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Master thesis in Medicine

Analysis of Inflammatory processes in the human Entorhinal and Frontal cortex during normal aging and Alzheimer's disease

Student
Nathalie JAUSLIN

Director
Dr. Beat M. Riederer (PD & MER)
Center for Psychiatric Neurosciences
CHUV - LAUSANNE

Co-Director
Dr. Geneviève Leuba (PD & MER)
Center for Psychiatric Neurosciences
CHUV - LAUSANNE

Expert
Dr. Armand Savioz (PD)
Neuropsychiatric Center
HUG - GENEVE

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1. Abstract

Background: Alzheimer's disease (AD) is the most common cause of dementia in the world. This disease is characterized by neuronal degeneration as well as the accumulation of abnormal proteins. An increase of evidences shows that the disease is also accompanied by a reactive gliosis, which allows advancing the hypothesis that an inflammatory process occurs in the brain through an increase in microglia and astrocytes density. These cells respond dynamically to neurodegenerative lesions inducing a local activation of immune components and acquiring the ability to secrete pro-inflammatory cytokines such as IL-1β and TNF-α thereby triggering an immune cascade. It is assumed that chronic inflammatory processes in specific areas of the brain such as the entorhinal and frontal cortex could contribute to the worsening of the clinical symptoms of the disease. Moreover, some studies have suggested that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) may help to curb or even prevent AD.

Method: In order to understand the relationship between inflammatory processes and Alzheimer's disease, the inflammatory changes were investigated in human brain tissues of the entorhinal and the frontal cortex from control and AD subjects by using one-dimensional electrophoresis and immunostaining combined with chemiluminescence. A polyclonal GFAP antibody was used to detect the reactive astrogliosis, a monoclonal AD2 antibody to evaluate the accumulation of pathological tau protein and a monoclonal β-amyloid antibody, which reacts with the component of senile plaques. Three antibodies were used to detect specific inflammatory proteins, a monoclonal TNF-α antibody, a monoclonal IL-1β antibody and a monoclonal NF-κB antibody. Quantitative and statistical analyses were then performed to compare the respective concentrations of these proteins in the two brain regions between a control group and moderate and severe Alzheimer's disease cases.

Results: The results highlighted a disparity in the pathological accumulation of tau and β-amyloid proteins not only according to the stage of the disease but also depending on the brain region. It also appears that the reactive astrogliosis and the inflammatory processes, found in significant quantities at the beginning of AD, do not persist indefinitely and that there is a decrease of these proteins when the disease reaches an advanced stage.

Conclusions: Alzheimer's disease is marked by the presence of a significant amount of glial cells as well as a high density of inflammatory molecules. However, these components are involved early in the development of the disease and do not seem to be correlated with the worsening of the cognitive decline. Therapies focused on inflammatory processes, as for example NSAIDs, should then be administered as soon as possible when the symptoms are still mild or even not yet clinically diagnosed. New diagnostic techniques are therefore necessary to detect the disease in the very early stages and the development of targeted treatments remains one of the major challenges of current research about Alzheimer's disease.

Keywords: Alzheimer's disease, neuroinflammation, astrocytes, microglia

2. Introduction

As longevity increases, the age-related diseases become more prevalent and particularly disorders of cognition. Best-known under the term of dementia this condition can be caused by a set of diseases and traumas that affect the brain as for example neurodegenerative disorders or strokes. In 2012, according to the Swiss Alzheimer's Association estimations, approximately 8% of the people over 65 and more than 30% of those over 90 live with a dementia [1, 2]. As the number of elders in our society increases, dementia has become a major concern for the aging population and will become one of the biggest challenges over the next thirty years. It is now estimated that about 110'000 people live with dementia in Switzerland and the prevalence of this condition is expected to triple by 2050 [3, 4].

According to the World Health Association, dementia refers to a syndrome, usually of chronic course in which there is a memory disorder associated with at least one other cognitive impairment such as attention, language, visuospatial skills or problem solving [1]. These changes must be of sufficient severity to affect daily functional activities and are beyond what should be observed during normal aging [4]. The clinical manifestation of dementia may vary according to the degree of severity of the disease. The signs and symptoms of dementia can generally be classified into three stages [4, 5, 6]: 1) The first stage in which the disease often goes unnoticed is characterized by a tendency to forget appointments or to get lost in familiar places. Symptoms usually appear gradually and patients and their relatives often consider these manifestations as a normal part of aging, which makes the diagnosis of dementia difficult as that moment. 2) The second stage corresponds to a worsening of signs and symptoms. The patients suffer significant omissions regarding recent events or forget for example the names of their relatives or the names of very well known personalities such as presidents. There is also a greater difficulty with communication. 3) The end-stage of dementia is characterized by an almost total dependence and is marked by a strong inactivity. The memory problems are very important and the physical signs and symptoms become more evident. Symptoms can range from loss of consciousness of time and space to the difficulty to communicate and move. There are also behavioural changes with greater impulsivity and aggressiveness.
Although dementia mainly affects the elderly it is not a condition of normal aging and always represents a pathological process. Unfortunately the current state of knowledge does not enable us to prevent, stop or cure this condition [1]. However, different treatments contribute to improve the quality of life of these patients [1, 4].

Dementia requires a major commitment of the relatives but also of the institutions and other care services. This disease has indeed not only physical and psychological consequences but has also a significant impact on social and economic conditions [5]. It is now considered the third leading cause of medico-social dependencies after cardiovascular diseases and cancers [1, 2]. Therefore, it is important to make an early diagnosis in order to provide specific support and help improve the quality of life of the patients and their families. This could also help delay the institutionalization often necessary in the terminal state of the disease.

There are many causes of dementia including Alzheimer's disease, fronto-temporal dementias, diffuse lewy-body disease, vascular and mixed dementia [6]. The final etiological diagnosis of cognitive disorders can only be confirmed by post-mortem neuropathological analysis [5, 7]; however, clinical diagnosis can detect the presence of dementia with acceptable specificity and sensitivity [7]. The clinical assessment of cognitive disorders is based on the definition of dementia according to the International Classification of Diseases: the CIM-10 [8] and the DSM-IV TR [9]. According to these classifications, the concept of dementia excludes an acute confusional state, a psychiatric disorder such as depression, only one single cognitive deficit and a mental retardation [8, 9]. The first step of the diagnosis is to collect a detailed history of symptoms and the rapidity of their progression. This history can be hold from the patient but an updated version of the family also plays a central role to clarify the exact course of the disease

[4]. This first evaluation may be facilitated by the use of standardized assessments tools such as the Informant Questionnaire on Cognitive Decline in the Elderly (IQCDE) [10]; the Nurse's Observation Scale for Geriatric Patients (NOSGER) [11]; the Bayer-Activities of Daily Living Scale (BADL) [12] and the Instrumental Activities of Daily Life Scale (IADL) [13]. Additional testing may also be used such as the Mini Mental State Examination (MMSE) [7], the clock test [14], the Mementool [7] and the Mini Geriatric Depression Scale (GDS) [7]. These screening tests do not allow a precise diagnosis but they highlight the probability of dementia and are good tools to assess the severity of the cognitive impairment. Thus, abnormal results to these tests should encourage pursuing further and specialized investigations [7].
The next step is to clarify the etiologic entity suspected to be the cause of the cognitive impairment. The neuroradiological investigations of the brain are essential for the differential diagnosis [4, 7]. The magnetic resonance imaging (MRI) is the best technique to establish a diagnostic hypothesis mainly based on the exclusion of other conditions that can cause similar symptoms, such as a tumour for example [4]. This approach also allows to assess the importance of vascular changes in the brain and to estimate brain atrophy [4]. The diagnosis however generally remains a diagnosis based on probabilities and can only be confirmed on a neuropathological level.

Among all the causes of dementia, Alzheimer's disease (AD) is by far the most common one and is believed to cause 60 to 70% of all cases [1]. Only probable clinical diagnosis of AD is currently available mainly based on the precise characterization of cognitive and behavioural deficits but there is unfortunately no absolute correlation between clinical signs and the underlying pathology [3]. More over, other causes of dementia can also appear in addition of the characteristics of AD making more difficult the etiological diagnosis. That is why at present the ultimate and precise diagnosis of AD is not possible without the post-mortem neuropathological confirmation [15]. This study focuses on this very common type of dementia.

3. Alzheimer's disease

3.1 Clinics

A German psychiatrist, Emil Kraepelin, first described the symptoms of this disease in the early 1900s [16]. It is however Alois Alzheimer, a German psychiatrist, neurologist and physician who discovered the neuropathological features underlying the disease [16]. It was in 1906 that he discovered a peculiar disease in the cerebral cortex of August Deter, a woman who suffered memory, language and other psychological disorders [17].

Alzheimer's disease is clinically characterised by an amnesiac syndrome – with loss of short-term and long-term memory as well as episodic and semantic memory – associated with a deterioration of higher cognitive functions such as visual and spatial disorders as well as problems with reasoning or calculating difficulties [5, 6, 7]. These troubles are the sign of a cortical involvement [5].

A pre-symptomatic state called mild cognitive impairment (MCI) is thought to be an intermediate state between normal cognition and dementia [18]. MCI criteria typically include a measurable deficit in cognition in at least one domain but not fulfilling the criteria of dementia and having no major impact on activities of daily life [18, 19]. The exact distinction between normal aging and MCI is difficult in clinical practice because of their similarities. It is normal with aging to experience memory problems and other cognitive disorders but the concept of MCI was originally developed to highlight the memory impairment and its possible precursor status of AD. Petersen et al have actually highlighted the development of certain forms of cognitive impairment to a more advanced disease making the MCI a probable early onset of dementia [20].

As for the other dementias, clinical diagnosis of Alzheimer's disease is based on a series of diagnostic criteria making it more or less probable or in some cases almost certain. It is recommended to use the diagnostic criteria for Alzheimer's disease according to the DSM-IV-TR [9] and the NINCDS-ADRDA [21, 22]. These criteria are mainly based on the clinical symptoms of the disease affecting the cognitive functions but they also consider the somatic and psychiatric disorders, the personality and social interaction changes as well as the family problems. Based on the results of these evaluations and diagnostic criteria a probable diagnosis of Alzheimer's disease can be established.

In the absence of biological or neuroimaging markers of AD, clinicians must rely on the clinical symptoms. The scales GDS9 (Global Deterioration Scale) [23] and CDR10 (Clinical Dementia Rating) [24] provide a multidimensional assessment of cognition, function and behaviour and thus generally allow specifying the stage of the disease. Three major clinical stages of Alzheimer's disease are defined [5, 6, 25] and are represented in the FIG.1 below. The initial stage of the disease is marked by a greater intellectual passivity with a loss of interest in daily activities, a failure of memory and anomic aphasia characterized by a difficulty for the patient to find his words. The second stage of the disease is marked by a progression of cognitive impairment including hallucinations and delusions [25]. Talking becomes very difficult and simple acts of everyday life are problematic. This stage is often considered the most difficult to support for the family [6]. The last clinical stage of the disease is marked by a loss of contact with the world and mutism [5]. The patient becomes totally dependent on the caregivers and is affected by psychomotor disorders, which make him very vulnerable to infections that are generally common causes of death [5, 6]. The progression of the disease is never experienced exactly the same way from one person to another and some symptoms may occur at any time of the disease. However, this classification allows families and caregivers to assess the progression of the disease and plan necessary measures to cope with future needs.

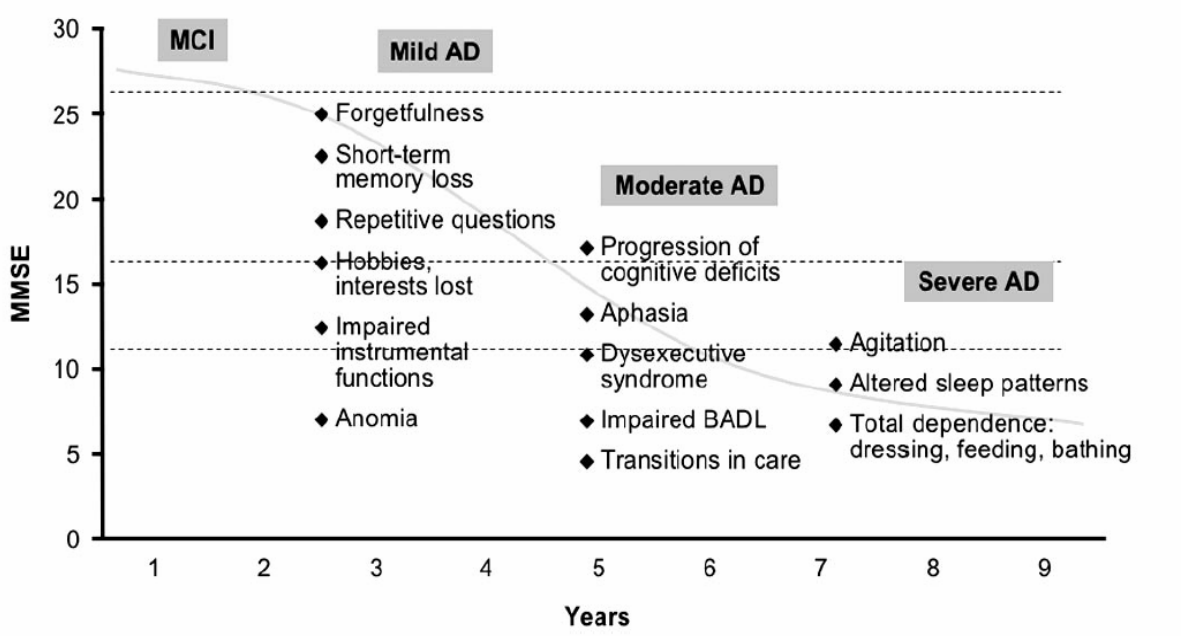

FIG. 1: Progression of symptoms of Alzheimer's disease [25].
MMSE: Mini Mental Status Examination. MCI: Mild Cognitive Impairment. AD: Alzheimer's disease. BADL: Basic Activities of Daily Living.

3.2 Neuropathology

From a neuropathological point of view Alzheimer's disease is a chronic neurodegenerative disorder defined by a massive and progressive cell loss in the brain [26] and characterized by the deposition of neuritic plaques and neurofibrillary tangles in many regions of the brain [27]. The first brain areas to be affected by neurodegeneration and neuronal cell death are mainly the entorhinal cortex and the hippocampus, which are key areas in the storage of information [28, 29]. With the progression of the disease, degeneration gradually reaches other cortical regions. Neuritic plaques and neurofibrillary tangles represent the pathological hallmarks of AD, and are respectively related to the accumulation of the beta-amyloid peptide (Aβ) and to cytoskeletal changes, which will be discussed in more detail later [26].

The pre-symptomatic phase or mild cognitive impairment (MCI) already presents some structural alterations in limbic regions, which includes the hippocampus [19]. This condition is thought to last for approximately 7 to 10 years until the overproduction and accumulation of abnormal structures in the brain reach a critical level and the symptoms become more important [19]. The morphological changes observed during this stage can also be seen in normal aging. It is therefore not certain that all people with MCI develop Alzheimer's disease and this overlap with normal aging makes the diagnosis of this disease more difficult [30].

The initial state of the disease is an early-stage pathology ranging from mild neuronal dystrophy to early-stage Braak pathology [28, 29, 31]. This phase may last for several years according to individual brain reserve. The second phase or moderate AD is established when the progression of neuronal damage extends to the adjacent brain regions with a worsening of clinical symptoms and a decline of cognitive functions [28, 29, 31]. The third and clinically defined dementia phase of AD is characterized by severe cognitive and functional impairment.

The Braak staging is a method described by Braak and Braak in 1991, and is used to classify the degree of the pathology in Alzheimer's disease based on the distribution pattern of neurofibrillary changes [31]. It is most often used in research and is determined by performing an autopsy of the brain. Braak and Braak noticed that the disease always starts at the same place and that the neurofibrillary degeneration develops sequentially [29, 31]. They distinguished six stages that they associated with different cognitive functioning. Stages I and II are used when neurofibrillary tangles are confined mainly to the transentorhinal region of the brain reaching the second layer of the entorhinal cortex and the hippocampal CA1 sector [31]. Stages III and IV when there is a severe involvement of limbic regions such as the hippocampus. The number of neurofibrillary tangles increases in the affected areas and extends without the amygdala and the temporal neocortex [31]. The last stages, V and VI, are marked by cortical destruction and an extensive neocortical involvement [31]. Figure 2 shows the distribution pattern of neurofibrillary tangles described by Braak and Braak.

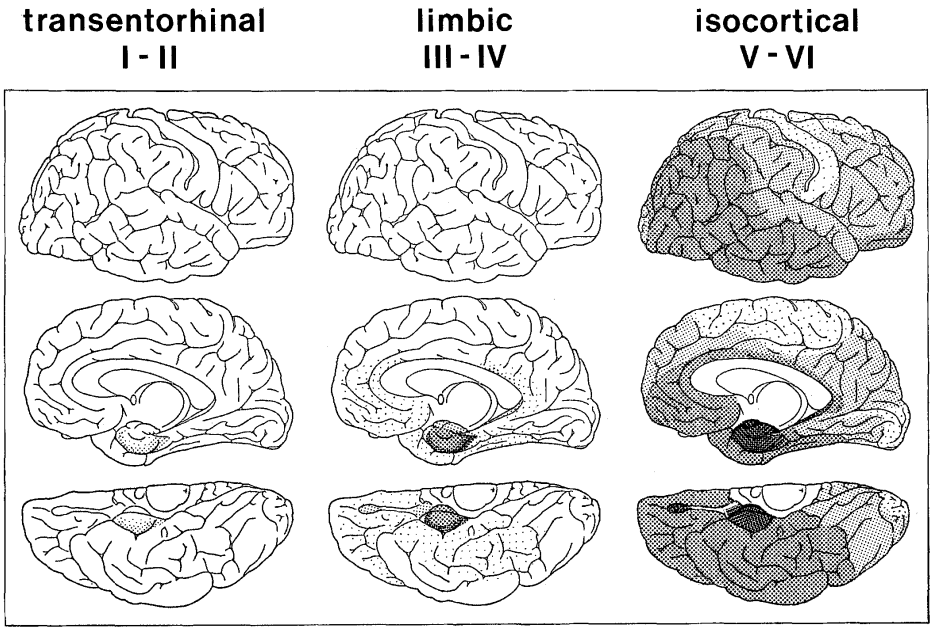

FIG. 2: Distribution pattern of neurofibrillary tangles.
« *Six stages (I-VI) can be distinguished. Stages I-II show alterations, which are virtually confined, to a single layer of the transentorhinal region. The key characteristic of stages III-IV is the severe involvement of the entorhinal and transentorhinal regions. Stages V-VI are marked by isocortical destruction. Increasing density of shading indicates increasing severity of neurofibrillary changes.* »
[29]

3.3 Etiology and risk factors

The exact cause of AD is still not known, but today, it is assumed that there is an interaction of several factors [26, 27]. It has been shown that it is possible to preserve a good mental shape by having a good physical and mental condition, which helps decreasing the risk of developing the disease [6]. However, a number of risk factor is beyond our influence, one of the most important one is age. The risk of developing a cognitive impairment, still low at the age of 60, doubles every five years thereafter [1]. Indeed, while AD is not an inevitable consequence of aging, its frequency increases dramatically as people age into their 70's and beyond. It is also assumed that other factors – not yet identified – may trigger or accelerate brain lesions [15].

Genetic variations play an important role in the autosomic form of the disease, which represents 2-3% of all AD cases [32]. A possible family component was suggested early in the discovery of this condition and evidences for a genetic component for AD were developed by a variety of sources [32, 33, 34]. More over, many studies have found an important frequency of the disease in relatives of patients suffering from AD [32, 33]. It was then highlighted that a familiar component was associated with a very early onset of the disease [31]. Since this discovery, a number of mutations in different genes have been identified, which may play a role in the incidence of familial AD. One of these genes encodes for the precursor of the protein beta-amyloid (APP), while the other ones, encode for transmembrane proteins, Presenilins 1 and 2, which are implicated in multiple cellular processes including the Aβ peptide pathway [34]. Another gene, encoding for the apolipoprotein E, has also been associated with familial Alzheimer's disease [34]. In this case, it is not a mutation but a genetic polymorphism, which makes these people more prone to the disease [31]. The apolipoprotein E gene exists in three different forms or alleles (E2, E3, E4), each producing a slightly different protein. It is only the presence of the E4 allele, which is a risk factor for the disease [31].

Although genetic factors may explain a part of the development of the disease, most patients with AD have no relatives suffering from this condition. This shows that other risk factors come into play. Age is often mentioned as the principal risk factor but other processes as for example inflammatory and oxidative events may play an important role in the course of the disease. The purpose of the present study is to better understand the role of inflammatory processes in the development of the sporadic form of AD.

4. Accumulation of altered proteins: physiopathology of Alzheimer's disease

Analyses of brain tissue of patients with Alzheimer's disease have shown many molecular lesions, but the characteristic anomaly, which predominates, is the accumulation of misfolded proteins resulting in cerebral dysfunction [35]. Two major components present significant abnormalities in Alzheimer's disease tissue: the β-amyloid peptide and the tau protein [27].

The major assumption about AD is the Aβ hypothesis, which states that the accumulation of a β-amyloid peptide could trigger a complex cascade downstream that could result in cognitive deficits [30]. The Aβ peptide is generated from a sequential proteolysis of a transmembrane protein precursor, the amyloid precursor protein (APP), which is ubiquitously expressed in neuronal and non-neuronal cells. APP can be processed by three proteases, the α-, β-, and γ-proteases [27]. According to the pathway that leads to the hydrolysis of the APP, the final result will be different. Studies have shown that alterations in these processes could cause the accumulation of an abnormal Aβ product, which then aggregates into amyloid plaques and may play a central role in the pathogenesis of the neurodegeneration [26, 27, 35].

The cleavage by the proteases can follow two different pathways. In the first one, also called the non–amyloidogenic pathway, the amyloid precursor protein is cleaved by the α-secretase. In this case, the cleavage of the APP occurs within the Aβ region, which can no longer be produced [27, 36]. The second one, called the amyloidogenic pathway leading to the Aβ peptide generation, is mediated by the β-secretase which leaves intact the Aβ region within the membrane. Then, a subsequent cleavage by the γ-secretase liberates the Aβ peptide in the extracellular space, which aggregate into insoluble oligomers [27, 36]. This, further originate fibrillary Aβ species that accumulate into senile and neuritic plaques, that might lead to synaptic damages and ultimately to neurodegeneration [26]. The exact mechanism by which such damages are caused is not fully understood, but various hypotheses explain them, as for example mitochondrial dysfunctions, lysosomal failure and alterations in signalling pathways [35]. The figure below illustrates these processes.

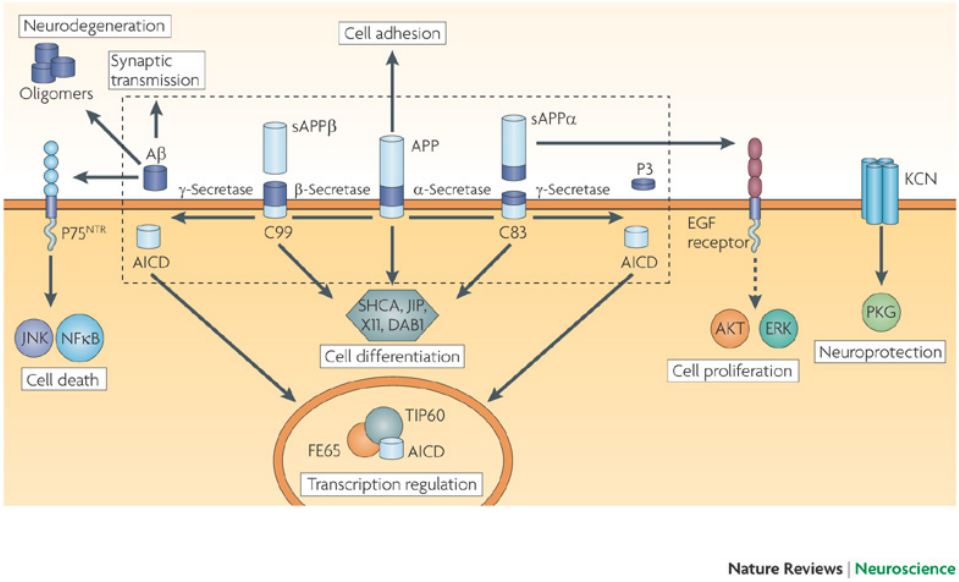

FIG. 3: Processing of Amyloid Precursor Protein.

« *On the right side, cleavage by α-secretase initiates non-amyloidogenic processing. A large amyloid precursor protein (sAPPα) ectodomain is released, leaving behind an 83-residue carboxy-terminal fragment. C83 is then digested by γ-secretase, liberating extracellular p3 and the amyloid intracellular domain (AICD). On the left side, amyloidogenic processing is initiated by β-secretase releasing a shortened sAPPα. The retained C99 is also a γ-secretase substrate, generating Aβ and AICD. AICD is targeted to the nucleus signalling transcription activation. Soluble Aβ is then prone to aggregation.* » [36]

Experimental evidences indicate that these abnormal signalling pathways might also exacerbate the promotion of abnormal tau phosphorylation and aggregation [36]. Tau protein is a microtubule-associated protein mainly localized in the neurons, which normally promotes the assembly and stability of microtubules and vesicle transports. When tau is being hyperphosphorylated, it turns into an insoluble form and lacks its affinity for microtubules and self-associates into paired helical filament structures that are called neurofibrillary tangles [27]. Theses aggregates of abnormal tau molecules are cytotoxic and impair normal brain functions [27]. Recent studies seem to demonstrate that tau protein may alone cause different types of dementia even in the absence of amyloid pathology [30]. These processes of accumulation of altered proteins, along with an increased oxidative stress and the decrease of the mechanism of clearance of proteins - all of which are also related to aging - may accelerate the accumulation of Aβ peptide and hyperphosphorylated tau proteins in Alzheimer's disease [27, 35].

Although these pathological processes are widely proven in Alzheimer's disease, it has been highlighted that these events may also appear during normal aging. Delrieu et al. have been able to demonstrate that 16 to 33% of normal patients also present some amyloid lesions [37]. Moreover, it seems that there is no great relationship between memory performances and the amyloid burden and that clinical symptoms would be better correlated to the synaptic dysfunction and neuronal loss [38].
Therefore, the mechanism underlying the formation of amyloid plaques and their alleged role in the physiopathology of AD is currently being questioned [30].

5. Inflammatory hypothesis

In Alzheimer's disease, brain regions affected by neuronal degeneration show an increase in astrocytes density suggesting a reactive astrogliosis [39, 40]. Reactive gliosis is a complex response that occurs following an injury or a damage of the brain tissue for example during neurodegenerative diseases. This gliosis is characterized by the proliferation and transition to an active state of the glia, i.e. astrocytes and microglia, and by an increased permeability of the blood-brain barrier [41]. Given that these activated astrocytes are localized preferentially around senile plaques several hypotheses support the idea that other factors, including inflammatory processes and oxidative stress, may play a key role in the pathogenesis of Alzheimer's disease [39, 42, 43, 44].

The main role of inflammatory processes in the central nervous system is to maintain homeostasis by fighting infections and preventing microorganisms to cause too much damage to the tissues. The immune cells present in the brain are microglia and astrocytes whose main role is to phagocytose and degrade toxic products [40, 45, 46]. Microglia is a population of brain-resident cells that have the same characteristics of macrophages whose normal function in the brain is immune surveillance. They are the first cell type to respond to any perturbation or injury within the central nervous system [40]. Astrocytes on the other hand are the most frequent cells of the brain. They have multiple functions that allow neurons to mature and specialize for the tasks of information processing but it has been shown that they can also play an important role in inflammatory processes [39, 40]. These two types of immune cells normally remain in a quiescent stay until an infection or an injury activates them [47]. Once activated, they acquire the ability to secrete a variety of pro-inflammatory molecules such as interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α) [47, 48, 49, 50, 51]. This acute inflammatory response is essential to promote the migration and activation of other components of the immune system to the compromised area and to respond immediately to the injury [44].

A chronic response occurs when the harmful stimulus persists over time. The inflammatory process transforms into a long-standing and often self-perpetuating neuroinflammatory response, which in the end could have detrimental consequences for neurons [40, 44]. Fuster-Matanzo and colleagues showed that chronically activated microglia and astrocytes could induce necrosis in adjacent neurons by releasing highly toxic products such as reactive oxygen intermediates, nitric oxide and inflammatory cytokines [52]. Thus, chronic neuroinflammation may have a double effect, it can be useful for neuroprotection – as a normal physiological process essential in the defence against microorganisms or external aggression and to promote tissue repair – but an excessive inflammatory response may in some cases be harmful and interfere with normal brain functions [42, 47]. This chronic inflammation cycle could play a role in the development or the progression of neurodegenerative diseases such as Alzheimer's disease [42, 44, 53].

It is thought that in this pathological condition the inflammatory mediators such as cytokines and chemokines could alter the normal structure of the brain [54]. We know for example that a variety of brain lesions result in an inflammatory response, which exacerbate brain damage by increasing oxidative stress [44, 55]. More over studies in animals have shown that prolonged brain inflammation may interfere with the normal processes of the brain such as impairing functions of the hippocampus, the brain region involved in the generation and storage of memory [56]. Other neurodegenerative diseases, such as amyotrophic lateral sclerosis or Parkinson disease, also show signs of inflammation at the onset of symptoms and it is supposed that inflammation could have an important role in the pathogenesis of these disorders [57]. All these observations led to an "inflammatory hypothesis", which suggests that the inflammatory factors produced by the activated immune cells of the brain could damage neurons, axonal processes and synapses leading to the symptoms of Alzheimer's disease [44, 48, 49, 52]. Another argument in favour of this hypothesis comes from the results obtained by Russo and colleagues

showing that genetic polymorphisms of certain inflammatory cytokines and their receptors can modulate the risk of neurodegenerative diseases [48]. Furthermore, they have shown that modulation of these inflammatory molecules in animal cell models could be effective in the fight against the disease process [48].

Chronic inflammation often begins insidiously as a smouldering and asymptomatic response. Thus, it may happen several years before the damage caused by inflammation is important enough to interfere with the proper functioning of the brain and the disease becomes clinically detectable. Alzheimer's disease is characterized by a long pre-clinic period during which the brain is confronted to increasing amounts of toxic Aβ peptides [19]. It is supposed that amyloid deposition begins 10 to 20 years before the appearance of clinical dementia [40]. Increase of glial cells and expression of inflammatory cytokines have been observed in AD brains and these immune cells are found in large concentrations around β-amyloid plaques suggesting a local increased activity of inflammatory processes in these specific regions [52]. It is also assumed that the activation of these inflammatory processes may activate caspases leading to the cleavage of nuclear and cytoskeletal proteins including tau protein and thus promoting the formation of neurofibrillary tangles [38].

More over it has been demonstrated that an inflammatory environment may also influence the temporal and spatial relationship in the neural stem cell niche affecting the neurogenesis, i.e. the formation of new neurons [48]. Damaged neurons can normally be repaired by the activation of neuronal stem cells. Inflammation could then have an important impact on the efficiency of brain functions and repair, which are essential for patients with Alzheimer's disease who need to adapt and maintain their brain functionality despite the neuronal loss. Besides, neurogenesis normally contributes to the improvement of learning and memory that are the main cognitive deficits in AD [52].

The results of these studies support a role of inflammation in the development of neurodegenerative disorders such as Alzheimer's disease. Furthermore, they support the idea that chronic inflammation may be one of the earliest events in the disease and may last for several years before clinical symptoms appear. The chronic release of pro-inflammatory molecules is likely to maintain a prolonged cascade of proteins secretion that may contribute to the neurodegeneration [49]. It is however not clear whether these immune changes are a consequence of the pathological amyloid aggregation or contribute to its pathogenesis. Two different models support this inflammatory hypothesis [54, 52]. The main hypothesis claims that the inflammatory cells are chronically activated as a response to the accumulation of Aβ deposition [52]. The other assumption is that the initial events in the pathogenesis of the disease are due to inflammatory events occurring in the periphery, such as for example vascular inflammation and atherosclerosis causing a consequent accumulation and deposition of amyloid proteins [57]. The mechanism by which cytokines could stimulate Aβ production is complex and not well understood but a study suggested that the cleavage of the APP protein could be disturbed [49]. Some models supporting this hypothesis suggest the existence of a cycle in which inflammatory changes lead to the accumulation of Aβ protein, which then stimulates glial cells to produce more inflammatory mediators [50]. Although this discussion remains open, it shows some evidence on the role of inflammatory processes in the development and/or the progression of the disease.

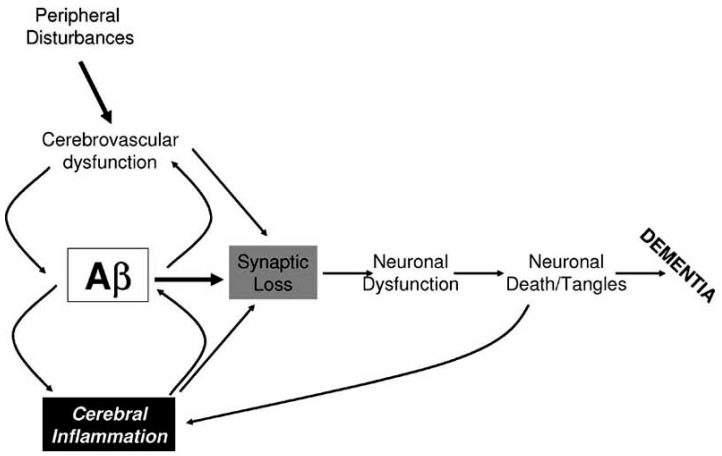

FIG. 4: Potential interactions of Aβ peptide, vascular inflammation and cerebral inflammation in precipitating Alzheimer's disease pathogenesis.
« *This scheme shows that peripheral disturbances (e.g. high levels of cholesterol, vascular inflammation, atherosclerosis) may cause brain dysfunction by initiating an inflammatory pathway. This will reduce transport of oxygen and glucose, creating conditions of oxidative stress that can increase Aβ production and aggregation, and neuronal stress directly. Deposition of Aβ can also lead to cerebral inflammation, which can feedback to increase production of Aβ. The contribution of cerebral inflammation in the progression of pathological changes remains unclear, but it is thought that inflammatory factors can mediate many of the neurotoxic events occurring in AD.* » [57]

Supporting this inflammatory hypothesis, some studies have suggested that the use of non-steroidal anti-inflammatory drugs (NSAIDs) may help to prevent Alzheimer's disease by modulating the factors involved in neuroinflammation [54, 57, 58]. It has been demonstrated by these studies that a neuroprotective effect against the development of AD could be obtained by a long-term use of NSAIDs [54, 58]. The use of these drugs in patients with established Alzheimer's disease has shown some beneficial effects on cognition not only by suppressing the inflammatory effects but also by decreasing the production of toxic Aβ peptides [58]. In addition, it has been demonstrated that patients with inflammatory diseases such as rheumatoid arthritis or osteoarthritis have less risk of developing AD. This reinforces the idea of a possible protective effect of anti-inflammatory drugs [57].
However, these therapeutic studies have, for the moment, shown positive results only in a small proportion of patients and other randomized studies present inconsistent results. Indeed, recent clinical trials with NSAIDs based on the hypothesis that these drugs could prevent the neurodegenerative disorders of the central nervous system show no significant difference in cognitive decline between NSAIDs use and placebo [59, 60, 61, 62, 63]. It was even demonstrated that the use of NSAIDs could have an adverse effect in advanced disease stages [64]. Thus, although the inflammatory component of Alzheimer's disease has been established by various studies, its exact role in the disease has not yet been clearly elucidated, and clinical trials of NSAIDs have not yielded the expected results. It is then not possible to conclude that anti-inflammatory treatments alone are a valid approach for AD.

6. Aims of the project

This project tries to better understand the differences between the normal aging of the human brain and the processes leading to Alzheimer's disease. As described earlier, many hypotheses exist to explain the pathogenesis of this disease, however, the relevance of theses hypotheses is constantly being discussed and questioned and the disease remains unfortunately unexplained and without effective treatments [30]. Diagnosis and therapeutic strategies might greatly benefit of a new approach based on the molecular pathogenesis of the disorder [15]. Thus the detection of very early and possibly preclinical stages of the disease by highlighting specific molecules becomes necessary for the efficacy of future targeted therapeutic strategies.

For the present study, attention was focused on the inflammatory hypothesis based on the idea that the accumulation of microglia and astrocytes around amyloid plaques activate a local cytokine-mediated response, which may damage neurons and exacerbate the pathological processes underlying the disease [39, 50, 65]. The release of cytokines and chemokines will cause the subsequent activation of other components of the immune response, which have the potential to further damage neurons and amplify any ongoing inflammatory response [40, 42, 44]. It is assumed that a chronic inflammation in specific areas of the brain could contribute to the worsening of the symptoms of AD.

This project consists on the analysis of different inflammatory molecules as well as the detection of characteristic proteins of AD in brain tissues from the entorhinal and the frontal region from control, moderate and severe Alzheimer's disease patients. These two brain regions were chosen for their importance in the development of the disease based on the model of Braak and Braak which states that the disease starts in a region of the entorhinal cortex then spreading to the Frontal cortex and other cerebral areas [28, 29].

The three molecules selected to target the inflammatory processes are TNF-α, IL-1β and NF-κB and were chosen for their important role in inflammation based on scientific literature [45, 48, 49, 52, 66, 67, 68]. It has indeed been demonstrated in these studies that activated microglia and astrocytes in AD brains might contribute to cell death by releasing pro-inflammatory cytokines such as TNF-α, IL-1β and INF-γ. More over it is assumed that these pro-inflammatory cytokines activate some protein kinases involved in tau hyperphosphorylation [48]. Another study suggested that the continuous presence of immune activators such as Aβ might stimulate the chronic state of inflammation in AD brains and showed that cytokines such as TNF-α, IL-1β and IL-6 can be directly cytotoxic, when chronically produced at high concentrations [49]. It was also suggested that these pro-inflammatory cytokines might in combination stimulate the production of Aβ peptide [49]. The transcription factor family, NF-κB, has been identified as playing a major role in the initiation of the innate and adaptative immune responses by regulating the expression of several genes involved in inflammatory and immune responses. Many studies suggest that this molecule should be considered as an important mechanism in inflammatory processes and autoimmune diseases [66, 67, 68]. More over some anti-inflammatory drugs and immunosuppressive treatments inhibit NF-κB [67]. Ascolani and colleagues investigated the expression of a number of genes involved in the inflammatory and oxidative response of a well-defined small cohort of sporadic AD patients [69]. They discovered that the expression of various NF-κB target genes were increased in peripheral blood mononuclear cells suggesting that NF-κB activation could be a potential new hallmark of AD progression [69].

The analyses and investigations of this research focus on three main issues:

1. The first question is to know whether significant inflammation is found in AD brains and if so at what stage of the disease the inflammation does play a role?

2. The second issue is to examine the cerebral distribution of the inflammation between the Entorhinal and the Frontal cortex and to determine if there is a significant difference between these two areas. The severity of the disease varies between these two brain regions and thus allows determining whether the inflammation is related to AD either as a primary and early event or as a secondary and late stage process.

3. The third question investigates whether there is a significant correlation between inflammation and the age of subjects.

The results of this study could help to better apprehend the role of neuroinflammation in the development of Alzheimer's disease and its symptomatology and to assess whether the development of new-targeted therapies against inflammatory processes could slow down the progression of the disease.

7. Materials and Methods

7.1 Tissues

A total of 20 brains were studied, eight normal control cases and twelve sporadic confirmed AD cases without knowing familial history, all going from 59 to 87 years of age. Clinical diagnosis was confirmed by neuropathological post-mortem examination. In addition, Alzheimer's cases were separated into two groups depending on the severity of the disease: six cases with moderate AD (Braak stages II-III-IV on post-mortem examination) and six cases with severe AD (Braak stages V-VI on post-mortem examination). The cause of death and the post-mortem delay (PMD) are also indicated.

Case	Sex	Age	PMD (hours)	Group	Cause of death
1	F	88	13	Control	Myocardial Infarction
2	F	79	12	Control	Bronchopneumonia
3	F	81	12	Control	Pulmonary cancer
4	M	72	7	Control	Heart failure
5	F	59	18	Control	Septic choc
6	M	85	14	Control	Prostatic cancer
7	M	73	7	Control	Heart failure
8	F	75	6	Control	Unknown
9	F	95	9	AD moderate	Septic choc + BP
10	F	80	6.5	Braak II	Pulmonary Embolism
11	F	92	12	AD moderate	Bronchopneumonia
12	F	88	7	Braak III	Bronchopneumonia
13	F	87	22	Braak II	Bronchopneumonia+ AOP
14	F	90	22	Braak IV	High digestive haemorrhage
15	M	84	2.5	AD severe	AD + Vascular dementia
16	F	78	6	Braak VI	AD + Diabetes + HTA
17	M	72	9	Braak V	Unknown
18	F	78	4	AD severe	AD + Diabetes + HTA
19	F	83	23	Braak VI	Unknown
20	F	87	12	AD severe	Unknown

BP: Bronchopneumonia; AOP : acute pulmonary oedema; HTA: Hypertension

7.2 Antibodies

Firts Antibodies	Used to detect a specific protein on the autopsy tissue
Anti-GFAP	Rabbit polyclonal antibody Anti-Glial Fibrillary Acidic Protein – GFAP (1:2'000 dilution) from Sigma (G-9269). GFAP is a major intermediate filament protein and a major cytoskeletal structure of astrocytes.
Anti-AD2	Mouse monoclonal antibody AD2 (1:10'000 dilution) from BioRad (56484) reacts with the Tau protein and more specifically with the upstream flanking region of the microtubule-binding domain when Ser396 and Ser404 are phosphorylated. These are phosphorylation sites, which remain stable in neurofibrillary lesions even in autopsy tissues thus making AD2 useful for the detection of the neurofibrillary tangles in AD brains.
Anti-β-amyloid	Mouse monoclonal antibody β-amyloid peptide 1-42 (1 :1'000 dilution) from DAKO. β-amyloid is the major component of amyloid plaques in association with Alzheimer's disease.
Anti-TNF-α	Mouse monoclonal antibody TNF-α (1:2'000 dilution) from Abcam (ab1793). Tumor necrosis factor-alpha is a pleiotropic inflammatory cytokine, which serves a variety of functions during inflammation as for example neutrophil proliferation.
Anti-NF-KB	Mouse monoclonal antibody NF-kB (1:100 dilution) from LabForce AG (SC-8414). Nuclear factor kappa-light-chain-enhancer is a protein complex responsible for cytokines production in response to stimuli such as stress, free radicals, irradiation and inflammatory processes.
Anti-IL-1β	Mouse monoclonal antibody IL-1β (1:100 dilution) from LabForce AG (SC-32294). Interleukin-1 beta also known as catabolin is a cytokine protein, which is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.
Second Antibodies	Used to detect the first antibodies
ECLTM Rabbit IgG	HRP-Linked (1:2'000 dilution) from GE Healthcare (NA-934).
ECLTM Mouse IgG	HRP-Linked (1:2'000 dilution) from GE Healthcare (NA-931).

7.3 SDS-PAGE

SDS (Sodium Dodecyl Sulfate) is the most common dissociating agent used to denature native proteins to individual polypeptides. The purpose of this method is to separate proteins according to their size and no other physical feature. SDS is a negative charged detergent that can dissolve hydrophobic molecules. When molecules are incubated with SDS, the membranes are dissolved so all the proteins contain only primary structure and all of them are covered with many negative charges. This allows the proteins, when placed in an electric field, to migrate towards the anode. If we want the proteins to separate by size, we need to put them into an environment that allows different sized proteins to move at different rates. The environment

chosen to separate the proteins by size is polyacrylamide, which is a polymer of acrylamide monomers, which turns into a gel made of a labyrinth of different sized pores. We can then use electricity to pull the proteins through the gel. This entire process is called PolyAcrylamide Gel Electrophoresis (PAGE).
Gels were prepared with acrylamide solution (39% acrylamide stock solution (Merk), 1% N.N-methylendiacrylamide (Merk), stacking buffer (3% Tris (Sigma), 0.2% SDS (BioRad), pH: 6.8, separating buffer (9.1% Tris (Sigma), 0.2% SDS (BioRad), pH: 8.8), ammonium persulfate (APS solution of 10% (Sigma)) and tetra-methyl-ethylene-diamine (TEMED) (Eurobio). Defined amounts of brain sample were then separated by a 3.6% - 15% gradient SDS-PAGE. The acrylamide gradient gel of 3.6% - 15% allows separating different proteins according to their molecular weight in the molecular range of 300 KD to 15 KD. The electrophoresis was done with 140V for around 3h in a running buffer (0.3% Tris (Sigma), 1.44% Glycine (Biosolve), 0.1% SDS (BioRad), and deionised water). After the electrophoresis, the gels were either stained by Coomassie blue or transferred to nitrocellulose membrane for Western Blotting [70].

7.4 Coomassie blue staining

After the electrophoresis, the gels were incubated in the Coomassie brilliant blue dye over night. This allows visualizing the general protein composition of the brain regions that are studied and to correct for uneven protein loading. The same samples were prepared for Coomassie blue staining and for Western blots on brain tissue from the entorhinal and frontal cortex. The gel usually contains a marker of known molecular weight, which allows determining the molecular weight of the proteins in the solution.

7.5 Western Blot and Chemiluminescence

Western blot is an analytical technique used to detect specific proteins in the given sample. In order to make the proteins accessible to antibody detection they are moved from within the gel onto a membrane made of nitrocellulose. The method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the nitrocellulose membrane. It consists on placing a nitrocellulose membrane (Reinforced NC, OptitRan BA-S 85) on top of the gel, and a stack of filter papers (GB003, Schleicher & Schuell) on top of that. The entire stack is placed in a buffer solution (24.9mM Tris (Sigma), 191.8mM Glycine (Biosolve), 20% methanol (Brenntag), 0.01% SDS (BioRad) and deionised water) by orientating the nitrocellulose membrane towards the anode. The transfer was done with 25V overnight. The proteins moved from within the gel onto the membrane while maintaining the organization they had within the gel. As a result, the proteins are exposed on a thin surface layer, which facilitate their detection. The nitrocellulose membrane is chosen for its non-specific protein binding properties (i.e. binds all proteins equally well). After the proteins were transferred to a nitrocellulose membrane, they were stained with monoclonal and polyclonal antibodies specific to the target protein. Detection is typically performed using peroxidase-linked antibodies to catalyse a chemiluminescent reaction.

The first step of chemiluminescence is the saturation of the membrane, which helps to avoid non-specific binding of the antibodies. To do so, the membrane was blocked for 30min to 1h with 5% milk in PBS-Tween20. Thus, when the primary antibody is later added, there is no room on the membrane for it to attach other than the specific binding site - the epitope of targeted proteins. After being washed for 15min with PBS (1.34g Na_2HPO_4, 9.06g NaCl (Merk), pH: 7.4), the membrane was incubated during 1h with a solution containing the first antibody (ABI, PBS, 2.5% milk, 0.15% Tween20), which will detect the protein we are looking for. After several washings in PBS, we incubated the membrane during 1h with the second antibody linked to a peroxidase enzyme that allows detection. Finally, after several washings, the membrane was

incubated for exactly 1min in the presence of the detection solution (Amersham, ECL Western Blotting Detection Reagents, GE Healthcare). This solution contains a substrate that triggers a chemiluminiscent reaction in contact with the peroxidase-conjugated antibody. For the chemiluminescence detection, the membrane was then exposed to light sensitive films (Kodak Biomax MR) for several seconds, minutes and sometimes hours, and the films were developed with the KODAK X-OMAT 1000 Processor. For further analysis, the films were scanned with an Image Scanner PowerLock 1120 UDS (GE Healthcare).

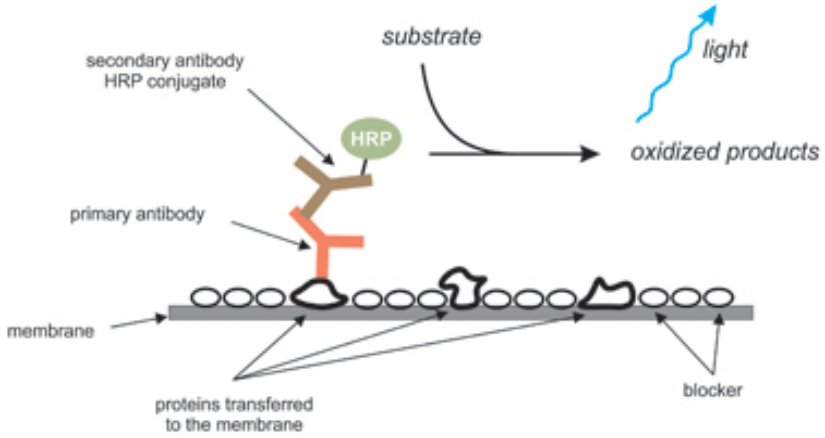

FIG. 5: Chemiluminescence method for western blot detection.
« *In this detection method, a primary antibody is added first, either a monoclonal or a polyclonal antibody, to bind to the antigen. Then a labelled secondary antibody is used directed against the primary antibody and reacts with the substrate.* » [71]

7.6 Quantification by Densitometry and Statistical analyses

After scanning the films, the immunoblots were analysed by using the quantification BioLab software (1-D Main, American Applied Biotechnology) to evaluate the intensity of the bands. Before performing the statistical analyses, the data were all normalized and adjusted to the levels of β-actin. The results were then entered into an Excel database from which graphs were designed. Statistical analyses were done by using the R project for statistical computing to compare the differences in the molecular density among the groups. Three comparisons were made. The first one was between the control cases and the moderate AD patients. The second between control and severe AD cases and finally a comparison was made between the two groups of Alzheimer's disease. The One Way Anova test and the Tukey's HSD test were used to determine if the sets of data were significantly different from each other. Statistical significance was considered for a p-value < 0.05. A result is statistically significant when it is unlikely that it could be obtained by a simple chance. Usually the threshold of 0.05 is used, which means that the observed result has less than 5% chances to be obtained randomly. It is therefore considered statistically significant.

8. Coomassie blue gels and Western blots

The Coomassie blue gels show equal loading of the proteins on the entorhinal (Fig 6.B) and the frontal samples (Fig 7.B). Some proteins seem more abundant in some areas as for example sample 9 of the entorhinal cortex or samples 13 and 14 of the frontal cortex but all the samples were normalized and adjusted to the levels of β-actin (Fig 6/7.A).

For each brain region a series of Western blots were prepared and immunostained with antibodies for GFAP, AD2 and β-amyloid to asses the degree respectively of astrogliosis, pathological tau protein and amyloid plaques. Antibodies directed against TNF-α, NF-kB and IL-1β – three molecules having an important role in the cascade of reactions triggered by inflammatory cells - were used to highlight inflammatory processes. The samples are aligned from left to right starting with the control group and then the AD cases. Patients 9 to 14 of the entorhinal cortex (Fig 6) and patients 8 to 13 of the frontal cortex (Fig 7) were identified as cases of moderate Alzheimer's disease (Braak stages II to IV). The rest of the AD patients are cases of severe AD (Braak stages V to VI).

GFAP immunoreactivity in the entorhinal cortex (Fig 6.C) is present everywhere with a trend towards increased GFAP immunoreactivity in moderate AD cases. However, the more the disease progresses (severe AD group) the more the detection of GFAP seems to fade. The results obtained in the frontal cortex (Fig 7.C) are more difficult to interpret on the immunoblot, which seems very homogeneous although the results suggest a slight increase of the GFAP detection in the severe AD group.

The immunodetection by AD2 (Fig 6/7.D) - a monoclonal antibody directed against pathological hyperphosphorylated tau proteins - shows no detection in the control tissues, except for two controls in the entorhinal cortex where we can distinguish a slight detection for patients 7 and 8. In contrast, tissues from patients with Alzheimer's disease show a large disparity of results with a significant increase of AD2 levels in the severe AD group in both brain regions.

Results with monoclonal β-amyloid antibody vary somewhat between the two brain regions studied. In the entorhinal cortex (Fig 6.E), the immunoreactivity reflects a distribution of β-amyloid to all samples, controls and AD patients, with however a significant decrease of the immunoreactivity in the AD cases. The results obtained in the frontal cortex (Fig 7.E) are almost the opposite suggesting an increment of this protein in AD cases with a more significant increase in the severe Alzheimer group.
The amyloid detection is at about 100 kDa, which could indicate that the antibody used recognizes the amyloid precursor protein that is about 90 kDa and not the Aβ fragment. The cleavage of β-amyloid precursor protein (APP) generates multiple proteins, soluble β-amyloid precursor protein alpha and beta (sAPPα, sAPPβ) and Aβ peptide. Previous studies have shown that sAPPα and sAPPβ possess neurotrophic properties, whereas Aβ is neurotoxic [72]. The detection here could be the neurotrophic part of the APP, the sAPPalpha or the sAPPbeta proteins. This could explain the decrease observed in cases of Alzheimer's disease of the entorhinal cortex [72].

The three antibodies used for the detection of specific inflammatory molecules show rather homogeneous results and will be discussed as one inflammatory process. The entorhinal cortex (Fig 6.F-H) does not appear to show an increase in inflammation in the AD cases compared to control ones. On the contrary, the results suggest rather a decrease in inflammatory proteins with the progression of the disease. On the other hand, the results obtained in the frontal cortex (Fig 7.F-H) show an increase of the immunosignal with the development of the disease. This increase is even more important in cases with severe AD.

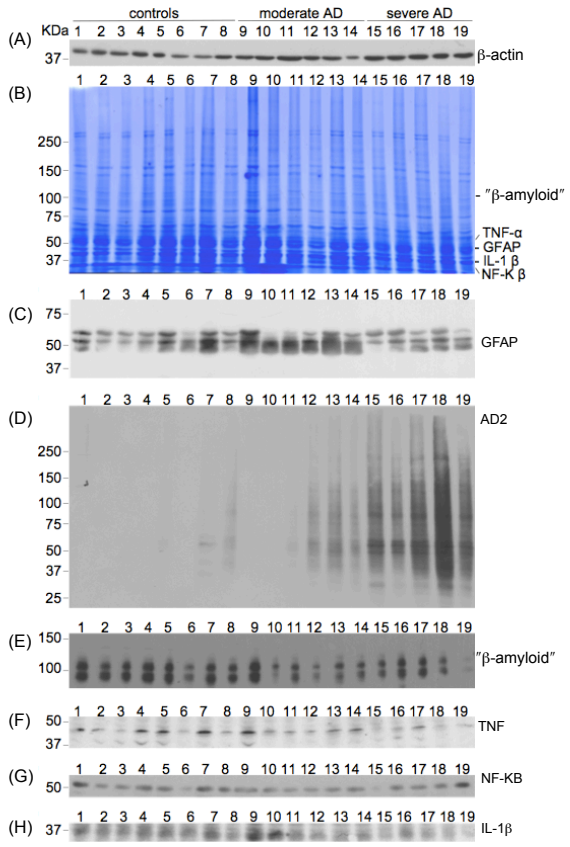

FIG. 6: **One-DE gels 3.6-15% gradient gels with 20μg of entorhinal cortex sample per well**.

The first 8 are controls without neurological pathology. The next 11 samples are the AD patients. This group is separated into two other groups, the first 6 samples are moderate AD and the last 5 are severe AD. All the samples were normalized to the levels of β-actin (A). The samples were then prepared for Coomassie blue stain (B) and for Western blots immunostained with (C): polyclonal anti-GFAP; (D): monoclonal anti-AD2; (E): monoclonal anti-"β-amyloid"; (F): monoclonal anti-TNF-α; (G): monoclonal anti-NF-kB; (H): monoclonal anti-IL-1β. After incubation with secondary peroxidase-conjugated antibodies detection was made by enhanced chemiluminescence. Molecular weights are indicated in KDa to the left, while relevant protein candidates are indicated to the right.

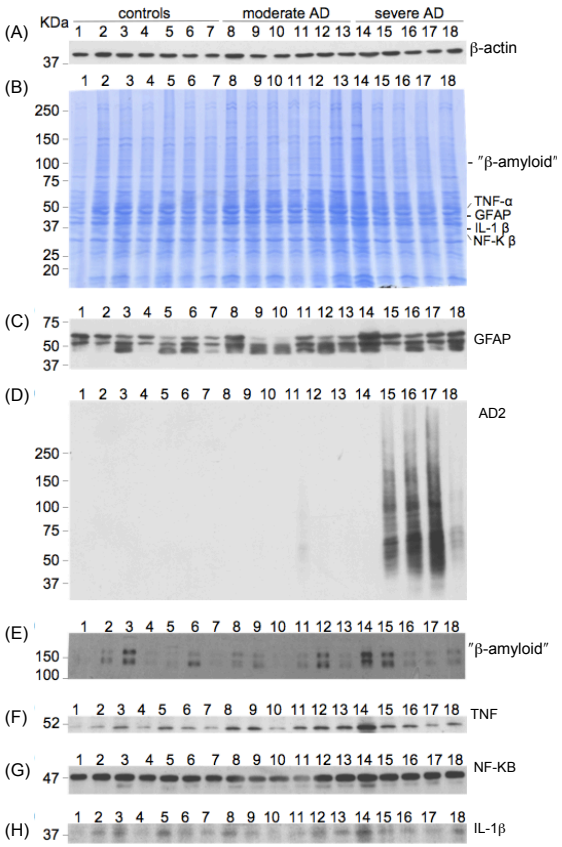

FIG. 7: **One-DE gels 3.6-15% gradient gels with 20μg of frontal cortex sample per well.**

The first 7 are controls without neurological pathology. The next 11 samples are the AD patients. This group is separated into two other groups, the first 6 samples are moderate AD and the last 5 are severe AD. All the samples were normalized to the levels of β-actin (A). The samples were then prepared for Coomassie blue stain (B) and for Western blots immunostained with (C): polyclonal anti-GFAP; (D): monoclonal anti-AD2; (E): monoclonal anti-"β-amyloid"; (F): monoclonal anti-TNF-α; (G): monoclonal anti-NF-kB; (H): monoclonal anti-IL-1β. After incubation with secondary peroxidase-conjugated antibodies detection was made by enhanced chemiluminescence. Molecular weights are indicated in KDa to the left, while relevant protein candidates are indicated to the right.

9. Statistical analyses and proteins quantification

9.1 Analysis of inflammation according to the stage of the disease and to the cerebral distribution between the entorhinal and the frontal cortex.

In order to know whether inflammation is found significantly in patients with AD, the three groups were compared with each other for each cerebral region by the One way Anova test. The antibodies used to detect the inflammation are GFAP for the reactive gliosis, TNF-α, IL-1β and NF-κB. The test attempts to reject the null hypothesis, which states that the three groups have the same inflammatory composition. In case of rejection of the null hypothesis the three groups were compared in pairs with the Posthoc Tukey's test. The threshold for rejection of the null hypothesis is a p-value < 0.05.

Entorhinal Cortex

With this analysis it is possible to reject the null hypothesis for IL-1β, β-amyloid and AD2 in the entorhinal cortex and identify a significant difference in the levels of these markers between the three groups. The FIG.8 shows the distribution of astrocytes and inflammation according to the severity of the disease in the entorhinal cortex. The analysis identifies a significant difference in the proteins composition between the three groups only for IL-1β with less quantities of this protein in the group with severe AD compared within the two other groups (p-value = 0.00507). The data available do not provide sufficient evidence that the other markers are differentially expressed between the three groups (p-value > 0.05). The FIG.9 shows the distribution of AD2 and β-amyloid according to the severity of the disease in the entorhinal cortex. The statistical analysis identifies a significant difference in the proteins composition between the three groups. (p-value < 0.05). The analysis of AD2 immunoreaction allows stating that patients with severe AD have higher AD2 levels than those with moderate AD or without the disease and that patients with moderate AD have higher AD2 levels than the controls. The β-amyloid analysis allows to state that patients with severe AD have less amyloid amounts than the two other groups and that patient with moderate AD have less amyloid amounts than the control group.

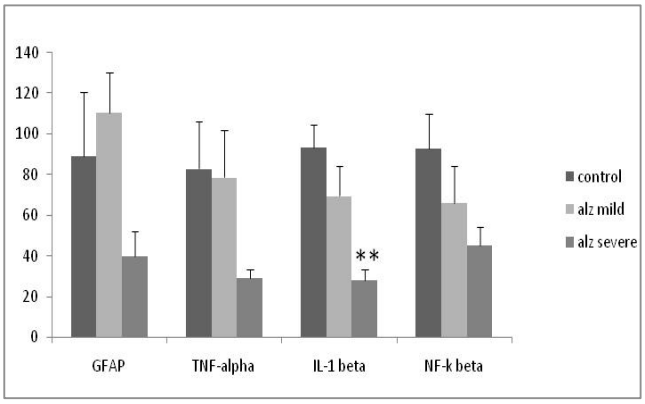

FIG. 8: Immunoreaction intensity of markers for astrocytes and inflammation according to the severity of the disease in the entorhinal cortex.

Statistical analysis by the One Way Anova test of GFAP, TNF-α, IL-1β and NF-κB between severe AD, moderate AD and controls.

The values on the left represent the staining density of immunoblotting bands. One asterisk means there is a statistical significance (p < 0.05) while very high statistical significance (p < 0.01) is reported with two asterisks.

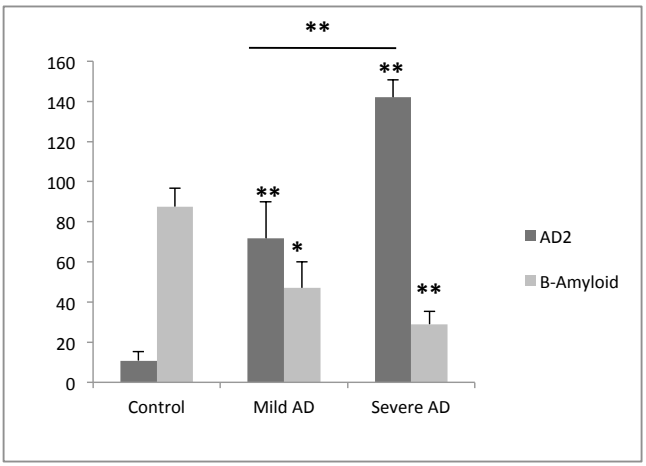

FIG. 9: Immunoreaction intensity of markers for AD2 and β-amyloid according to the severity of the disease in the entorhinal cortex.

Statistical analysis by the One Way Anova test of AD2 and β-amyloid between severe AD, moderate AD and controls.

The values on the left represent the staining density of immunoblotting bands. One asterisk means there is a statistical significance ($p < 0.05$) while very high statistical significance ($p < 0.01$) is reported with two asterisks.

Frontal Cortex

In the frontal cortex the statistical analyses allow rejecting the null hypothesis for GFAP, β-amyloid and AD2 and to highlight a significant difference in the levels of these markers between the three groups. The FIG.10 shows the distribution of astrocytes and inflammation according to the severity of the disease in the frontal cortex. The analysis identifies a significant difference in the proteins composition between the three groups only for GFAP with higher levels of this protein in the severe AD group compared to the other ones (p-value = 0.016). The data available do not provide sufficient evidence to state that the inflammatory markers are differentially expressed between the three groups (p-value > 0.05). The FIG.11 shows the distribution of AD2 and β-amyloid according to the severity of the disease in the frontal cortex. It is possible to identify a significant difference in the proteins composition between the three groups. (p-value< 0.05). The AD2 analysis allows stating that patients with severe AD have higher AD2 levels than those moderate AD or without the disease. The β-amyloid analysis allows stating that patients with severe AD have higher amyloid amounts than the two other groups.

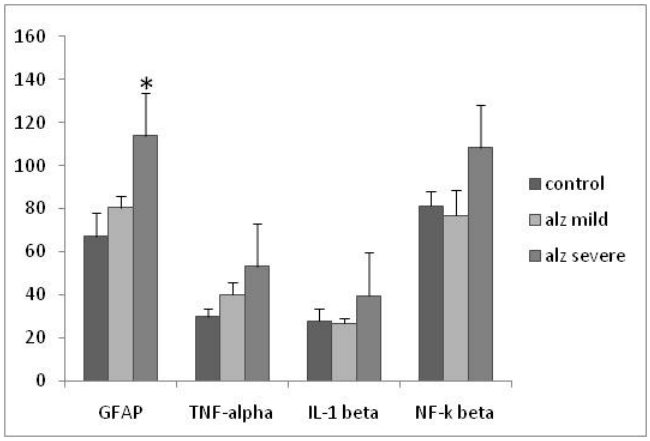

FIG. 10: Immunoreaction intensity of markers for astrocytes and inflammation according to the severity of the disease in the frontal cortex.

Statistical analysis by the One Way Anova test of GFAP, TNF-α, IL-1β and NF-κB between severe AD, moderate AD and controls.

The values on the left represent the staining density of immunoblotting bands. One asterisk means there is a statistical significance ($p < 0.05$) while very high statistical significance ($p < 0.01$) is reported with two asterisks.

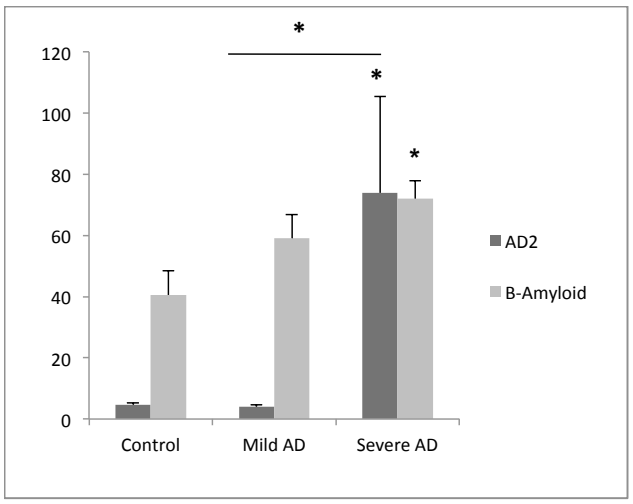

FIG. 11: Immunoreaction intensity of markers for AD2 and β-amyloid according to the severity of the disease in the frontal cortex.

Statistical analysis by the One Way Anova test of AD2 and β-amyloid between severe AD, moderate AD and controls.

The values on the left represent the staining density of immunoblotting bands. One asterisk means there is a statistical significance ($p < 0.05$) while very high statistical significance ($p < 0.01$) is reported with two asterisks.

9.2 Analysis of inflammation according to the age of subjects

The association between the age of subjects and the values of markers was estimated by the Kendall's tau (τ) correlation coefficient. This coefficient is used as a statistical hypothesis test to establish whether two variables (age and a specific antibody) may be regarded as statistically dependent. Statistical analyzes do not allow to conclude to any correlation between age and inflammation.

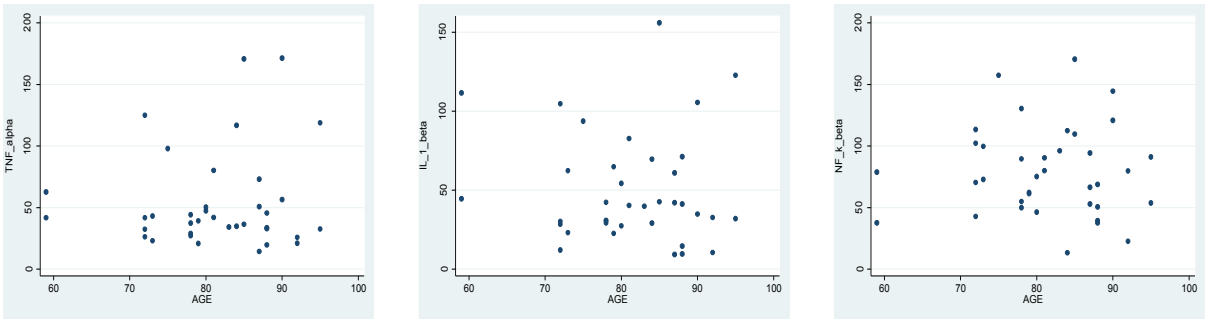

FIG. 12: Distribution of inflammation according to the age of subject.

Statistical analysis by the Kendall's tau correlation coefficient of the association between the age of subjects and the values of inflammatory markers. No statistical correlation was found and the p-value was > 0.05 for the three proteins.

10. Summary of results

The aim of the study was to investigate the inflammatory changes in the entorhinal and the frontal cortex of human brains from patients with and without Alzheimer's disease. A total of 20 brains were analysed – 8 controls, 6 moderate AD and 6 severe AD – using one-dimensional electrophoresis and immunostaining combined with chemiluminescence. The results highlighted a disparity in the pathological accumulation of tau and β-amyloid proteins not only according to the stage of the disease (moderate and severe AD) but also depending on the region of the brain (entorhinal and frontal cortex). There is also a large difference in GFAP composition between the two brain regions. It appears that the reactive gliosis is higher in patients with moderate AD than controls in the entorhinal cortex but starts to decrease with the progression of the disease whereas it increases in patients with severe AD in the frontal cortex. It was identified that inflammatory processes are present since the beginning of the disease and that

they become more important with the progression of it in the frontal cortex but that they diminishes in the entorhinal region when the disease reaches a severe stage.

11. Discussion

Inflammation and Alzheimer's disease

The purpose of the present study was to corroborate the hypothesis that the pathogenesis of AD is also accompanied by a reactive gliosis supporting the idea that inflammatory processes occur in some brain regions. The results obtained in this study suggest that immune responses in the central nervous system play a role in the early development of Alzheimer's disease but it seems that the worsening of cognitive symptoms is associated to other components.

The increase in glial fibrillary acidic protein, GFAP, is a well-established feature of Alzheimer's disease and serves as a good marker to indicate neurodegenerative changes [39, 45, 47, 49, 52, 73] The analysis of this study confirms that it is an important component at the beginning of the disease showing that GFAP levels are very high in the moderate AD group. The evolution of this protein varies however according to the progression of the disease and brain region. For example, in the entorhinal cortex the more the disease advances the more GFAP levels seem to lose their importance. The results of a previous study based on an immune therapy are in accordance with these findings showing a decline of active microglia levels in patients with severe AD and thus highlighting the possibility of a reduction of the reactive gliosis in the later stages of the disease [57]. On the other hand, the frontal cortex shows an increase in this protein density with the progression of cognitive decline. According to Braak and colleagues, Alzheimer's disease starts in a region of the temporal lobe, which includes the hippocampus and the entorhinal cortex and spreads to the rest of the brain areas, particularly in the frontal cortex, only later in time [28]. We can therefore assume that while the disease reaches a very advanced stage in the entorhinal cortex – when all the neurons are dead and the glia seems not active anymore - it progresses in the frontal cortex where active neurodegeneration and astrocytes can be found in great quantities. This observation is in agreement with the study of Porchet et al. suggesting an association between the degree of neuronal death and the amount of astrocytes [39]. Glial cells are the protectors of the central nervous system and among all their functions they have the capacity to respond to all forms of brain injury by transforming to an active state and secreting pro-inflammatory cytokines [45, 52, 73]. These reactions are a normal physiological response of the body against external or internal aggression and can therefore also be encountered during normal aging [39]. This might explain why of GFAP was also found in the control group.

However, this ability of immune cells to activate themselves and to secrete cytokines to protect the brain from aggressions can also have deleterious effects and play a key role in the pathogenesis of neurodegenerative diseases. Indeed, astrocytes and microglia, the major immune cells in the cerebral tissue, are involved in the maintenance of the synaptic plasticity, which allows the modulation of connections between neurons [47, 74]. This neural plasticity is essential to the development of learning and memory and allows explaining the many forms of memory present in all individuals with a central nervous system [75, 74]. This fine and precise modulation of neuronal connections is achieved through these same proinflammatory cytokines such as TNF-α and IL-1β [74]. During neurodegeneration, the immune cells involved secrete larger amounts of cytokines, which consequently alters the normal and necessary balance to maintain synaptic plasticity [74]. Thus, neuroinflammation could also participate in the pathogenesis of neurodegenerative diseases by altering the synaptic processes that underlie learning and memory.

A number of studies also demonstrated that highly toxic products such as reactive oxygen intermediates and inflammatory cytokines such as TNF-α and IL-1β produced and secreted by astrocytes might induce a subsequent cascade of pro-inflammatory gene transcription that can lead to neuronal loss [42, 48, 55, 65, 76]. Inflammation plays an important role in brain injuries by decreasing the permeability of the blood-brain barrier allowing changes in the cellular and inflammatory profile of the brain tissue [55]. Chronically activated immune cells and inflammatory cytokines are then believed to play a central role on neurodegenerative disorders by inducing neuronal death [49]. The results of the present study are compatible with that hypothesis revealing a similar evolution between GFAP and inflammatory molecules in AD brains. It also revealed that in the entorhinal cortex neuroinflammation is very important in the moderate AD group, with high levels of TNF-α, IL-1β and NF-kB, while it decreases in the severe AD group when the amount of astrocytes diminishes. The inflammatory components also follow the same increasing trend as GFAP in the frontal cortex when the disease spreads into that brain area. In agreement with the idea that the evolution observed in the frontal cortex reflects the earlier events that occurred previously in the entorhinal area, the results show an increase of the inflammatory components in this brain area. This observation again strongly suggests an early involvement of inflammatory processes in the pathogenesis of AD. These results are in agreement with many studies that also highlighted the role of immune responses in the early stages of the disease [39, 40, 76, 77, 78].

Inflammation could also play a role as a potential mediator of oxidative stress and dysfunction of the ubiquitin proteasome system [55]. The main function of this ubiquitin proteasome complex is to control and to degrade unneeded or damaged proteins. The toxicity of inflammation is principally mediated by the release of reactive oxygen and reactive nitrogen species by glial cells as a defence mechanism against injury or neurodegeneration [49]. Unfortunately, these free radicals can cause severe alterations in the structure and function of proteins leading to a dysfunction of the proteasome system and to neuronal cells death [42, 55]. It has indeed been shown that alterations of the oxidative processes and the formation of excess free radicals during neuroinflammation can lead to cellular dysfunctions and may play a pathogenic role in early AD [55, 65]. Thus in Alzheimer's disease aggregation of abnormal proteins could be induced by an activation of inflammatory processes, reactive gliosis and oxidative damage [42, 49].

As mentioned in several studies the inflammatory and oxidative characteristics of AD may also be present during normal aging [49, 50, 53, 55, 65]. The status of oxidative processes and inflammation can be modified by the normal senescence and normal cells degeneration also leading to a cellular and ubiquitin proteasome system dysfunction [42, 55, 65]. In addition, the antioxidant defence mechanisms decrease during normal aging with a consequent failure to repair and degrade damaged proteins [55]. The present study corroborates these overlap between normal and pathological aging with similar results between the control group and the ones affected by the disease. These similarities make it even more difficult to understand the pathological processes of Alzheimer's disease. More over, clinical trials using these inflammatory and oxidative processes as therapeutic strategies in AD have not yielded convincing results [54, 56, 57, 64, 79]

Pathological hallmarks of Alzheimer's disease: beta-amyloid and neurofibrillary tangles

The deregulation of the APP metabolism and therefore the deposition of senile plaques, in particular beta-amyloid protein, have been assumed to have a toxic effect on the surrounding cells and thereby be potent activators of the innate immune responses [30, 65, 80]. These senile plaques are supposed to have a crucial role in the development of the disease and to be closely related to neuroinflammation [40]. Morales and colleagues demonstrated that endogenous damage signals – probably the senile plaques – can activate microglia and astrocytes, inducing NF-kB with consequent release of cytokines mediators such as TNF-α and IL-1β [81]. The well-

established association of glial cells and inflammation related proteins around amyloid plaques reinforce this hypothesis [39, 42, 65]. In the present study, the results of the β-amyloid protein corroborate this idea revealing a quite similar evolution of its density to that of GFAP. First, they show that Aβ density is higher at the onset of the disease, e.g. among the moderate AD patients. Second, it seems that the amount of protein amyloid in the entorhinal cortex decreases with the progression of the disease as well as GFAP levels. These observations are consistent with the results published by Costanza and colleagues [30], which showed that levels of amyloid deposits, detected by positron emission, increase rapidly at the beginning of the degenerative process but seem to stabilize and reach a plateau when the disease progresses and the clinical symptoms appear. Then, when the disease reaches a more advanced state, the density of the Aβ protein appears to be very little associated with the evolution of the cognitive decline [81]. Finally the frontal cortex, later hit by the disease, also follows the same trend as GFAP showing an increase in the amyloid density with the progression of the disease. It can be assumed that this region is reaching the plateau observed by Costanza et al. and that subsequently the levels of β-amyloid will also begin to decrease. This corroborates the hypothesis suggested by several studies that although Aβ deposits may play a key role in the neuronal death and the reactive gliosis, the severity of cognitive decline in the advanced stages of the disease may be very loosely bound to the amyloid load [30, 38, 81, 82]. As the other proteins, the amyloid components are also found in the control group providing the evidence that all these pathological hallmarks can also be present during normal aging. This statement was already suggested by many other studies [30, 37, 81], which revealed a significant overlap between controls and patients with AD making more difficult to underlie the exact pathological hallmarks and the molecular pathways leading to the disease.

This Aβ hypothesis also suggested that the amyloid protein may be the first trigger of the Alzheimer pathological cascade. However, as mentioned earlier, amyloid density is inversely proportional to the severity of the cognitive decline. Some studies pointed that total neurofibrillary tangles (NFT) counts may be more strongly associated to neuronal loss than amyloid amounts [30, 55, 81, 82]. The presence of these aggregates of abnormal tau molecules in AD brains has indeed been widely described in literature and these studies suggest that tau density would be better correlated with the symptoms of the disease than the presence of the amyloid burden [30, 38, 81]. The results obtained in this study also corroborate these observations showing an increasing evolution in the two brain regions of the AD2 antibody with the progression of the disease being first very low in the moderate AD group to finally significantly increase in the group with severe AD. Although amyloid deposits and astrogliosis have been clearly demonstrated in the brain of patients suffering from AD [30, 39, 42, 65, 80], it appears that NFT is an event more strongly associated with neuronal loss and clinical manifestation of the disease than the amyloid amounts.

The link between these different assumptions underlying the etiology of the disease – tau protein, beta-amyloid and inflammation – has not yet been clearly elucidated and needs to be further explored. Some evidences suggested however that the Aβ and its inflammatory cascade might precede the development of both NFT formation and cognitive impairment [77]. As presented in a study the amyloid deposits may activate, in addition to astrocytes and microglia, some caspases, essential proteases in cell death, which in consequence may lead to the disruption of the nuclear cytoskeletal components such as tau protein [38]. This may then be critical to NFT formation and to the clinical progression of dementia. Other neuropathological studies also highlighted that a neuroinflammatory response in the cerebral neocortex could parallel the early stages of AD pathology and precedes the late stage, tau-related pathology [15]. More over it is supposed that amyloid deposition begins 10 to 20 years before the appearance of clinical dementia [40]. A study published in 1998 had already highlighted this feature of the very advanced stages of Alzheimer's disease. Indeed, Thal and colleagues had noticed in the terminal stages of the disease, that the frequency of plaques was significantly reduced while a progressive

increased in alteration of tau protein could be correlated to a sharp deterioration in dementia [83]. The results of the present study do confirm this concept of evolution of the Alzheimer pathogenesis. The amyloid deposition and the inflammatory components seem to start very early in the disease, perhaps even before the appearance of the first symptoms, suggesting an initial role of these proteins in the disease. Then as the density of Aβ and neuroinflammation seem to stabilize and to decrease, the quantities of tau protein increase and the symptoms of the disease worsen with more important deterioration of cognitive decline.

The results also show a slight signal of AD2 on the control group. This may be the evidence that the hyperphosphorylation of tau protein may also be encountered during normal aging, as is the case of the other components, and that these pathological characteristics may then not be unique to dementia. These observations are in agreement with many studies, which highlighted that NFT and amyloid deposits are also observed in normal aging [15, 27, 35, 55, 65, 81, 82]. Giannakopoulos and his colleagues pointed out that in case of AD the distribution in the brain areas and the respective densities of these components are significantly different and more abundant than in normal aging brains [82]. This pathological cascade suggests that AD may be due to several factors that in relationship with each other could lead to the neurodegenerative processes and to the severe cognitive impairment.

Although research has permitted to highlight some of the characteristics underlying the neurodegeneration, the exact cause of AD remains unknown. In addition, the role of neuroinflammation is still not well understood. Indeed, it is not clear whether neurodegeneration could be caused by neuroinflammation or if this immune cascade may only be a reflection of a desperate attempt to repair brain cell damage. This study allows however to corroborate some hypothesis already presented in the past and to focus on the role of inflammation including proinflammatory cytokines such as TNF-α, IL-1β and NF-κB in association with a reactive gliosis at the beginning of the disease. It also highlighted the significant difference between brain regions with a probable onset of the disease in the entorhinal region, as described by Braak and Braak in the 90s [28] and similar but later changes in the frontal cortex and probably also in other brain regions.
It is however important to note that this study is limited to a small number of samples and it would be wise to conduct a similar study on a larger scale by including a larger number of patients in order to draw more significant conclusions. In this study TNF-α, IL-1β and NF-κB were used as inflammatory cytokines but many other components of neuroinflammation are also very important and it would be judicious to use other markers of inflammation such as for example IL-6, INF-γ, the C-reactive protein or the complement.

12. Conclusions and Perspectives

Alzheimer's disease represents a major public health concern not only by its frequency in the world population but also by its ambiguous relationship with the physiology of normal aging. It has indeed been highlighted that the characteristic features of this disease are also present in normal brain in most of the elderly after 70 in the absence of cognitive decline [30]. It has also been established now that age is the biggest risk factor for non-genetic AD [30]. It is however presently not well-understood why and the similarities of this disease with normal aging hinder to define what differentiates normal brain senescence from the physiopathology leading to Alzheimer's disease. Why the Aβ protein aggregates into senile plaques remains unclear and its link with the other pathological components of AD has not been elucidated. Although research has managed to highlight certain particularities of the physiopathology of the disease, post-mortem measurements of the classic pathological hallmarks only explain to a limited extends the expression of dementia in the population and numerous other potentially modifiable factors also seem to contribute to the clinical presentation of dementia [84]. Nevertheless we can agree on the fact that accumulation of β-amyloid and pathological tau proteins as well as inflammatory

processes take place in the pathogenesis of AD. What is not well defined is the link between these different components and the pathways leading to cell death and cognitive decline. Several assumptions and potential explanations have been proposed but it appears difficult to come to an agreement. For example Russo et al. showed that a variety of cytotoxic substances released by activated microglia might cause neuronal damage by enhancing oxidative stress and activating cell-death pathways [48]. They also highlighted that an over-activation of microglia can result from a variety of injury signals, such as oxidative stress molecules, Aβ peptide, ischemia or brain trauma, which all promote erroneous signalling cascades and can induce proinflammatory cytokines production. However it has been suggested that increase in Aβ amounts may be also due to other processes. For example Lynch and his colleagues have speculated that AD pathogenetic cascade is not due to a single factor but to a collection of events, which accumulates with the already-existing age-related changes [50].

Whatever initiates the pathological cascade, it appears from the similarity of their evolutions, that Aβ and GFAP are related and that they play a key role at the very beginning of the pathogenesis while both have their levels decrease with the progression of the disease and are not correlated with the worsening of the cognitive decline. These observations are in line with many experimental and epidemiological data from animal and cell culture models showing that Aβ deposits and activated glial cells can produce factors that are toxic for neurons and that a protective effect could be obtained only by a very early and prolonged used of anti-inflammatory drugs [44, 57, 54]. Thus the overproduction or decreased degradation of potentially toxic peptides such as Aβ and the subsequent activation of inflammatory processes may then just be initial steps in a long cascade of detrimental changes. This assumption is also supported by Eikelenboom et al. who showed that a neuroinflammatory response in the cerebral neocortex parallels the early stages of AD and precedes the late stage tau-related pathology [77]. As mentioned earlier, the presence of the tau pathological protein seems much more associated with the cognitive decline than the deposition of amyloid burden. Numerous articles of the current literature agree with this chronological order of the pathological cascade. For example, a study highlighted that whereas the degree of tauopathy correlates strongly with cognitive decline in AD, genetic, pathologic and biochemical evidence implicates the aggregation of Aβ as a critical, early trigger in the chain of events that leads to tauopathy, neuronal dysfunction and dementia [53].

In conclusion although the physiopathological cascade leading to neuronal death and major cognitive decline that characterize Alzheimer's disease is still not well understood, it appears that the hypothesis of inflammatory processes taking place at the beginning of the disease is an idea more and more accepted in the scientific community. More over a new emerging idea from recent research seems to indicate that therapies focused on inflammatory processes could provide a new therapeutic approach to the disease and that they should be administered as soon as possible when the symptoms are still mild or even not yet clinically detected. However, the results of these clinical trials have so far yielded only few conclusive results and still need to be further analysed. New diagnostic techniques are therefore required to detect the disease as early as possible. The understanding of the relationship of the pathological components of the disease still remains one of the major challenges of current research and appears as a necessity to help developing new-targeted treatments.

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