhinal cortex in some cases (stage B), and eventually spread into all isocortical areas including sensory and motor cortex and subcortical areas. Neurofibrillary changes begin in the transentorhinal regions (stage I-II), spread to hippocampus (stage III-VI), and finally reach isocortical regions (stage V-VI) (Braak & Braak, 1997; Thal *et al*., 2002).

Mutations in APP (Goate *et al*., 1991), presenilin (PS) 1 and 2 (Rogaev *et al*., 1995; Sherrington *et al*., 1995) account for approximately 5% of AD cases implying that the

majority of AD cases are sporadic, with unknown cause. The fact that FAD mutations influence the production and processing of Aβ and leads to early onset AD supported the amyloid cascade hypothesis (Hardy & Higgins, 1992), which proposes that accumulation of Aβ is the initial cause for the downstream pathological events including NFT formation, neuronal loss, and subsequent dementia.

Amyloid cascade hypothesis has been questioned due to gaps in providing a complete description of AD pathogenesis. Recent studies showed that, regardless of the presence or absence of Aβ, cognitively normal elderly with markers for neuronal injuries did not differ in glucose hypometabolism, hippocampal atrophy, and in conversion rates to AD. This is an argument against the role of Aβ as an initiator of downstream pathological events in AD (Chetelat, 2013; Knopman *et al*., 2013). Furthermore, the extent of NFTs and neuronal loss correlate better with the severity of dementia than the plaque load in the brain (Giannakopoulos *et al*., 2009). The absence of NTFs in mouse models for AD despite loads of Aβ in the brain (*see Section 1.3.1*) and the failure of clinical trials with Aβ-lowering interventions suggest that amyloid-independent pathological pathways may also occur in AD (Armstrong, 2014).

Dysregulation of other cellular processes such as mitochondrial dysfunction (Lin & Beal, 2006), increased oxidative stress, deficits in glucose metabolism, disturbance in clearance mechanisms *i.e*. autophagy and ubiquitin systems (Butterfield *et al*., 2014), and dysregulated inflammatory processes (Morales *et al*., 2014) have been implicated in the pathogenesis of AD. Thus sporadic AD is a multifactorial disease, which may originate from distinct underlying pathologies involving amyloid-dependent and -independent processes in a complex interplay between genetic and environmental factors.

1.1.4 Inflammation in the brain in Alzheimer disease

Evidence for the presence of inflammation in post mortem brain from AD patients was described for the first time in early eighties, when amyloid plaques were shown to be associated with immunoglobulins (Igs), complement components (C) 1q, C3 and C4

(Eikelenboom & Stam, 1982). Subsequent studies showed increased microglial activation, especially adjacent to the amyloid plaques (McGeer *et al*., 1987; McGeer *et al*., 1988), and increased production of the pro-inflammatory cytokines interleukin (IL)-1 and IL-6 (Griffin *et al*., 1989). Thus, the activity of multiple immune pathways including cytokines, complement system, membrane attack complex (MAC), chemokines, and acute phase proteins are increased in the AD brain (for extensive review see (Akiyama *et al*., 2000)). *In vitro* studies showed that Aβ *per se* contributed to inflammation by binding to receptors for advanced glycation end-product (Yan *et al*., 1996), scavenger receptors (El Khoury *et al*., 1996; Paresce *et al*., 1996), toll-like receptors (TLRs) (Walter *et al*., 2007; Reed-Geaghan *et al*., 2009), to activate microglia and astrocytes. Activated glial cells produce a wide range of inflammatory mediators including complement factors, cytokines, reactive oxygen species (ROS), secreted proteases, excitatory amino acids, and nitric oxide (NO) (Akiyama *et al*., 2000; Lyman *et al*., 2014) that can cause mitochondrial dysfunction (Wilkins *et al*., 2014), synaptic dysfunction, inhibition of neurogenesis, and neuronal death (Lyman *et al*., 2014). In addition, Aβ was shown to activate the classical (C1q) and alternative (C3) complement pathways (Tuppo & Arias, 2005), and to enhance the production of tumour necrosis factor α (TNFα), IL-1β and IL-6 (Del Bo *et al*., 1995; Pan *et al*., 2011). Stimulation with pro-inflammatory cytokines, TNFα, interferon γ (IFNγ), IL-1β and IL-6, in turn increased the levels of Aβ by enhancing βAPP processing (Dash & Moore, 1995; Blasko *et al*., 2000; Yamamoto *et al*., 2007) to favour the amyloidogenic pathway or by enhancing the expression of APP (Ringheim *et al*., 1998), thus resulting in a self-perpetuating vicious circle (Del Bo *et al*., 1995).

Activated microglia surrounding the amyloid plaques are found in the neocortex of patients with low Braak stages of AD-pathology, preceding the later stages that are characterized by neurofibrillary changes (Arends *et al*., 2000; Vehmas *et al*., 2003; Hoozemans *et al*., 2006).

Studies in transgenic animal models of AD, by confirming the inflammatory aspects found in AD patients (Apelt & Schliebs, 2001; Abbas *et al*., 2002; Patel *et al*., 2005), not only supported the role of inflammatory processes in AD pathogenesis, but also highlighted inflammation as an early event in AD. For instance the increase in inflammatory response in 3xTgAD mice (Janelsins *et al*., 2005) and Tg2576 mice (Tehranian *et al*., 2001) precedes

amyloid pathology. Studies in humans have shown that mild cognitive impairment (MCI) (*see section 3.1.3*) patients had increased levels of inflammatory markers in the CSF as compared to control subjects (Brosseron *et al*., 2014), with parallel increase in microglial (Okello *et al*., 2009; Fan *et al*., 2014) and astrocyte (Carter *et al*., 2012) activation in the brain as revealed by *in vivo* imaging studies, thus supporting the idea that inflammation is an early phenomenon in the course of AD. Interestingly, increased microglial activation was correlated with glucose hypometabolism in both AD and MCI patients (Fan *et al*., 2014), suggesting that microglial activation may be linked to synaptic dysfunction in these patients. In addition, these studies provide evidence that findings from *in vivo* studies in humans are in agreement with the findings obtained in mouse models for AD, which increase the reliability of models for translational purposes.

There is evidence that infiltration of peripheral immune cells belonging to the innate immune arm, such as neutrophils (Baik *et al*., 2014), monocyte/macrophages and acquired immune arm, such as T-cells, occurs in the AD brain (Togo *et al*., 2002). The role of different T helper (Th) cell subsets has been well characterized in multiple sclerosis, but emerging data suggest a role for Th-17, Th-9 and Th-1 cells in the development of chronic inflammation in AD (Saresella *et al*., 2011; Gonzalez & Pacheco, 2014) although the knowledge in this field is limited.

1.1.5 Systemic inflammation in Alzheimer disease

Beyond the presence of inflammatory mediators in the brain of AD patients, several lines of evidence support the role of inflammation in AD pathogenesis. Genome-wide association studies revealed that polymorphisms in several genes encoding inflammatory proteins were associated with a risk for AD. Of particular interest are the genetic variants that influence the innate immunity such as triggering receptor expressed on myeloid 2 (TREM2), adenosine triphosphate - binding cassette subtype family A member 7 (ABCA7), and CR1 (Wilkins *et al*., 2014). TREM2 has been shown to suppress cytokine activation and to polarize microglia towards a phagocytic phenotype (Humphries & Kohli, 2014; Wilkins *et al*., 2014), ABCA7 plays role in lipid transport across the membrane and regulates phagocytosis by macrophages

(Hollingworth *et al*., 2011), whereas CR1 activates complement pathway and phagocytosis (Wilkins *et al*., 2014). Interestingly, all these genes affect phagocytosis or the clearance mechanisms of the cell.

Epidemiological studies in the middle of the 1990s revealed that chronic usage of non-steroidal anti-inflammatory drugs (NSAIDs) reduced the risk of developing AD (McGeer *et al*., 1996), suggesting that systemic inflammation may play a role in AD. Furthermore, several risk factors for AD, including aging, obesity, diabetes, hypertension, and smoking (Reitz *et al*., 2010), are associated with increased systemic inflammation (Yaffe *et al*., 2004). Evidence for low-grade systemic inflammation has been found in the plasma and CSF of AD patients, with a shifted balance towards a pro-inflammatory profile (Ahluwalia & Vellas, 2003). According to a meta-analysis, increased levels of IL-1β, IL-6, IL-12, IL-18, TNFα, and transforming growth factor (TGF) β were found in the plasma of AD patients, whereas only increased TGFβ was found in the CSF (Swardfager *et al*., 2010). Early studies focused on investigating the relationship between peripheral inflammatory markers and cognitive decline in old adults to assess predictive capacity of inflammatory markers for future dementia (Bettcher & Kramer, 2014). Some studies found that elevated levels of acute phase proteins such as C-reactive protein (CRP), α-1-antichymotrypsin, and cytokines including IL-6 and TNFα, were associated with increased risk for AD in elderly subjects (Engelhart *et al*., 2004; Dik *et al*., 2005; Dziedzic, 2006). Other studies found no association between baseline inflammation and future risk for AD or other dementia (for review see (Bettcher & Kramer, 2014)), or even inverse correlation, especially in older ages when increased baseline levels of CRP were negatively associated with cognitive decline in the oldest old (median age 77 years) (Lima *et al*., 2014). Similar findings have been reported for cardiovascular factors that confer risk at midlife but show an inverse association with cognitive decline in later ages (van den Berg *et al*., 2007). It is possible that the early and late stages on the continuum towards AD have different underlying mechanisms.

High plasma and CSF levels of soluble TNF-receptor I (TNFRI) in MCI were related to increased risk of conversion to AD (Buchhave *et al*., 2010; Diniz *et al*., 2010). With regard to association between the levels of inflammatory markers and cognitive decline, AD patients

with high serum TNFα levels at baseline had increased cognitive decline compared to those with low serum levels of TNFα (Holmes *et al*., 2009). In addition, acute inflammatory events such as infection and trauma (Holmes *et al*., 2009) or delirium (Cunningham, 2011) were associated with further increase in cognitive decline. Leung et al. found an association between the cytokine levels in plasma and disease severity when AD patients were divided based on their cognitive decline into slow, intermediate and fast decliners (Leung *et al*., 2013). Interestingly, the levels of anti-inflammatory cytokines IL-4 and IL-10 were increased in the fast decliners, whereas the pro-inflammatory cytokines IL-2 and IFNγ, but also IL-4 levels were higher in intermediate decliners compared to the slow decliners, indicating the heterogeneity in the AD population.

Changes in peripheral immune cells occur in AD (Singh *et al*., 1986; Song *et al*., 1999). Shad et al. found that the levels of monocytes were higher in the serum of AD patients when compared to the normal range described for monocyte counts, and the AD patients with that showed normal counts of monocytes had higher levels of neutrophils (Shad *et al*., 2013). Other studies on the functional state of peripheral immune cells described increased levels of mitochondrial lipid peroxidation markers in leukocytes from MCI patients and an increase in mitochondrial oxidative stress proteins in leukocytes from both MCI and AD patients (Sultana *et al*., 2013). Blood transcriptome analysis of MCI and AD showed perturbation of mitochondria, ribosomes, and the immune system (Han *et al*., 2013). Elevation of mitochondrial stress markers in neutrophils (Vitte *et al*., 2004), increased activation of platelets (Casoli *et al*., 2010), and perturbation in phagocytic processes of macrophages (Fiala *et al*., 2005), have been reported to occur in AD. When comparing $A\beta_{1\text{-}42}$ stimulated Th-1 responses in healthy elderly subjects with healthy middle age and healthy young controls, the levels of IFNγ producing Th-1 responses declined progressively with age, whereas those of IL-10 increased (Loewenbrueck *et al.*, 2010). Interestingly, IL-10 responses were higher in AD patients and in patients with Down syndrome compared to healthy elderly subjects. While IFNγ producing Th-1 responses was decreased in aged healthy controls, the same responses were diminished in AD patients and patients with Down syndrome (Loewenbrueck *et al.*, 2010), indicating a failure in peripheral immune system in AD distinguished from that

of aging. Altogether these studies highlight the complexity of immunological changes in AD in which the failure is not only confined to the brain, but peripheral failures may also have substantial contribution.

1.1.6 The dual nature of inflammation in Alzheimer disease

Many of the mediators of inflammation, including IL1-β, IL-6, IL-10, and TNFα, are expressed in the brain, and at physiological levels play important roles in processes such as neurogenesis and in learning and memory (Erta *et al*., 2012; Estes & McAllister, 2014). It can be argued that an increase or decrease in the levels of inflammatory mediators may lead to pathological conditions or disturbance in brain function. Likewise, microglia in the brain display both pro-inflammatory and non-inflammatory functions. In normal conditions, "resting" microglia monitor the microenvironment and synapses by extending and retracting processes, and this surveillance function plays an important role in maintenance of brain homeostasis. It has been shown that microglia, in addition to its role in synapse remodelling, promote beneficial responses by inducing neurogenesis in the hippocampus and dentate gyrus, releasing growth factors, and phagocytosis of debris after stroke (Polazzi & Monti, 2010). Increasing evidence suggest a complex role for inflammation in AD. Thus, in addition to the detrimental role of inflammatory mediators in AD as discussed in the previous chapter, the protective role of inflammation in AD is supported by other studies. Overexpression of IL-6 (Chakrabarty *et al*., 2010b) and IL-1β (Shaftel *et al*., 2007) in a mouse model for AD was shown to result in microglial activation and reduced amyloid deposition by enhancing phagocytosis (Chakrabarty *et al*., 2010b). Although the 3xTgAD mice (*see section 1.3.1 and Table 1*) exhibit increased levels of TNF-α, as also seen in AD, blocking of TNFα signalling by knocking out the cognate receptors for TNFα (TNFRI and TNFRII) in 3xTgAD mice was associated with increased amyloid deposition and enhanced tau phosphorylation (Montgomery *et al*., 2011). Moreover, microglia from TNFR deficient 3xTgAD mice showed defective phagocytosis, thus highlighting the protective role of TNFα in facilitating clearance mechanisms. In these studies, the elevated levels of inflammatory markers are present prior to AD-pathology, thus underscoring the impact of the temporal aspect on the detrimental or

beneficial outcome of inflammation. The inflammatory milieu could be another factor in determining the outcome of inflammation. The induction of Th-2 responses with elevated anti-inflammatory cytokine levels in an already established pro-inflammatory milieu in 3xTgAD mice led to beneficial responses by offsetting the pro-inflammatory milieu (Jung *et al*., 2012). The pro-inflammatory cytokine IFNγ plays an important role in neurogenesis at physiological levels in brain. Although a decline in neurogenesis has been observed in wildtype and APP-Tg mice in an age-dependent manner, a moderate IFNγ overexpression in APP-Tg mice showed almost two-fold higher neurogenesis (Baron *et al*., 2008), suggesting that small increases in IFNγ could compensate for age-related decline in neurogenesis.

Activation of complements occurs at early stages in AD and increases in advanced stages of the disease (Zanjani *et al*., 2005). The messenger ribonucleic acid (mRNA) levels of complement proteins were higher in brain than in liver, indicating local synthesis in the brain (Yasojima *et al*., 1999). Analysis of post-mortem brain tissue from AD patients showed a significantly higher number of amyloid plaques associated with staining for MAC, and this correlated with synaptic loss (Lue *et al*., 1996), suggesting a detrimental role for complement. In contrast, the risk allele in CR1 associated with AD has an additional binding site for C3b/C4b, and may dampen the complement pathway and C3b mediated opsonisation and clearance of Aβ (Brouwers *et al*., 2012). The protective role of complement in Aβ clearance and phagocytosis was reported in mouse models of AD. Blocking of the complement system by overexpression of soluble Crry (complement inhibitor in rodents) (Wyss-Coray *et al*., 2002) or C3 deficiency (Maier *et al*., 2008) in a mouse model for AD increased the levels of Aβ, which was not due to increased production of Aβ but rather reflected the role of complement in the clearance mechanism. In the same studies, blocking complement was associated with decreased neuronal loss in the hippocampus. Both Crry overexpression and C3 deficiency leads to reduced levels of C5a. It is possible that the protective effect of complement blockage is due to reduced levels of C5a or hindering of MAC formation. This is supported by findings from 3xTgAD and Tg2576 mice in which anti-C5a antibodies decreased inflammation and improved behaviour (Fonseca *et al*., 2009), indicating the complex nature of immune processes.

Additional examples of the dual role of inflammation in AD could be derived from the clinical trials involving NSAIDs. Despite the reduced risk for developing AD associated with long-term medication with NSAIDs (McGeer *et al*., 1996), clinical trials in patients with established AD were not therapeutically beneficial (Leoutsakos *et al*., 2012). Leoutsakos et al. stratified the patients in the preclinical AD group that received naproxen or celecoxib into three phases based on their cognitive decline (Leoutsakos *et al*., 2012), meaning that patients with a fast decline in cognition probably would be closer to AD than those with little or no cognitive decline. Interestingly, naproxen was beneficial in fast decliners, but had no effects in non- or little decliners, or in slow decliners. In contrast, celecoxib was beneficial in slow decliners, but harmful in the fast decliners. Celecoxib is a selective inhibitor of cyclooxygenase (COX)-2, and its expression peaks in neurons in Braak phase (NFT) 0-II, probably representing an adaptive response, which subsequently declines (Hoozemans *et al*., 2008). Thus, blocking of COX-2 in early phases may therefore be harmful whereas blocking of COX-1 by naproxen (in addition to COX-2) may be beneficial, since the microglial COX-1 expression increases in later Braak phases III-IV (Hoozemans *et al*., 2008). Stratification of AD patients based on pro-inflammatory endotypes (PIE), which was characterized by plasma TNFα and CRP levels, showed that 12 months treatment with naproxen had a positive effect on cognition in the subset of patients with high baseline PIE, whereas opposite effects of the treatment were observed in those with low baseline PIE (O'Bryant *et al.*).

1.2 Allergy

1.2.1 Airway allergy - Overview

Allergy is a chronic inflammatory disease, often with onset already during childhood or adolescence, affecting more than 20% of the Western population. Asthma, allergic rhinitis and atopic dermatitis are among the most commonly encountered chronic allergic diseases, often referred to as atopic disorders. More than 300 million people suffer from asthma worldwide and the global prevalence of asthma ranges from 1-18% in different countries (Bateman *et al.*, 2008). Asthma is a heterogeneous disorder, the exact cause is not known but

genetic factors (atopy, history of asthma in the family), environmental factors (air pollution, viral infections, allergen exposure, occupational exposure), and life-style factors (food, smoking), influence the disease (Alberi, 2013).

The allergic cascade crucial for development of asthma appears in three phases namely the induction phase, early phase and late phase. During the induction phase, the allergens enter the airways, are processed and brought to lymph nodes by antigen-presenting cells, and are presented to T- and B-cells. Activation of T-cells - especially Th-2 cells - leads to production of cytokines, and anti-inflammatory cytokines of which IL-4, IL-5, IL-9 and IL-13 are the most important in the development of asthma. IL-4 and IL-13 play a role in the development of IgE antibodies, IL-5 is important for the production and mobilization of eosinophils, and IL-9 is important for mast cell development. In the early phase, lasting between 30 and 60 min, mast cells secrete inflammatory mediators (histamine, proteases, prostaglandins, leukotrienes, cytokines, growth factors) after activation by allergen, leading to constriction of airway smooth muscles, vascular leakage, mucus production, enhanced airway hyperresponsiveness, and recruitment of inflammatory cells. During the late phase reaction 4 - 6 h later, activated Th-2 cells release IL-4, IL-5, and IL-13, with subsequent IgE isotype-switching in B-cells, eosinophil activation, and release of pro-inflammatory mediators. Various cells such as eosinophils, neutrophils, dendritic cells, T-cells, macrophages, endothelial cells, airway smooth muscle cells, and bronchial epithelial cells, and the mediators released by these cells, contribute to airway remodelling *i.e.* airway wall thickening, subepithelial fibrosis, globlet cell hyperplasia, airway smooth muscle hyperplasia and hypertrophy, and epithelial hypertrophy (for review see (Bloemen *et al.*, 2007; Diamant *et al.*, 2013)).

Extensive research in the field of asthma have led to subtyping the disease into endotypes (subtype of the disease with distinct pathogenic mechanisms), which could prove useful for designing therapies tailored to the individual's biology. Agache et al. characterized five endotypes of asthma:

a) allergic asthma, often with onset at childhood, driven by Th-2 responses and recruitment of eosinophils in the airways; the presence or exposure to the allergen drives the Th-2 inflammation; is often steroid-responsive
b) intrinsic (non-atopic) asthma, which represents one third of all adult asthmatics, shares similarities with allergic asthma, but with higher levels of the pro-inflammatory cytokines IL-2 and IFNγ, but no change in IL-4 in the bronchoalveolar lavage (BAL) fluid; allergens have no obvious role in driving the inflammation; can be both steroid-responsive and steroid-resistant
c) non-eosinophilic asthma, characterized by the presence of neutrophils in the airways instead of eosinophils; steroid-resistant
d) aspirin-intolerant asthma; affects 5 - 10% of asthmatics, more common in non-atopic asthmatics with greater sensitivity to leukotrienes; steroid-responsive
e) extensive remodelling asthma, characterized by minimal inflammation, but extensive airway remodelling, with production of angiogenic and lymphangiogenic factors; steroid-resistant (Agache *et al.*, 2012)

The response to existing asthma treatments, including β_2-agonists and steroids, shows individual variation, and non-eosinophilic asthmatics usually respond poor to glucocorticoids. Treatment based on the underlying disease mechanism has proven efficient, for example in the case of anti-cytokine therapy with Mepolizumab (anti-IL-5 antibody) in severe asthmatics with persistent eosinophilia (Agache *et al.*, 2012; Lin *et al.*, 2013).

1.2.2 Allergy and the brain

There is a wide literature on the effects of systemic inflammation on the central nervous system (CNS) associated with bacterial and viral infections, generally accompanied by Th-1 responses. However, studies on the effects of systemic inflammation associated with Th-2 responses, such as allergic diseases, on the brain are emerging. It is clear that asthma symptoms are accentuated during periods of increased stress or emotions, and this was shown in a study of undergraduate asthmatics who, during the final examination week, showed larger airway inflammation and airway obstruction in response to allergen challenge,

compared to the stress-free period (Liu *et al*., 2002). In studies of food allergies, mice orally challenged with ovalbumin (OVA) showed increased anxiety-like behaviour as shown by the time spent in the open arms of elevated plus maze (EPM). The neural correlates for these behavioural changes included increased activity in specific brain regions; the paraventricular nucleus of the thalamus, the central nucleus of amygdala, and the nucleus of the solitary tract (Basso *et al*., 2003; Costa-Pinto *et al*., 2005). OVA-sensitized mice also showed preference for the otherwise aversive lit chamber, instead of the dark chamber where they were previously exposed to the allergen (Costa-Pinto *et al*., 2007). In human asthmatics, asthma-specific words induced increased activity in the anterior insular cortex (AIC), following allergen challenge. The AIC activity correlated with increase in the number of eosinophils in the sputum (disease severity) (Rosenkranz *et al*., 2012). Hypoxia resulting from asthma may have secondary effects on brain and cognition (Alberi, 2013).

The components of the nervous system (sympathetic, parasympathetic and sensory) can modulate the immune processes involved in the development of allergic reaction, either directly or indirectly. For example, the sympathetic nervous system (SNS) modulates antigen uptake and processing in dendritic cells (DCs) through adrenergic receptors expressed on DCs. Similarly, the SNS modulates Th-1 and Th-2 balance in T-cells towards an anti-inflammatory profile and enhance humoral responses by increasing Ig production from B-cells (Forsythe, 2012). Immune cells such as eosinophils, neutrophils and macrophages, but also endothelial cells, express nicotinic acetylcholine receptors (AChRs), through which the parasympathetic nervous system exerts its anti-inflammatory action (Kolahian & Gosens, 2012). Thus, it is not surprising that stress exacerbates the course of disorders such as asthma and AD in which inflammation is associated with its pathogenesis. Peripheral sensory neurons regulate inflammation locally by modulating the responses in immune cells through release of neuropeptides such as substance P, neuropeptide Y and vasoactive intestinal polypeptide (Forsythe, 2012). Interestingly, neurons have been shown to express receptors for Igs, supporting the idea that the cross-talk between the immune and nervous system is bidirectional. Dorsal root ganglion (DRG) neurons express high affinity receptors for IgG (FcγRI or CD64) and for IgE (FcεRI) (Andoh & Kuraishi, 2004b). The formation of IgG-

antigen complex on sensitized DRG neurons induced an increase in intracellular calcium levels and the release of substance P from these neurons (Andoh & Kuraishi, 2004a). Furthermore, it was shown that sensitized DRG neurons not only bound IgE, but also Ig light chain (IgLC), and that IgLC-binding increased the intracellular calcium levels (Rijnierse *et al*., 2009).

1.2.3 Allergy and Alzheimer disease

The first report that investigated the association between atopic allergic diseases (allergic rhinitis, allergic dermatitis, and allergic asthma) and AD showed that atopy was associated with a 16% increase in the risk for AD (Eriksson *et al*., 2008). In addition, asthma was associated with reduction in survival time. Subjects with asthma lived on average 1.8 years less than non-asthmatics (Eriksson *et al*., 2008). Subsequent epidemiological studies on airway disorders, such as asthma and chronic obstructive pulmonary disorders (COPD), in the elderly have shown a positive association to cognitive impairment and/or dementia (Rusanen *et al*., 2013; Singh *et al*., 2013; Chen *et al*., 2014; Peng *et al*., 2014; Singh *et al*., 2014). Rusanen et al. found that mid-life (age range 39 - 64 years) asthma and COPD, respectively, were associated with an almost two-fold increase in the risk for cognitive impairment, whereas late-life (age range 65 - 80 years) diagnosed asthma and COPD were associated with reduced risk (Rusanen *et al*., 2013). However, in the study of Chen et al., although asthma was associated with a more than two-fold increase in the risk for developing any dementia or AD, subgroup analysis showed that both mid- (age range 45 - 64 years) and late-life (≥ 65 years) asthma were associated with almost two-fold increase in risk for any dementia, whereas only late-life asthma was associated with almost three-fold increase in risk for AD (Chen *et al*., 2014). Other factors, such as asthma exacerbations (Peng *et al*., 2014) or duration of COPD (Singh *et al*., 2013), were associated with an increased risk for MCI. The mechanisms of interaction between allergic diseases and AD have not been studied. As discussed above, allergic diseases are heterogeneous, with different inflammatory profiles, and consequently the interaction of allergy with AD can be very complex.

1.2.4 Neuroimmune communication

Following systemic infection, the body activates the innate immune response by inducing the production of inflammatory mediators such as cytokines, chemokines, and prostaglandins at the site of infection to eliminate the pathogen. This response is followed by production of other mediators that promote phagocytosis of the pathogen and debris, and induce repair to re-establish homeostasis. If the inflammation is unresolved by innate immunity, the body mounts up the adaptive immune response, which in contrast to innate immunity, is highly specific and involves activation of T- and B-cells.

The neuroimmune communication and the impact of systemic inflammatory responses – for example induced by bacterial or viral antigens – on the brain has been studied extensively. Peripherally produced cytokines such as IL-1β, IL-6 and TNFα, in response to LPS infection have been shown to induce sickness behaviour *i.e.* fever, nausea, reduced appetite, loss of interest in social and physical activity, fatigue, and fragmented sleep (Dantzer *et al*., 2008). Systemic administration of lipopolysaccharide (LPS) was shown to increase the production of IL-1β and TNFα and other inflammatory mediators in the brain (van Dam *et al*., 1992; Laye *et al*., 1994; Quan *et al*., 1999; Eriksson *et al*., 2000). Several pathways are implicated in facilitating neuroimmune communication. One pathway involves the activation of the vagus nerve by locally produced cytokines. The second pathway is through activation of resident macrophages in circumventricular organs (CVOs). As these structures do not have a BBB, the pro-inflammatory cytokines produced by macrophages could enter the brain parenchyma. The third pathway involves the transport of cytokines (excessively produced) across the BBB by means of saturable transport systems (Dantzer *et al*., 2008). In the fourth pathway, the activation of IL-1 receptors on perivascular macrophages and endothelial cells of brain venules, by circulating cytokines, leads to production of prostaglandins (E2 series) that diffuse into the brain, and play role in activation of hypothalamic pituitary axis and cytokine induced fever (Konsman *et al*., 2002; Dantzer *et al*., 2008).

1.3 Animal models

1.3.1 Mouse models for Alzheimer disease

Several types of transgenic (Tg) mouse models for AD have been developed by incorporation of human genes with FAD mutations into the mouse genome. The neuropathological features of some of the commonly used Tg strains are presented in *Table 1*. Although manifestation of AD-like pathology and related pathological processes occur at different time points (*see Table 1*), the bottom line is that the Tg mice mirror some features of human AD including amyloid accumulation, abnormal hyperphosphorylation of tau, neuroinflammation, cognitive deficits, and impairment of synaptic plasticity. Development of amyloid pathology differs from strain to strain, and aspects including number and types of FAD mutations incorporated into the mouse genome, site of integration in the deoxyribonucleic acid (DNA), type of promoter driving the gene expression, and the genetic background of mice may contribute to some of the observed differences. Moreover, the isoforms of APP used for overexpression may account for some differences. Although the APP695 isoform is predominantly expressed by neuronal cells in the brain, Kunitz-type serine protease inhibitor-containing isoforms of APP (APP751) is found on glial cells and a slight increase in APP751 with aging has been reported in humans (though not on glial cells), and may play a role in amyloid formation (Rockenstein *et al*., 1995). The expression levels of human APP differ between the strains (*See Table 1*), and several studies show an association between transgene expression levels and development of plaque pathology (Sturchler-Pierrat *et al*., 1997; Chishti *et al*., 2001; Ronnback *et al*., 2011). Although, none of the Tg mice represent the full spectrum of human AD, they have been important *in vivo* tools for studying AD pathogenesis, to provide insights into disease mechanism, and to evaluate disease-modifying therapies and biomarkers. An ideal AD model should mimic most, if not all, aspects of AD that include the pathogenesis of the disease *i.e* its etiology and progression of the pathology. The available animal models for AD suffer from major limitations: (i) sporadic AD accounting for more than 95% of AD cases, does not have familial mutations, (ii) mouse models for AD do not show neurodegeneration – a substantial hallmark in AD, (iii) the tangle pathology – another hallmark of AD – is absent

in these mice despite the presence of hyperphosphorylated tau, unless the tangle pathology is driven by a mutated tau gene (3xTgAD and APPSWE-Tau) that is not present in human AD. In conclusion, mouse models for AD are mostly models of amyloidosis, raising major concerns when extrapolating data from models to human conditions, and may probably explain why the majority of compounds proving successful in Tg mice failed in human trials (for review see (Franco & Cedazo-Minguez, 2014)). Nevertheless, a new rat model of AD developed on mutant APP and PS1 genes, showed age-related amyloidosis, tangle pathology, gliosis, behavioural deficits, and neuronal loss in hippocampus and cortex, and may offer a more human-like model of AD, compared to mouse models (Cohen *et al*., 2013).

1.3.2 Mouse models for asthma

Asthma is a predominantly human disorder although cats and horses develop airway allergy analogous to the human condition (Mullane & Williams, 2014). Models of asthma, acute and/or chronic, have been developed in several species including rodents and non-human primates (Mullane & Williams, 2014; Mercer *et al*., 2015), but the most commonly used and extensively investigated model is the OVA-mouse model based on (i) sensitization to and (ii) subsequent airway challenge with the allergen, namely OVA. The nature of inflammatory response, and the development of associated changes, depend on several factors including choice of strain, route and dose of sensitization, the nature of adjuvant, the type of allergen, and the route and duration of the challenge (Kumar *et al*., 2008). The route of sensitization influences the strength of the immunological response since mice sensitized intraperitoneally (i.p.) showed higher levels of allergen-specific IgE levels compared to intranasal or mucosal sensitization (Zhang *et al*., 1997; Zhu & Gilmour, 2009). An adjuvant, such as aluminum hydroxide ($Al(OH)_3$), is used to boost the humoral immunity towards Th-2 skewing (Eisenbarth *et al*., 2008; Kool *et al*., 2008), one of the features of allergic (atopic) asthma (Agache *et al*., 2012). The mouse strains affect the type of allergic inflammation in terms of cytokine profile, immune responses and airway responsiveness (Zhang *et al*., 1997; Zhu & Gilmour, 2009; Kelada *et al*., 2011). For instance, increased

levels of eosinophils are found in the BAL of Balb/c mice following allergy, while an increase in neutrophils in addition to eosinophils is found in C57BL6 mice (Whitehead *et al*., 2003; Sarlus *et al*., 2012). Furthermore, other intrinsic differences between the strains such as alveolar size (Soutiere *et al*., 2004) and lung mechanics (Tankersley *et al*., 1999), may also affect the nature of allergic inflammation.

The OVA-mouse models have played a pivotal role in investigating the molecular mechanism of allergic inflammation by recapitulating significant clinical feature of human asthma including lung eosinophilia, Th-2 driven cytokines (IL-4, IL-5, IL-13), increased mast cell activation, airway hyperresponsiveness and remodelling (Mercer *et al*., 2015). Despite showing patterns of human-like asthma and responding positively to glucocorticoids and to β2-receptor agonists, the models of asthma have substantially failed in translating to human conditions (Mercer *et al*., 2015), most likely due to the heterogeneous nature of asthma, with several phenotypes with different etiology and distinct cellular and molecular mechanisms (Agache *et al*., 2012). Airway inflammation in intrinsic asthma shares similarities with allergic asthma – often with onset in childhood – but IL-2, IFNγ and not IL-4 are increased in the BAL fluid, whereas neutrophilic asthma – often with adult onset – is characterized by the absence of eosinophilia and increased Th-17 response (Agache *et al*., 2012). Thus, extrapolating to a heterogeneous disorder such as asthma from mouse models that largely represent the allergic/eosinophilic phenotype (Kumar & Foster, 2012), can possibly succumb into failure. In order to increase the success for translation, the model used should be appropriate for the outcome that is being studied (Kumar & Foster, 2012; Mercer *et al*., 2015). For example differences at the molecular level, such as β1-receptor predominance on mouse airways compared to β2-receptor in humans, or different subsets of mediators released by mast cells of mouse *vs* human, may influence outcomes for the translation approaches (Mercer *et al*., 2015). Thus, guinea pigs will be a suitable model for studying outcomes involving β-receptors in airways compared to mice (Mercer *et al*., 2015).

Table. 1 Characteristics of some of the commonly used Tg mouse models for AD

Name	Transgene	Promoter	APP expression level	Amyloid pathology	Tau pathology	Gliosis	Cognitive impairment	Synaptic loss	Primary Reference
3xTgAD	APP695 (K670N/M671L) PS1 (M146V) Tau (P301L)	Thy1.2	6-8-fold	6 months	12 months	Yes	4 months	Unknown	(Oddo *et al*., 2003)
Tg2576	APP695 (K670N/M671L)	Hamster PrP	6-fold	11 - 13 months	Absent	Yes 10 - 16 months	6 - 12 months	4.5 months	(Hsiao *et al*., 1996)
APPSwe-Tau	APP69 (K670N/M671L) Tau (P301L)	Hamster PrP	Similar as Tg2576	6 - 7 months	3 - 6 months	Yes 3 months	Unknown	Unknown	(Lewis *et al*., 2001)
5xFAD	1st transgene: hAPP695 (K670N/M671L) (Swe) I176V(Florida) V717I (London) 2nd transgene hPS1 M146L L266V	Thy 1		1.5 - 2 months	Absent	Yes	4 - 5 months	Yes Progressive 9 - 12 months + Neuronal loss	(Oakley *et al*., 2006)
TgCRND8	hAPP965 (K670N/M671L) (Swe) V717F Indiana	Hamster PrP	5-fold	3 months	Absent p-tau at 7 - 12 months	Yes 2.5 - 3 months	3 months		(Chishti *et al*., 2001)
APPPS1	hAPP695 (K670N/M671L) PS1 (l166P)	Thy 1	3-fold	1.5 months	Absent. p-tau positive neurites observed	Yes 1.5 months	7 - 8 months	Yes 4 months Neuronal loss at 17 months	(Radde *et al*., 2006)
APP23	hAPP751 (K670N/M671L)	Thy 1	7-fold	6 months	Absent. p-tau positive neurites observed	Yes Associated with plauqes at 6 months	3 months	Neuronal loss 14 - 18 months Synaptic loss absent at as old as 24 months	(Sturchler-Pierrat *et al*., 1997)
Tg-Arc-Swe	hAPP695 (K670N/M671L) (E693G)	Thy 1.2	3-fold	5 - 6 months	Absent	Yes	4 - 8 months	Unknown	(Lord *et al*., 2006)
PDAPP (line 109)	hAPP 695, 751, 770 V717F (Indiana)	hPDGF-β	10-fold	6 months	Absent p-tau at 14 months	Yes associated with plaques	3 months	Synaptic loss present at 8 months	(Games *et al*., 1995)

In addition, the branching pattern of the lungs in mice, and the diameter of the airways in relation to the body size differ substantially between mice and humans (Kumar *et al*., 2008). Despite such differences, the mouse models for asthma have yielded valuable information regarding the pathogenesis of asthma.

In conclusion, animal models are valuable tools for studying *in vivo* mechanisms as long as we are aware of their limitations and the heterogeneous nature of the disease we are investigating. Different features of the disease are presented by different models, *e.g*. the Tg2576 model for AD (Hsiao *et al*., 1996) can serve as a good model for studying amyloidosis, whereas 3xTgAD mice (Oddo *et al*., 2003) can also allow for studying tau pathology (but not AD-like tau pathology). Similarly, mouse models of asthma show features that are relevant for the eosinophilic or allergic asthma phenotype in humans (Kumar & Foster, 2012). Thus, one could argue (in our case) that the "true" effects of asthma on AD pathogenesis may (i) depend on and (ii) possibly be limited to those aspects of disease that are represented by our model.

2 AIMS

The main aim of this thesis was to study the effects of peripheral inflammation associated with allergy on the brain in presence or absence of AD-like pathology.

The specific aims were to investigate:

Paper I — the effects of airway-associated allergy on inflammatory markers and AD-related proteins in the brain using naive mice.

Paper II — the effects of airway-associated allergy on gene expression in the brain of naive mice.

Paper III — the effects of airway-associated allergy on the brain with regard to inflammatory markers, AD-related proteins, and behaviour using 3xTgAD mice and their background strain.

Paper IV — whether presence of allergy influences the levels of immunoglobulin classes, and the levels of pro- and anti-inflammatory cytokines, in the CSF and serum from patients with AD, MCI, and subjective cognitive impairment (SCI).

3 METHODOLOGY

The detailed description of the experimental procedures used in **Paper I-IV** is provided in the respective paper. In this section, methods will be summarized with a brief account of the advantages and limitations for some of the models and methods.

3.1 Methods for studying the pathogenesis of Alzheimer disease

3.1.1 Mouse models

We have used male mice in all studies, which may show less variation compared to female mice due to hormonal changes. However, variation is unavoidable because male mice, by nature, will establish hierarchy and the less dominant mice will be more stressed. Balb/c mice (**Paper I**) are good Th-2 responders and were used for development and validation of the allergy protocol but further analyses were performed in C57BL6 strain (**Paper I, II**), which is used as background strain for many transgenic animals. 3xTgAD mice and age-matched male wildtype hybrid mice B6129SF1/J (Bg) were used in **Paper III**. The 3xTgAD mice (Oddo *et al*., 2003) carry transgenes encoding mutated human amyloid APP_{Swe} and tau_{P301L} on $PS1_{M146V}$ knock-in background (*see Table 1*). The mice show intraneuronal Aβ and increased inflammation in entorhinal cortex at the age of three months (Janelsins *et al*., 2005) and develop amyloid plaques and tangles at the age of six and twelve months, respectively (Oddo *et al*., 2003). Behavioural characterization of these mice has revealed that memory deficits appear already at three months of age (Billings *et al*., 2005b) and at six months 3xTgAD mice show increased anxiety-like behaviour (Gimenez-Llort *et al*., 2007). We chose the 3xTgAD model in order to study the effects of allergy on both Aβ and tau phosphorylation as these mice develop both pathologies. For comments on validity of the AD mouse model, *see section 1.3.1*.

3.1.2 Human subjects

In addition to mouse models, we have analysed (**Paper IV**) samples obtained from SCI, MCI, and AD (*see Introduction*) cases.

3.1.2.1 Subjective cognitive impairment

SCI is defined by memory complains and is common in elderly (Jessen *et al.*, 2007). However, these patients do not show impairment on objective cognitive task, but some probably represent an early stage prior to developing MCI. Recently, it was shown that individuals with SCI who were experiencing memory problems had a higher risk to develop AD compared to those without memory problems, and the risk was similar to those of early MCI (Jessen *et al.*, 2014). Patients with SCI represent an interesting study population and longitudinal follow-up of these patients may provide opportunities to reveal early changes associated with AD.

3.1.2.2 Mild cognitive impairment

Subjects with MCI were diagnosed according to following criteria (Winblad *et al*., 2004):

- Not normal, not demented (does not meet criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV), and the International Classification of Mental and Behavioural Disorders (ICD-10) for dementia syndrome)
- Cognitive decline reported by the patient or informant and measurable impairment objectively on cognitive tasks or evidence of decline on objective cognitive tasks
- Preserved basic activities of daily living with minimal impairment in complex instrumental functions.

In the modified criteria (Albert *et al*., 2011), the assessment of biomarkers (such as CSF and imaging) is incorporated for the research criteria, which makes MCI a valuable source to study early changes in the course of AD. However, there are limitations with the use of

MCI as prodromal stage for AD. MCI represents a very heterogeneous group (Winblad *et al*., 2004), not only due to etiological reasons, but also there may be subgroups in MCI with different conversion rates to AD. When dividing MCI groups into late and early MCI, the former was associated with higher risk for developing AD (Jessen *et al*., 2014). Thus, there is a need for "markers", which could allow stratification of this group in order to achieve a more homogenous subgroup. MCI constitute a very interesting study population for the detection of early changes associated with AD.

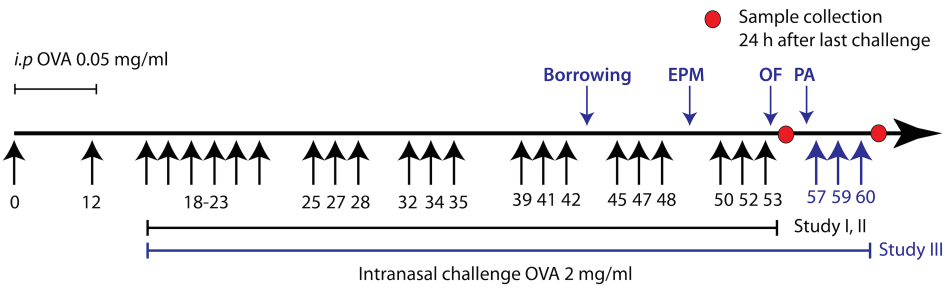

Fig. 1. Mice were given intraperitoneal (i.p.) injection of ovalbumin (OVA) on day 0 and day 12 and subsequently challenged intranasally. The black arrows indicate days. In study III (indicated by blue lines), behavioural tests including borrowing, elevated plus maze (EPM), open field (OF), and passive avoidance (PA) were performed on the indicated gaps when mice were not challenged with OVA. Samples were collected 24 h after the last challenge as indicated by the red circle.

3.2 Experimental protocols in vivo

3.2.1 Allergy provocation protocol

All the mice were subjected to the chronic airway allergy protocol based on OVA sensitization followed by OVA-challenge (*see Section 1.3.2*). Briefly, mice were sensitized by receiving an i.p. injection of 200 μl suspension containing 10 µg OVA diluted in 4 mg/ml $Al(OH)_3$ in phosphate-buffered saline (PBS)-alum on day 0 and 12. The animals were challenged daily from day 18 to day 23, and then 3 times per week during an additional 5-week (**Paper I, II**) or 6-week (**Paper III**) period, by intranasal instillation of

100 µg OVA diluted in 50 µl PBS-alum (Fig. 1). Intranasal instillation was performed under light anesthesia with a controlled flow of 4% isoflurane and 96% O_2, using a Univentor 400 anesthesia unit. Control animals underwent the same treatment but received PBS-alum instead of OVA.

3.2.2 Behavioural studies

A battery of behavioural tests (**Paper III**) was chosen to assess the effects of allergy-induced inflammation on emotionality/anxiety-like behaviour (*i.e.* elevated plus maze (EPM), open field (OF)) and hippocampus-dependent behaviour (*i.e.* EPM, burrowing, passive avoidance (PA)), (see (Billings *et al.*, 2005a; Cunningham *et al.*, 2009)). The behavioural tests considered least stressful, and presumably giving less long-lasting effects to the animal, were performed first (*i.e.* burrowing), while more stressful tests were performed at the end (*i.e.* passive avoidance). The tests were performed in the order described below.

3.2.2.1 Burrowing test

The burrowing test assesses a typical behaviour for rodents and consists of digging. The substrate offered for digging is a higher quantity of pelleted food, which the animal naturally tends to remove from the tube it is presented in. Cytotoxic lesions in hippocampus and prefrontal cortex (to lesser extent) in mice reduces the burrowing activity (Deacon, 2006). Systemic inflammation induced by LPS at sub-pyrogenic levels has been shown to decrease the burrowing activity (Teeling et al., 2007). Approximately 2 h prior to the start of the dark phase, the mice were placed individually in a cage with a burrowing tube containing 200 g food pellets. The quantity of food pellets displaced after 2 h and after the dark phase (approx. 16 h) was calculated by weighing the pellets that remained in the tube (displaced food = total food - remaining food).

3.2.2.2 Elevated plus maze

EPM test is a classical test for the assessment of anxiety-like behaviour in rodents (File *et al.*, 2005). The test was developed to provide a measure of anxiety that was unaffected by changes in overall motor activity. In EPM, opposition is created between the natural desire of the mouse to explore novel environment (positive motivation to enter the open arm) and the aversion to open areas (negative motivation to enter the open arm) in the apparatus. The EPM apparatus consisted of plus-shaped maze with two of the arms protected by transparent walls designating the close arms of the maze while the other two arms served as open arms. The maze was located 40 cm above the ground so that the "open arms combine the elements of unfamiliarity, openness and elevation" (File, 2001). The height above the floor is important because mice could jump off the maze if it is close to the ground. The EPM provides measures of two independent factors. The % of entries to open arms (number of open arms visits in relation to total number of entries on close and open arms) and % of time spent in open arms (time spent in open arms in relation to total time spent on close and open arms) reflected the measures of anxiety. On the other hand, the numbers of entries to the close arm and total arm entries reflected the measure of motor activity (File, 2001). Although the intensity of light affects the performance in OF, EPM does not rely on aversion to bright light (File *et al.*, 2005).

3.2.2.3 Open field

The OF test is a classical test in which the behaviour of the mouse is dependent on variables such as locomotor function, exploratory drive and the fear/anxiety-like state (Stanford, 2007). The problem with OF is that it cannot differentiate between which variables that drives the behaviour, thus the information given by this test is inconclusive. It is a robust screening test but needs to be combined with other tests. The OF test consisted of a square plexiglas arena (approx. 35 x 35 cm). Each mouse was released in one corner, and could thereafter freely explore the environment for 30 min. Horizontal and vertical activities were detected by infra-red beams and photoreceptor cells.

3.2.2.4 Passive avoidance

The PA test is a classical conditioning test in which the mice learn to associate the dark compartment, where they receive an aversive stimulus (for example electric foot shock), with an aversive experience. After the conditioning, the animal makes during the memory testing a choice to enter the dark compartment where it received the foot shock or stay in the lit chamber. The latency to enter the dark compartment is measured. The test is hippocampus-dependent but other cortical areas have been shown to contribute to aversive contextual association (Baarendse *et al.*, 2008). The short-term memory was tested 1.5 h and the long-term memory 24 h after the mice received the shock.

3.3 Biochemical and morphological analyses

3.3.1 Patient samples

CSF and serum samples (**Paper IV**) from SCI, MCI, and AD patients with or without allergy were obtained from the Memory Clinic at Karolinska University Hospital, Huddinge, Sweden. CSF is secreted by the choroid plexus of all four ventricles, flows along the ventricular system and subarachnoid space and plays a key role in volume transmission *i.e.* distributing substances present in CSF within the brain and clearing the brain metabolites (Agnati *et al*., 1995). This makes CSF valuable material for providing insights about the brain although the substances present in CSF might not exactly mirror the processes occurring inside the brain. Blood analysis is interesting for studying systemic changes and is less invasive compared to CSF. However, analysis of both CSF and blood may represent global changes, thus local changes may not be detected with CSF and blood analysis.

To comment on the study population, approximately 1800 patients visited the Memory clinic at Karolinska University Hospital, Huddinge between years 2007 and 2012, and approximately 20% of these patients had allergy according to the medical records, which could be representative with regards to the incidence of allergy. However, the Memory clinic at Karolinska University Hospital is located at the South of Stockholm and may be biased in terms of socioeconomic and ethnic groups compared to other parts of Stockholm or Sweden.

These aspects cannot be overlooked when generalising data for a whole population. Furthermore, patients with allergies represented a rather heterogeneous group as allergic diseases included several types of allergies that were either self-reported or diagnosed by physician. The effects of different types of allergies on brain might not be the same and the reliability of self-reports for different conditions may vary. It is possible that allergies are under-reported at the memory clinic and therefore the presence of allergic patients in the groups without allergy cannot be excluded. We did not have information about the duration of allergies, which could lead to variation among subjects with allergy.

3.3.2 Allergy confirmation in bronchoalveolar lavage

The aim of collecting BAL fluid from mice was mainly to confirm allergy (**Paper I, III**). The animals were sacrificed 24 h after the last antigen challenge. After collecting the brain tissue, the lungs were dissected out and the trachea was cannulated with a catheter. The lungs were carefully flushed twice with ice-cold PBS while the lobes were manually massaged to ensure even fillings of the lungs. The recovered BAL fluid was centrifuged and the cells were spinned onto glass slides, air-dried, fixed in ethanol, and stained with the May-Grünwald/Giemsa method. The number of eosinophils, macrophages, neutrophils and lymphocytes were counted on the basis of morphology.

3.3.3 Antibody-based techniques

3.3.3.1 Western blotting

Western blot is an antibody-based semi-quantitative method where proteins are separated with regard to their molecular mass in an electric field and subsequently visualized as bands with the aid of target-specific antibodies. Information about the molecular weight (MW) of the band on the blot could serve as a checkpoint to confirm that antibody has bound the correct protein. However, proteins are subjected to post-translational modifications, which influence their MWs and therefore the MW observed on the blot could be sometimes different than the theoretical MW. Western blots were performed on brain homogenates from mice (**Paper I, II, III**). Bound antibodies were detected with enhanced chemiluminescence

(ECL) using charged-couple device camera and the optical density of bands was measured using Multi Gauge (version 3) software.

3.3.3.2 Immunohistochemistry

Immunohistochemistry is a valuable technique for studying protein expression. It is a semi-quantitative technique as western blot but with the advantage of providing information about the localization of the protein of interest in the tissue or cell being studied. For IgG, IgE, CD138 staining (**Paper I**) and CD64 staining (**Paper III**), the sections were incubated for 30 min with 5% normal serum and subsequently incubated with primary antibodies at 4 °C overnight. For visualization with fluorescence (CD138) (**Paper I**), the sections were incubated with flourophore-conjugated secondary antibody for 1 h, at room temperature (RT). For visualization with diaminobenzidine (DAB) (**Paper I, III**) the sections, after incubating for 1 h at RT with appropriate biotinylated-secondary antibodies, were exposed to streptavidin-horse radish peroxidase complex for 30 min at RT. The immunoreactivity was visualized by incubation with DAB in the presence of H_2O_2 for 3 min. The slides were analysed under light microscopy (Nikon Eclipse E800) and photographed. For CD64 staining, antigen retrieval was performed, which due to protein denaturation may result in epitope unmasking.

3.3.3.3 Cytokine assays

The levels of pro- and anti-inflammatory cytokines in mouse homogenates (**Paper I, Paper III**) and human CSF and serum (**Paper IV**) were analysed with multiplex assays developed by Meso Scale Discovery (MSD) technology, which is a modified version of enzyme-linked immunosorbent assay (ELISA). The MSD assay relies on the binding of the antigen to a capture antibody followed by detection with a tagged secondary as in traditional ELISA but uses electric signal in addition to ECL signal to minimize background signal. The main advantage of MSD technique is the simultaneous measurement of multiple analytes and a volume requirement of 25-50 µL per sample. Another advantage is the wide range of the

standard curve compared to traditional ELISAs. The disadvantage of this method with regard to cytokine measurement, at least in our experience, was the reduced sensitivity compared to traditional ELISAs.

3.3.3.4 Immunoglobulin assay

The levels of IgM, IgA, IgG subclasses and the total IgG levels were analysed in the CSF and serum (**Paper IV**) using a 7-plex human isotyping panel and a single-plex (for total IgG) developed by MSD.

3.3.4 DNA-based techniques

3.3.4.1 Microarrays

We performed microarrays (**Paper II**) to obtain an overview of genes/pathways that were changed by allergy in the brain. Microarrays were performed in the hippocampus and frontal cortex using Affymetrix whole-transcript expression analysis in association with Bioinformatics and Expression Analysis Core Facility (BEA), Karolinska Institute. Briefly, the mRNA was extracted, reverse-transcribed to single stranded cDNA and hybridized to the probes on microarray chip. The fluorescence of the bound cDNA to the corresponding probes was measured. The array plate contains more than 770,000 oligonucleotide probes (25-mere) that cross-examine more than 28,000 annotated genes.

Parallel analysis of all genes expressed in a tissue makes microarrays a powerful tool for studying the complex networks of biological processes. Mircroarrays could be useful in multiple medical applications *e.g.* understanding the molecular characteristics of diseases, identification of new therapeutic targets and classification of sub-groups in a disease to identify individualized treatments (Tarca *et al.*, 2006). Microarrays generate large datasets and a plethora of factors contribute to the observed differences in gene expression between control and treated samples including:

a) Technical factors (related to sample and sample preparations)
b) Systemic factors (difference between arrays, reagents, instrumentation)
c) Biological factors (true variation)

In order to extract differential expression due to biological variation and to interpret the results, the microarray dataset is processed mainly in the following steps: (i) quality control to provide information on homogeneity (technical variation) of sample groups, (ii) preprocessing such as normalization to account for systemic variation and to enhance or extracts the meaningful characteristics of the dataset, (iii) detection of differentially expressed genes (DEGs) *i.e.* identify genes that are changed due to biological variation, and (iv) functional profiling of DEGs *i.e.* to extract biological knowledge (*e.g.* pathways, biological processes) using Gene Ontology approaches (Cordero *et al.*, 2007). In contrast to univariate measures, which are limited to studying single molecule at a time, the multivariate measures in microarrays allow to study whether multiple genes in a pathway/category are over-/under-represented among the DEGs, thus enhancing the reliability of the result (Blalock *et al.*, 2005).

Despite holding great promises, microarrays bring a number of problems. It is difficult to determine whether the changes in gene expression are functionally relevant, compensatory, or secondary to the process under the study (Blalock *et al.*, 2005). Due to high frequency of false positives and false negatives in large datasets, validation of microarray findings with reference methods such as polymerase chain reaction (PCR) is recommended (Rajeevan *et al.*, 2001; Wang *et al.*, 2006), although a robust correlation between Affymetrix arrays and PCR was reported (Lee *et al.*, 2008). In the brain, the changes in expression are relatively moderate and small changes may have biological significance (Soverchia *et al.*, 2005), thus making it difficult to distinguish true DEGs from noise. Therefore the dataset is more prone to include high rate of false positives and thus validation with PCR might be exclusively necessary.

3.3.4.2 Polymerase chain reaction

PCR is an alternative approach for studying gene expression that, as microarrays, relies on conversion of mRNA to cDNA (for review see (Kubista *et al*., 2006)). In contrast to microarrays, PCR allows for studying only one gene at time (per reaction well). PCR is more sensitive and, in our experience, more specific than microarrays. For example, to PCR reaction one could add target specific Taqman probes, which are short DNA sequences that span over exon regions to ensure that the amplified product in PCR reaction comes from the gene of interest. PCR was used (**Paper II**) as an alternative approach to validate the findings from microarrays. For quantification, the comparative cycle threshold (Ct) method with the arithmetic formula $2^{\Delta\Delta Ct}$ was used, which is only valid under the assumption that the amplification efficiency of the gene of interest and the reference gene is approximately equal. The reference gene – β-actin in our case – is used as an internal control for the amount of mRNA input in the PCR reaction.

One of the limitations regarding gene expression analysis (DNA-based methods) is that it does not take into account the post-transcriptional events. Protein is the functional unit of the cell and therefore information at mRNA level is not sufficient to describe the state of the cell.

3.4 Statistics

3.4.1 Univariate statistics

Statistical analysis was performed with SPSS software (IBM, Corporation, NY, USA) and the R software package (GNU General Public Licence) was used for graphic presentations. Normally distributed data was analysed with Student's t-test or one-way ANOVA followed by *post hoc* Bonferroni test or two-way ANOVA (**Paper I, II, III**), whereas non-normally distributed data was analysed with Mann-Whitney test (**Paper I, II, III, IV**). For all the studies, 95 % confidence interval and p-values < 0.05 was used.

3.4.2 Multivariate statistics

Multivariate analysis (MVA) (**Paper II**) was performed with SIMCA P+ software package (UMETRICS AB, Umeå, Sweden). The data were preprocessed by unit variance (UV) scaling and mean-centering. UV scaling allows comparison of completely different variables, such as behavioural activity in OF and cytokine levels obtained by ELISA. UV scaling uses the inverse standard deviation as a scaling weight for each variable to scale variables with higher level of variance with those with a lower variance. Mean-centering improves the interpretability of the data, by subtracting the average for each analysed variable, thus repositioning the dataset around the origin. The resulting data were fed into principle component analysis (PCA), and subsequently into orthogonal projection to latent structure-discriminant analysis (OPLS-DA).

PCA is an unsupervised multivariate technique mainly used to obtain an overview of the data by representing the multivariate data as a low-dimensional plane, usually consisting of 2 to 5 dimensions (Eriksson, 2006). The extracted information is projected in a set of new orthogonal variables called principle components (PCs). The direction that accounts for the largest variation in the datasets determines the direction of the first PC (PC1). The direction for PC2 is orthogonal to PC1, and accounts for the second largest variation. Subsequent PCs will describe the decreasing variability in a sequential manner, where each PC will be orthogonal to the direction of the previous PC. PCA gives an overview of the data by presenting the patterns of similarities in the observations (samples) and variables, as points in a score plot and a loading plot, respectively.

OPLS-DA is a supervised multivariate regression and prediction method used to describe the association between a quantitative data matrix X (in our case DEGs), and qualitative values in a Y vector (in our case control and allergy) (Wiklund *et al*., 2008). OPLS-DA separates the variation observed in X into two components: *(i)* the variation containing information related to Y and presented in the first predictive component (t1) of the score plot (in our case the variation related to the allergy), and *(ii)* the variation not related to Y and presented in the second (orthogonal) component of the score plot (t2) (in our case the variation unrelated to allergy). Separation of data unrelated to Y into orthogonal component facilitates the

interpretation of data (Holmes *et al*., 2006). The variables that are important for the class separation are represented in the loading plot with their corresponding jack-knifed intervals. The y-axis in the loading plot represents covariance (Wiklund *et al*., 2008), and variables with high covariance (positive and negative) are more likely to have impact on class separation.

The number of components in PCA and OPLS-DA depends on R^2 (estimation of fit) and Q^2 (estimation of prediction) values. The calculation of the Q^2 value was based on seven-fold cross validation (CV), in which 1/7th of the dataset (variables) was omitted randomly to create several parallel predictive models. The omitted data were then predicted by the respective model.

Jack-knifing is an approach used for finding the precision of an estimate. In PCA and OPLS-DA, jack-knifing uncertainty measures of scores and loadings are calculated from the set of multiple models generated from CV (Martens H *et al*., 2001). Confidence intervals that include zero have low reliability (Wiklund *et al*., 2008).

3.5 Ethics

The studies involving mice were approved by the Stockholm South Ethical Committee for animal experiments with the following ethical numbers: S200/07 (**Paper I**), S204/10 (**Paper II**), S158/12 (**Paper III**).

The study involving human CSF and serum samples (**Paper IV**) was approved by the Southern regional ethics committee of Stockholm with the ethical number 2011/680-31/1.

4 RESULTS AND DISCUSSION

4.1 Cell counts in the bronchoalveolar lavage

The analysis of BAL fluid was performed in Balb/c, C57BL6 (**Paper I**), 3xTgAD, and Bg (B6129SF1) mice (**Paper III**) to validate the allergy model. The number of eosinophils in the BAL was significantly increased in all mice confirming the presence of allergy (Fig. 2A). An increased number of eosinophils, or their molecular products, such as major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin, are found in allergic diseases, but most of the studies are performed in asthma (Wardlaw *et al*., 2000). In patients with mild asthma, the BAL fluid showed eosinophilia with normal neutrophil counts (Wardlaw *et al*., 1988), whereas the presence of neutrophils in the BAL is associated with severe asthma. However, both neutrophils and eosinophils have been reported to occur in the BAL of patients with allergic asthma (Frangova *et al*., 1996). In our model of airway allergy we found increased levels of neutrophils in response to OVA in all the strains except for Balb/c mice (Fig. 2B). The increase in eosinophils in the BAL of Balb/c mice and of neutrophils and eosinophils in the BAL of C57B6 mice are in agreement with previous work (Whitehead *et al*., 2003), but neutrophilia has been reported also in the Balb/c mice (Gueders *et al*., 2009). Eosinophils have been shown to increase 24 h post challenge in the BAL in several strains of mice (Whitehead *et al*., 2003), and in the blood in asthmatic patients (Djukanovic *et al*., 1990). We found that the total number of cells, reflecting eosinophils, neutrophils, macrophages, and lymphocytes in the BAL was significantly increased with allergy in all strains of mice (Fig. 2C), in agreement with studies in mice (Drent *et al*., 1993; Whitehead *et al*., 2003), but not in asthmatic patients (Smith, 1992). However, in other types of human allergic diseases such as extrinsic allergic alveolitis (EAA), the total number of cells in the BAL was increased, but was dependent on the time elapsed after antigen exposure (Drent *et al*., 1993). Compared to control subjects, patients with EAA had higher counts of eosinophils, neutrophils, macrophages, and lymphocytes in the BAL 2 - 7 days after antigen exposure, whereas no differences were found before 24 h, and at 8 - 30 days or 1 - 12 months after antigen exposure (Drent *et al*., 1993).

Strain-related differences were found in the total number of cells in BAL in the absence of allergy (Fig. 2C). The basal levels of eosinophils in the BAL of Bg mice, in the absence of allergy, were significantly higher than the basal levels in Balb/c and C57B6 mice, indicating strain-related differences also regarding eosinophils (Fig. 2A). However, Tg animals had significantly lower eosinophil cell counts and a lower trend in the neutrophils than Bg mice (their background strain), indicating differences induced by the transgene or pathology. During dissection, one could identify allergic animals from the "texture" of the lungs in Bg animals but this distinction was not clear from the lungs of the transgenic animals (unpublished observations). It would be of interest to study the lungs of patients with AD with regard to morphology, the presence of immune cells, and their molecular derivatives, especially knowing that respiratory infections such as pneumonia are frequently encountered in AD patients.

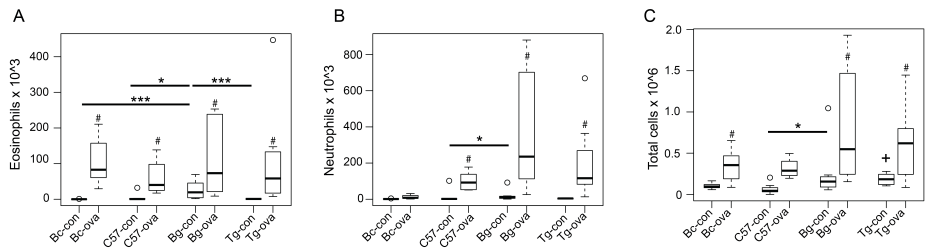

Fig. 2. Allergy-induced changes in immunocompetent cells in bronchoalveolar lavage (BAL) fluid from 3xTgAD and Wildtype mice. Allergy was induced in mice using ovalbumin (OVA) as allergen. Eosinophils (A), neutrophils (B), and total cells (C) (reflecting eosinophils, neutrophils, macrophages and lymphocytes) were counted based on their morphology. The boxplots indicate interquantile range (IQR), the upper whisker is 1.5 IQR + Q3, the lower whisker is 1.5 IQR - Q1, and the line in the middle shows the median. Con = control, OVA = allergic, Bc = Balb/c, C57 = C57BL6, Tg = 3xTgAD, Bg = background strain for 3xTgAD. The circles are outliers. * $P < 0.05$, *** $P < 0.001$. # indicates significant difference between allergy and controls animals within the strain, **+** indicates significant difference between Tg-con and C57-con as well as Tg-con and Bc-con mice.

Although we have confirmed the presence of allergy by performing analysis in the BAL, one of the limitation of our allergy model is that we have not performed the routine analysis to validate the allergy model *i. e.* to analyze the cytokine profile in the lungs, the presence of

IgE and IgG1 in the lungs, the inflammation in the lung sections, and the airway hyperresponsiveness.

4.2 Immunoglobulins in allergy and Alzheimer disease

4.2.1 Animal studies

Allergy was associated with a consistent increase in the levels of IgG and IgE in the brain of all the strains of mice (**Paper I, II, III**). At basal conditions, IgG-like immunoreactivity has been reported in C57BL6 and Balb/c mice on microglia, and also on macrophages and epithelial cells in the choroid plexus (Hazama *et al*., 2005). We found an increased IgG staining not only around the cerebral blood vessels, but also widely distributed in the brain parenchyma (**Paper I**). The same distribution pattern of IgG was found in a mouse model of prion disease following systemic inflammation induced by LPS (Lunnon *et al*., 2011), indicating that an increase in the brain levels of IgG is not unique to allergy. Assuming that the Igs observed in the brain enter from the periphery, there are several possible routes and mechanisms suggested, such as: a) leakage at sites lacking BBB as in the CVOs; or b) Ig-secreting cells infiltrating the brain (Hazama *et al*., 2005); c) a selective transporter at the BBB interface - the presence of a saturable transport system for IgG has been reported in the guinea pig (Zlokovic *et al*., 1990); d) neonatal FcR may play role in the transport of Ig across the BBB (Deane *et al*., 2005; Giragossian *et al*., 2013). To study whether Ig-secreting cells enter the brain, we stained the brain sections of mice with CD138, a marker for plasma cells, a major source of Igs (Kitamura *et al*., 1991). However, more recent studies have revealed neuronal Ig synthesis in both rat and human brain (Niu *et al*., 2011; Zhang *et al*., 2013a). The brain of allergic mice did not show any immunoreactivity for CD138, indicating that the Ig was not secreted locally by B-cells in the brain (**Paper I**). Infiltration of IgG-secreting plasma cells in the brain (Knopf *et al*., 1998), and formation of an IgG-OVA immune complex (IC) (Carare *et al*., 2013) in OVA-sensitized rodents only occurred when OVA was microinjected intracerebrally. In our studies, the formation of ICs in the brain is unlikely, as the traffic of big proteins such as OVA across the BBB presumably does not occur. It cannot be excluded that increased IgG levels in the brain of allergic mice may reflect a combination of both peripherally derived and locally synthesized IgG. It was recently shown that about 0.01% of

peripherally administered intravenous IgG (IVIG) in 3xTgAD mice reached the cerebral cortex, possibly by a saturable transporter (St-Amour *et al*., 2013). The functional relevance of increased Ig levels in the brain remains unknown. Zhang et al. showed that complement exposure (Zhang *et al*., 2013a), or administration of 6-hydroxydopamine (6-OHDA) increased the expression of IgG in primary neurons, and that primary neurons were dose-dependently protected against 6-OHDA-induced injury after treatment with neuron-derived IgG (Zhang *et al*., 2013b). It is thus possible that IgG plays a role in immunomodulation in CNS.

IgG is present in four subclasses in humans (IgG1 - IgG4) and mice (IgG1, IgG2a, IgG3b, IgG3), binds to FcγRs with various affinities, and regulates functions such as antibody-mediated cell cytotoxicity, phagocytosis of ICs, and production of pro-inflammatory mediators (Sanchez-Mejorada & Rosales, 1998; Karsten & Kohl, 2012). In the CNS, expression of FcγRs has been found on microglia, neurons, astrocytes, and oligodendrocytes (Okun *et al*., 2010). As a follow-up on our finding of increase IgG levels in the brain, we analysed the levels of FcγRs in Bg and Tg mice (**Paper III**). In the absence of allergy, Tg mice expressed higher levels of FcγRI (CD64) compared to Bg mice. Allergy significantly increased the levels of FcγRI both in Bg and Tg animals and the difference was more pronounced in the dentate gyrus than the cortex or other hippocampal areas. In contrast to other FcγRs, FcγRI binds monomeric IgG with high affinity (Karsten & Kohl, 2012), and is expressed on astrocytes and microglia (Zhang *et al*., 2013a). FcγRI expression was increased and NO production was decreased in microglia treated with complement in the presence of IgG (Zhang *et al*., 2013a), suggesting a protective role for IgG in the brain. Passive immunization against Aβ in Tg2576 mice was followed by an initial increase in FcγRII and III in the brain, and enhanced phagocytosis of Aβ (Wilcock *et al.*, 2004). However, the levels of FcγRs returned 3 months after immunization to the level in non-immunized mice. Similarly, post-mortem quantitative analysis performed seven years after immunization with $A\beta_{1\text{-}42}$ revealed significantly lower levels of CD64 and CD32 in immunized AD patients than in controls (Zotova *et al.*, 2013). Thus, the function of FcRs seems to be context-dependent similarly to other mediators of inflammation.

4.2.2 Human studies

Allergy influences the levels of Igs in human. Serum levels of IgE were increased in patients with atopic diseases (Berg & Johansson, 1969) with a parallel increase in serum IgG to the same allergen (Chapman *et al*., 1983), in agreement with our data in mice. Other classes of allergen specific Igs, such as IgM and IgG subclasses (IgG1 - 4), are produced in the serum of patients with allergy (Niederberger *et al*., 2002), although the levels of IgG subclasses vary in asthmatic individuals (Loftus *et al*., 1988). To study the influence of allergy on Igs in AD, we analysed the levels of IgM, IgA, total IgG, and its subclasses (IgG1 - 4), in the CSF and serum of patients with AD, MCI or SCI, with or without allergy. In contrast to the mice, there was no increase in the total levels of IgG due to allergy in any of the patient groups (**Paper IV**), although the occurrence of "allergen-specific" IgG in patients with allergy cannot be excluded. Previous studies in patients with allergy showed increased levels of IgE in serum during antigen exposure (Henderson *et al*., 1975), and decreased levels after antigen avoidance (Sensi *et al*., 1994). This indicates that the levels of Igs in allergic patients are dependent on antigen exposures. In the patient material that we analysed, data were not available for variables such as antigen exposure before sampling of blood or CSF. Furthermore, the allergic groups consisted of patients with different types of allergies, which may influence the results and conclusions. Analysis of the IgE levels in patients allergic to dust mite antigen during the avoidance period showed that changes in mucosal IgE levels, which represent the local area, were more rapid than changes in serum levels (Sensi *et al*., 1994). Therefore, blood and CSF analysis may be limiting in reflecting the local changes. We found a correlation between CSF and serum for IgG and IgA classes when the data from all patients were pooled, whereas no correlation was found for IgM, indicating possible local changes.

In mice, IL-4 drives the polarization of T-cells to Th-2 cells, and the predominant IgG subtype is IgG1, whereas the Th-1 response is driven by IFNγ and is associated with IgG2a (Tabira, 2010). In humans, this distinction is not clear. The majority of studies on the levels of Igs in asthmatics were performed in children or in young adults. Since the levels of Igs change with age (Ritchie *et al*., 1998), the results may vary in very young *vs* old ages. To

study the influence of allergy on Ig classes in AD, we analysed the levels of IgM, IgA, and the IgG subclasses, IgG1-IgG4, in CSF and serum of patients with AD, MCI, and SCI with or without allergy (**Paper IV**). In cases without allergy, IgG1 to total IgG ratio was higher in AD compared to SCI or MCI. Allergy was associated with lower levels of IgG1 to total IgG ratio and IgA in the CSF of patients with AD compared to those without AD, whereas IgM levels in serum were higher in MCI patients with allergy compared to those with allergy. The levels of IgA in serum increase with age, whereas those of IgM decrease with age (Ritchie *et al*., 1998). Aging has been shown to increase the levels of IgG1, IgG2, and IgG3 (Paganelli *et al*., 1992; Listi *et al*., 2006). It seems that Ig responses were influenced by the presence of allergy in the patients in our material.

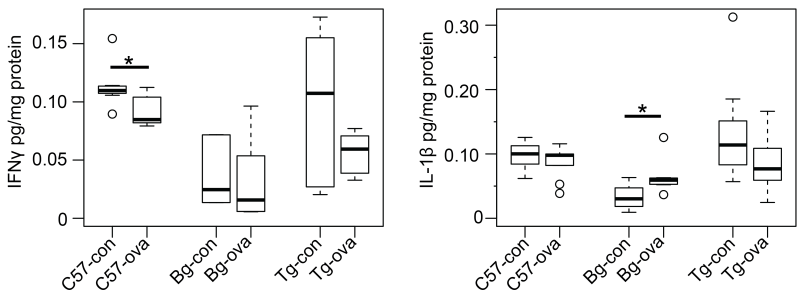

Fig. 3. Allergy-induced changes in cytokines in different mouse strains. Allergy was induced in mice using ovalbumin (OVA) as allergen. The levels of interferon (IFN)-γ (A), and interleukin (IL)-1β (B) were analysed in the hippocampus by using multiplex cytokine kit developed by Mesoscale. The boxplots indicate interquantile range (IQR), the upper whisker is 1.5 IQR + Q3, the lower whisker is 1.5 IQR - Q1, and the line in the middle shows the median. The age of Tg and Bg mice was approximately 7 months and that of C57BL6 mice was approximately 4 months at the end of the study. Con = control, OVA = allergic, C57 = C57BL6, Tg = 3xTgAD, Bg = background strain for 3xTgAD. The circles are outliers. * $P < 0.05$

4.3 The effects of allergy on the brain

Mild asthma in healthy elderly (≥ 55 years) was associated with increased decline in cognition (Caldera-Alvarado *et al.*, 2013), and asthmatics had 1.8 years shorter survival time compared to non-asthmatics (Eriksson *et al.*, 2008). In patients with mild to moderate asthma, 13 out of 21 subjects had abnormalities in their brain MRI scans (Parker *et al.*, 2011),

indicating that allergic inflammation such as asthma affects the brain. We investigated the effect of chronic OVA-induced airway allergy on brain in naïve mice (**Paper I, III**), with regard to cytokines and AD-related proteins (tau and APP). Allergy increased tau phosphorylation at Ser202/Thr205 (AT8) and Thr231/Ser235 (AT180 site) phosphorylation sites (Amniai *et al.*, 2009) in Balb/c and C57BL6 mice (**Paper I**), but not in Bg mice (**Paper III**), which showed a high variation both within the control group and the allergic group, ,. With regard to cytokines, allergy decreased the levels of IFNγ (Fig. 3) in C57BL6 mice, whereas increased IL-1β in Bg mice (Fig. 3). Interpretation of change in one cytokine is difficult, and there was no effect of allergy on phosphorylation of p38 and c-Jun N-terminal kinase (JNK), which are involved in production of cytokines. At low concentrations, IFNγ plays a role in neurogenesis (Butovsky *et al.*, 2006), and one could argue that reduction of IFNγ in the brain due to allergy may influence its physiological role in the brain. In a recent study, OVA-induced allergy was associated with reduced neurogenesis and reduced synapse density in the hippocampus (Guo *et al.*, 2013). Additional changes induced by allergy include the appearance of swollen, vacuolated, and damaged mitochondria, and reduced levels of c-fos and activity regulated cytoskeleton associated protein in the hippocampus (Guo *et al.*, 2013). Furthermore, the allergic mice showed impairment in Morris water maze (MWM) test, as indicated by impaired learning during the training sessions and poor memory during the probe trails (Guo *et al.*, 2013). However, treatment with Budesonide attenuated mitochondrial damage and increased neurogenesis in the allergic mice, without influencing the synapse density, or learning and memory (Guo *et al.*, 2013). With regard to inflammation in the brain, we did not observe any difference in microglial or astrocyte activation as analysed with ionizing calcium-binding adaptor molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP), respectively (**Paper I, III**). In contrast, Xia et al. found that OVA-induced allergy was associated with increased microglial activation as analysed by cd11b, increased levels of TNFα, IL-1β, and TGFβ, and decreased levels of IL-10, in the hippocampus (Xia *et al.*, 2014). A possible explanation to the discrepancy between the two studies may be the allergy induction protocol. Thus, our challenge model lasted 5 weeks, whereas Xia et al. used a challenge phase of 9 weeks and, probably even more important, included sessions with exposure to high concentration of OVA to induce aggravation (Xia *et al.*, 2014). Considering

the studies mentioned above, allergy seems to have negative consequences on the brain. It is difficult to conclude whether allergic disease may have positive or negative effects on the brain in the context of neurodegenerative diseases. Since inflammation may have both positive and negative consequences, the inflammatory environment in the brain, the timing, and the duration, may all be important factors in determining whether the effect of allergy on the brain is beneficial or detrimental.

By using microarrays, we further analysed the effects of allergy on gene expression in the brain. Allergy reduced the levels of IDE in hippocampus, frontal cortex and hypothalamus (**Paper II**). In addition, allergy was associated with reduction in the phosphorylation of insulin receptor (IR) (**Paper II**). These findings suggest that allergy has a negative effect on insulin signalling in the brain, which is involved in regulating peripheral fat and glucose metabolism (Koch *et al.*, 2008). Furthermore, metabolic diseases such as DT2 and obesity are associated with deficits of insulin signalling in the brain (Farooqui *et al.*, 2012). Asthma has been associated with obesity and DT2 (Meng *et al.*, 2014), and our findings warrant further studies on the link between asthma and metabolic disorders. These findings are also interesting in the context of AD, since both obesity (Letra *et al.*, 2014) and DT2 are risk factors for AD. Furthermore, IDE is involved in the cleavage of Aβ and its levels are reduced in AD brain (Hickman *et al.*, 2008). Taken together, these studies suggest that the presence of allergy may have negative consequences in subjects with predisposition to AD.

Several other interesting genes were found to be upregulated in the brain upon allergy (**Paper II**). Recently, it was shown that mice exposed to chronic but not acute stress (induced by stressors such as animal dander), increased the expression of hemoglobin genes in prefrontal cortex (Stankiewicz *et al.*, 2014). We found increased transcripts of hemoglobin genes in frontal cortex in mice exposed to allergy (**Paper II**), indicating that allergic diseases may result in increased stress on the brain. Several other interesting genes were altered in the microarray study, but for the reasons described below, it is necessary to validate each gene with other methods before any interpretation of the data. The complex architecture and function of the brain poses some challenges on microarray analysis of brain tissue. Primarily, the changes in gene expression in the brain are relatively moderate and consequently, small

changes may play a biologically significant role, but may be difficult to discriminate from noise. Therefore, the arbitrary cut-off level (> 2 fold change) may not apply for the brain. This means that the results may also include noise. Secondly, the intrinsic heterogeneity in the cell populations in the brain may dilute a significant fold change in the gene of interest if it is differentially expressed only in a portion of the cells. Lastly, an up-regulation of the gene in one cell population could possibly be counterbalanced by down-regulation of the same gene in the neighbouring cell population, and may thus end up undetected (Soverchia *et al*., 2005).

4.4 The effects of allergy in Alzheimer disease

4.4.1 Animal studies

As mentioned in the Introduction, epidemiological studies have shown that allergic diseases increased the risk for developing AD, and possible shared pathogenic pathways may be related to the observed association between AD and allergies. One such example is polymorphism in the IL-4 gene, which has been associated with increased risk for asthma (Micheal *et al.*, 2013), and was recently shown to increase the risk for AD in a Chinese population (Li *et al.*, 2014). The transgenic animal model of AD, 3xTgAD, was used to study the effect of allergy on the brain in the presence of AD-like pathology. The levels of inflammatory proteins, cytokines, C1q component C (C1qC), and decay accelerating factor (DAF), were analysed in the brain of 3xTgAD mouse strain and compared to Bg controls (**Paper III**). Allergy seemed to have differential effects in the presence or absence of AD-like pathology. In 3xTgAD mice, which had higher levels of inflammatory cytokines and C1qC levels in the brain, allergy decreased the phosphorylation of p38, increased the levels of mature brain-derived neurotrophic factor (BDNF) and DAF, which is a regulator of the complement system. BDNF is an important protective factor against development of dementia including AD (Weinstein *et al.*, 2014), and has been shown to modulate inflammation by decreasing the levels of inflammatory cytokines (Jiang *et al.*, 2010), and the activation of p38 (Tong *et al.*, 2012). However, BDNF was implicated in neuronal

hypersensitivity and dysfunction in the airways in asthma (Prakash & Martin, 2014). DAF is a membrane-bound complement inhibitor found on microglia, astrocytes, and to lesser extent on neurons (Kolev *et al.*, 2009). In AD brain, complement inhibitors were slightly increased compared to the complement proteins that were found to be substantially increased (Kolev *et al.*, 2009), thus rendering the brain vulnerable to complement damage. Our findings suggest that allergy induces beneficial responses in 3xTgAD mice. In Bg animals, allergy increased the hippocampal levels of IL-1β and of C1qC, without inducing changes in the complement inhibitor DAF. Furthermore, allergy decreased p-IR levels, and decreased the burrowing activity in Bg mice. Taken together, our results suggest that allergy induced beneficial responses in the presence of AD-like pathology, whereas the opposite was found in the brain in the absence of AD-like pathology.

Behavioural characterization revealed that at the age of four months, the 3xTgAD mice performed poorly in finding a hidden platform, and entering the dark chamber after 24 h, in the MWM and PA tests, respectively, indicating long-term memory deficits (Billings *et al*., 2005b). At the age of 6 months, their short-term memory as measured by finding the hidden platform 1.5 h after the last trial in MWM, and performance in open field, were further deteriorated (Billings *et al*., 2005b; Gimenez-Llort *et al*., 2007). Differences between 3xTgAD mice and wildtype mice in OF and PA have been reported previously (Clinton *et al.*, 2007; Gimenez-Llort *et al.*, 2007; Espana *et al.*, 2010). In our study, the behavioural tests PA, OF and EPM, did not reveal any differences, neither between genotypes nor due to allergy (**Paper III**). Allergy was induced at the age of 4 - 5 months in Bg and 3xTgAD mice, and the mice were sacrificed at 6 - 7 months. In a previous study, at 6 month time point, naïve 3xTgAD mice showed increased transcripts of TNFα and monocyte chemoattractant protein-1 in the entorhinal cortex, but not in the hippocampus (Janelsins *et al.*, 2005). Our studies showed increased levels of IL-1β, IL-8, and IL-12 in the hippocampus of the 3xTgAD mice (**Paper III**), indicating increased inflammation in the brain. There seems to be high variability between different colonies of 3xTgAD mice raised in different laboratories, which represent one of the main disadvantages of 3xTgAD mice. One such example is the discrepancy of the results between the original publication (Oddo *et al.*, 2003) and the study

of Mastrangelo et al. with regard to the development of AD-like pathology (Mastrangelo & Bowers, 2008).

4.4.2 Human studies

We extended our studies from a mouse model of AD to humans in order to investigate whether allergy affects the AD biomarkers tau and Aβ, and whether there was any relation between the presence of allergy and MMSE scores in AD, as well as in cases with MCI and SCI. Allergy was associated with reduced t-tau levels in the CSF of AD patients compared to those without AD (**Paper IV**). CSF levels of t-tau and p-tau reflect neurodegeneration (Alzheimer's, 2013), and p-tau levels may have more specificity for AD (McKhann *et al.*, 2011). Despite lower t-tau levels in AD patients with allergy, the MMSE scores were not different between the groups. MMSE is not sensitive enough to detect differences between AD groups, but useful in detecting cognitive disturbance (Harvan & Cotter, 2006). With regard to MCI cases, the MMSE scores were higher in the presence of allergy than in cases without allergy, and there were no differences in t-tau or p-tau levels (**Paper IV**). One could argue that changes observed in allergic patients may be due to the anti-inflammatory treatment, and not to allergy. It was described that AD patients treated with corticosteroids, but not with NSAIDs, had reduced numbers of amyloid plaques and NFTs in the cortex (Beeri *et al.*, 2012). In addition, the severity of dementia was higher in patients without anti-inflammatory treatment compared to those receiving NSAIDs or glucocorticoids (Beeri *et al.*, 2012). However, in the study of Beeri et al., the majority of patients received glucocorticoids as treatment for allergic diseases, thus it is difficult to conclude if the protective effect was mediated by the treatment itself, the presence of allergic disease, or the combination of treatment and allergy. In Tg2576 mice, dexamethasone treatment for 28 days decreased tau phosphorylation without changing t-tau levels, but impaired behaviour in fear-conditioning paradigms (Joshi *et al.*, 2012). In our study, we cannot exclude the potential bias due to anti-inflammatory treatment in the allergic group although nearly comparable cases without allergy received glucocorticoid treatment. However, this evokes another important issue *i.e.* "how much controls are the controls?" The effects of anti-inflammatory treatments (NSAIDs and steroids) were analysed in relation to MMSE and CSF biomarkers (p-tau, t-tau and Aβ)

in the entire dataset, and also in SCI, MCI, and AD groups separately. The MMSE scores were significantly higher in allergic AD patients who received steroid treatment compared to those without steroid treatment. There were no effects of any anti-inflammatory treatment on the levels of CSF biomarkers. In a recent study, it was shown that the asthma drug disodium chromoglycate reduced the brain levels of Aβ in a mouse model for AD by inhibiting Aβ aggregation and inducing microglial clearance (Hori *et al.*, 2014). Thus, the intake of different drugs in humans is challenging for the study design, especially if the study involves elderly people with many comorbidities.

Analysis of cytokine levels in CSF samples revealed no differences between SCI, MCI, and AD cases with or without allergy. However, in patients without allergy, the serum IFNγ levels were higher in MCI than in AD. Also, MCI patients without allergy had higher IFNγ levels than MCI cases with allergy (**Paper IV**). The effect of change in one cytokine is not immediately obvious, and many immune cells in periphery produce IFNγ. Studies in mice have shown both detrimental and beneficial role of IFNγ in the context of AD. Induction of IFNγ expression at very young age in mouse models of AD was associated with a decrease in AD-like pathology (Chakrabarty *et al.*, 2010a) and an increase in neurogenesis (Baron *et al.*, 2008; Mastrangelo *et al.*, 2009). In contrast, adoptive transfer of IFNγ expressing Th-1 cells at the age of 8 - 9 months (prior to plaque development) exacerbated AD-like pathology and impaired performance in the MWM in Tg2576 mice. These effects were reversed with IFNγ neutralizing antibodies (Browne *et al.*, 2013). With this background, it may be considered that reduced IFNγ levels in the serum of MCI patients with allergy could be beneficial. However, there was no correlation between serum IFNγ levels and MMSE scores in MCI patients with allergy. However, the interpretation of cytokine data is even more complicated considering that cytokine expression is a regulated process and thus prone to variation depending on time and physiological environment, even if we disregard variations due to technical reasons, or due to which body fluid is analysed. According to a recent meta-analysis, the data on several cytokines have given inconclusive results. Thus, in the case of TNFα levels in plasma and serum, up-regulation, down-regulation, or no change, has been reported when comparing AD patients and age-matched controls (Brosseron *et al.*, 2014). It

is conceivable that there are subgroups (or endotypes as described for asthmatics) of AD patients that have distinct cytokine profiles, which may explain the discrepancies in the results. In support of this view, Sudduth et al. found two populations within early AD patients: one with increased expression of pro-inflammatory markers (M1 type), and the other with increased levels of "anti-inflammatory markers" (M2 type) (Sudduth *et al.*, 2013). Interestingly, AD patients in M2 group had higher prevalence of vascular risk factors (Sudduth *et al.*, 2013). In the light of these findings, the previous reports on increased risk of cardiovascular disease in association with allergy (Mueller *et al.*, 2013; Park *et al.*, 2013), and our findings on deficits in insulin signalling in the brain induced by allergy, it is tempting to speculate that allergies (especially allergic diseases predominated by Th-2 responses) may be more prevalent within an AD subtype with "anti-inflammatory" profile.

To summarize the data from patients, we found beneficial responses of allergy in AD patients in terms of a reduction in t-tau levels in CSF of patients with AD, and decreased levels of IgA and IgG1 ratio. In MCI, allergy was associated with an increase in MMSE scores, and reduced levels of IFNγ in the serum.

5 CONCLUDING REMARKS

The aim of this thesis was to study the effects of allergy on the brain with focus on AD. In the first two studies, the effects of chronic airway allergy on the brain were investigated in the absence of AD-like pathology in mice. Subsequently, a mouse model for AD was used to study the effect of allergy on the brain in the presence of AD-like pathology. From these studies, we found that allergy induced presumably beneficial responses in the presence of AD-like pathology, whereas opposite responses were observed in the absence of AD-like pathology.

In studies on human samples, the results pointed towards possible beneficial effects of allergy on AD pathology, as seen by biomarkers (tau in CSF), cognition test (MMSE), and inflammatory markers in CSF and serum.

The key findings of this thesis could be summarized as follows:

- Allergy increased the levels of IgG and IgE in the brain. The increase in Ig was localized to blood vessels and in brain parenchyma.
- Allergy increased tau phosphorylation in the brain in the absence of AD-like pathology.
- Allergy induced deficits in insulin signalling in terms of reduced IDE levels and reduced phosphorylation of IR in the brain in the absence of AD-like pathology.
- Allergy increased the brain levels of mature BDNF, and of DAF, and decreased phosphorylation of p38, in the presence of AD-like pathology.
- Allergy impaired burrowing activity in mice in the absence of AD-like pathology.
- MCI patients with allergy had higher MMSE scores, higher serum IgM levels and lower serum IFNγ levels than those without allergy.
- AD patients with allergy had lower levels of total tau in CSF, lower levels of IgA and of IgG1 ratio in CSF.

To conclude - inflammation is a combination of extremely complex processes, which have the potential to become detrimental or beneficial for the host, depending on the type, timing, localization, and intensity of inflammatory response. The nature of the inflammation also

depends on the environment within the host. Therefore, the interpretation that allergy may have a beneficial role in AD may not be obvious, and further studies with better characterization of patients are necessary to investigate the role of allergy in relation to AD.

6 FUTURE RESEARCH DIRECTIONS

Epidemiological findings of an association between allergic diseases and an increased risk to developing AD suggest an interaction between the two diseases. Interestingly, the fact that both allergic diseases (Zhao *et al*., 2014) and AD (Driver, 2014) are inversely associated with cancers may strengthen the suggested positive association between allergy and AD. Study designs with "bridging approaches" as in this thesis requires collaboration between memory clinic and allergy clinic in order to obtain a better characterization of patients both with regards to allergies and AD. Therefore, the data derived from such studies will provide more precise information about the relationship between the two diseases.

Allergic diseases are often manifested at a young age, whereas AD has a late onset. Early immune stimulation in mice was shown to induce AD-like pathology in the brain later in life (Krstic *et al*., 2012). Therefore, it will be interesting to investigate the relationship between childhood allergies and the development of dementia later in life.

Allergic asthma has several phenotypes and it will be of interest to analyse the association of different asthma phenotypes with AD, both in an epidemiological setting, and in a prospective study design. In addition, proper functioning of the brain relies on sufficient oxygen supply and dysfunctional lungs may induce hypoxic conditions both peripherally and centrally. Therefore, it would also be of interest to correlate the severity of asthma with AD-pathology in the brain.

FAD accounts for only about 5% of the total AD cases, but represents a very interesting study population considering the fact that all individuals with FAD mutations will develop AD. It would thus be intriguing to investigate the association between allergy and AD in FAD cases at different ages. There are several questions that could be addressed. How prevalent are

allergic diseases in children with a FAD mutation? How does allergy affect the onset of AD in FAD cases? Th-2 stimulation in mouse models of AD in association with vaccinations was protective (Tabira, 2010), and our findings suggest a protective role of allergy. In FAD cases, Aβ plays an important role in the pathology, as in mouse models for AD - would allergic diseases with a predominant Th-2 phenotype, such as allergic asthma, be protective in FAD?

Allergy is positively associated with metabolic and vascular diseases, which are risk factors for AD. Thus, it will be of interest to investigate the prevalence of allergy in AD cases that show more vascular pathology. Such studies will allow identification of more homogenous subgroups within the AD population, and may lead to development of personalized therapies.

In the study of Rusanen et al., late-life asthma was associated with a decreased risk for AD, whereas, asthma was associated with an increased risk (Rusanen *et al*., 2013). Studies in mice can be used to address mechanistic questions regarding these associations. Since both timing and duration of inflammation influence AD-pathology differentially, the mouse models for AD offer appropriate tools to study the aspect of time. Studies on mice would allow investigation of how AD-like pathology evolves by analysing the brain at different old ages, after induction of allergy in early ages. Since timing of inflammation could affect the beneficial and detrimental role of inflammation, it would also be of interest to induce allergy in old mice and analyse the effects on the brain, both in wildtype and in mouse models for AD.

Final reflection

In conclusion, I will just add that in life there are more questions than answers, we have to endeavor ourselves in seeking answers, which however will generate more questions. In the case of studies on association between allergy and AD, in addition to the proposed designs above, approaches like microarrays or proteomics in mouse models of AD and patient material will provide a better insight into the different pathways that could be altered due to allergy.

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