Oligomeric Amyloid-β Peptide Uptake by Microglia Cells Grown on Substrates of Different Elasticity

BY

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THESIS

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This thesis is dedicated to my mom and my dad, Laura and Pippo, the guides of my life and the founders of my happiness. Thank you with all my heart for all the opportunities that you have given to me, for teaching me unconditional love, respect and for always believing in me. You are my inspiration and my motivation and without you I would never be what I am today.

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-β</td>
</tr>
<tr>
<td>Aβ$_{40}$</td>
<td>Amyloid-β (1-40)</td>
</tr>
<tr>
<td>Aβ$_{42}$</td>
<td>Amyloid-β (1-42)</td>
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<td>APP</td>
<td>Amyloid Precursor Protein</td>
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<tr>
<td>AVG</td>
<td>Average</td>
</tr>
<tr>
<td>C99</td>
<td>99-Amino-Acid C-Terminal</td>
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<tr>
<td>CCL</td>
<td>Chemokine C-C Motif Ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C Chemokine Receptor</td>
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<tr>
<td>CN+</td>
<td>Age-Matched Cognitively Normal Control Subject with Significant Brain Amyloid Load</td>
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<tr>
<td>CN-</td>
<td>Age-Matched Cognitively Normal Control Subject without Significant Brain Amyloid Load</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>cPLA$_2$</td>
<td>Cytosolic Phospholipase A$_2$</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EXP</td>
<td>Experiment</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>fAβ</td>
<td>Fibrillar Amyloid-β</td>
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<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3 – Hexafluoro-2-Propanol</td>
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<tr>
<td>H.S.</td>
<td>Hard Surface</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<td>MRE</td>
<td>Magnetic Resonance Elastography</td>
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<tr>
<td>NFTs</td>
<td>Neurofibrillary Tangles</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>P.I.</td>
<td>Protease/Phosphatase Inhibitor Cocktail</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin-1</td>
</tr>
<tr>
<td>PS2</td>
<td>Presenilin-2</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicilin-Streptomycin</td>
</tr>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>sAβ</td>
<td>Soluble Amyloid-β</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SGL</td>
<td>Subgranular Layer</td>
</tr>
<tr>
<td>SR</td>
<td>Scavenger Receptor</td>
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<tr>
<td>SVZ</td>
<td>Subventricular Zone</td>
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<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor</td>
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SUMMARY

Alzheimer’s disease (AD) is a neurodegenerative pathology without an accepted treatment, and its origin and evolution are still uncertain; it affects millions of people worldwide and so it has become a research priority in the last years to mitigate its impact on the society. Recently, some evidence has shown that the alterations in the mechanical characteristics of the brain tissues are implicated in the pathology. Thanks to new bio-imaging technologies, it is possible to study non-invasively the morphological changes in AD brain and correlate these alterations with cognitive performance; in addition, the morphological changes in AD brain tissue imply modifications also in the extracellular matrix (ECM) and in the mechanical features of the tissue. The changes in AD brain have been measured by magnetic resonance elastography (MRE) and it is found that the stiffness of the human brain tissue decreases in AD subjects and the reduction in the elasticity is correlated with the AD severity. As it is well known, the microenvironment surrounding the cells has several effects on the cell functions and proliferation. The main objective of this research is to investigate and evaluate how the mechanical properties of the cellular environment affect the AD-related microglial cell functions, specially the oligomeric amyloid-β peptide uptake; for this purpose, substrates with different stiffness are used to simulate AD and healthy brains, and the amyloid-β uptake by microglial BV-2 cells is measured; different levels of elasticity are used to simulate the different levels of severity of the pathology. The results demonstrate that the elasticity of the matrix influences the proliferation capabilities of the microglial cells, and it has effects on the amyloid internalization by these immune cells: a higher amyloid-β uptake is observed on softer substrate, that mimic the diseased brain tissue, compared to harder surface that represents a healthy brain.
1 INTRODUCTION

1.1 ALZHEIMER’S DISEASE

Alois Alzheimer was the first scientist who, in 1907, described the two principal hallmarks of the Alzheimer’s disease (AD) from the examination of the brain of a female subject that experienced symptoms of dementia. These two pathological alterations consist of: deposition of extracellular substances in some regions of the brain, also referred as amyloid plaques, and neurofibrillary tangles (NFTs) that occurs inside the neurons (LaFerla F. M., Green K. N. and Oddo S. 2007).

It was only in the middle and late 1980s that it was identified that the plaques are formed by accumulations of small peptides, known as amyloid-β (Aβ) (Glenner G. G. and Wong C. W. 1984), and that the NFTs are made up by aggregates of tau proteins, which are hyper-phosphorylated in the course of the pathology, due to a dysregulation of tau kinases (Grundke-Iqbal I. et al. 1986). In addition to these distinctive characteristics, also other numerous changes, both functional and structural, subsist in an AD brain, such as oxidative stress, inflammation, actin stress fiber formation and transmigration of monocytes across the blood brain barrier.

The consequences of the pathological alterations are neuronal and synaptic dysfunction which lead, at the time of death, to a loss of one-third of the weight of the brain (LaFerla F. M., Green K. N. and Oddo S. 2007).

Nowadays, thanks to the improvement in the bio-imaging technology, morphological changes
of AD brain are readily studied and correlated with the cognitive decline. The neuropathological peculiarities of AD, such as senile plaques and NFTs, occur both all over the hippocampal formation and in some parts of the cerebral cortex, and include both the subgranular layer (SGL) and sub-ventricular zone (SVZ) neurogenic areas (Lazarov O., et al. 2010); in SGL, a brain region in the hippocampus, and in SVZ, a region of the lateral ventricles, neural stem cells become neurons, thus if these areas have impaired functions also the neurogenesis is affected and this can lead to brain dysfunction that characterized the disease (Figure 1).

Figure 1 - Representative comparison between a normal brain (left) and a brain of a person affected by AD (right). Alzheimer’s disease affect specially the SGL, a region of the hippocampus, and the SVZ, which is part of the lateral ventricles. In these areas the neurogenesis happens, so impaired functions in these regions lead to neurodegeneration and cognitive decline. Reprinted with permission. Open source: by Garrondo (talk) SEVERESLICE_HIGH.JPG: ADEAR: "Alzheimer's Disease Education and Referral Center, a service of the National Institute on Aging."
Unfortunately, currently, there is not a drug or therapeutic agent able to target Aβ and that can induce positive results in clinical trials (Morris 2013); that’s why the research is focused on the observation of all alterations in AD brain to find a possible therapy to slow the evolution of the disease.

Alzheimer’s disease is a neurodegenerative pathology that affects wide areas of the hippocampus and the cerebral cortex and has an average clinical duration of 8-10 years (Masters C. L., et al. 2015); clinically, it is distinguished by the gradual deterioration of higher cognitive tasks involving memory, communication, learning, motor competences and perception, of millions of people in the world.

It is an age-related disease and the estimated prevalence in the population aged 65 or older is 10-30% with an incidence of 1-3%; the more prevalent type of the disease is sporadic AD (>95%), with a late onset (80-90 years old), due for the failure in the clearance of Aβ peptides from the brain parenchyma, while less patients (< 1%) have mutations in genes that can affect the processing of Aβ and so they can develop the pathology in younger age, around 45 years old (Masters C. L., et al. 2015).

Nowadays, the most frequent cause of dementia and the sixth-leading agent of death among people of age 65 or older is AD (Askarova S., et al. 2013). Today AD affects 46.8 million of people worldwide (Prince M., et al. 2015) and 5.5 million of Americans (Alzheimer's disease Association 2017); due to the increased life expectancy, AD is expected to impact on 13.2 million of Americans by 2050 if no therapies can be found (Zhu D., Bungart B. L., et al. 2015).

Hence, AD has become a research priority, in order to mitigate the increasing number of AD victims and also to help and find solutions for the multiple struggles of the victims’ families.
1.2 **Amyloid-β Peptide**

The role of Aβ peptides in AD is still controversial, but it is believed that these fragments are responsible for the progression of AD; their deposition into plaques in brain parenchyma can lead to neuronal death and speed the development of the pathology. The amyloid hypothesis consists in the affirmation that the amyloid plaques characteristic of AD could be provoked by the incorrect balance between the Aβ generation and elimination, which results in the accumulation of Aβ in different structures, such as monomers, oligomers, insoluble fibrils and plaques, in the central nervous system (CNS) (Mawuenyega K. G., et al. 2010).

Amyloid-β is derived from the endo-proteolysis of the amyloid precursor protein (APP), by the breakdown of APP by different enzymes systems called α-, β- and γ-secretase.

For α-secretase activity, three enzymes have been recognized (ADAM9, ADAM10, ADAM17), while for β- and γ-secretase the enzymes are, respectively, BACE1, that is an intrinsic type I membrane protein, that belongs from the pepsin group, and a system of enzymes constituted by presenilin-1 (PS1) and presenilin-2 (PS2) (LaFerla F. M., Green K. N. and Oddo S. 2007).

Amyloid precursor protein is a plasma membrane protein composed by 695-770 amino acids, (LaFerla F. M., Green K. N. and Oddo S. 2007) that is reported to possess functions in cell movement (Sabo S. L., et al. 2001) and cell adhesion (Breen K. C., Bruce M., and Anderton B. H. 1991). It can be also localized in other compartments, such as: trans-Golgi system, endoplasmic reticulum, and endosomal, mitochondrial and lysosomal membranes (Mizuguchi M., Ikeda K. and Kim S. U. 1992). Consequently, the liberation of Aβ could occur in several cellular compartments, depending on where the APP cleavage is performed; if the cleavage is inside the cell then intracellular Aβ is obtained, while if the cleavage is carried out at the plasma membrane then Aβ is released in the extracellular fluid.
Currently are known two different processing pathways for APP: the amyloidogenic pathway and the non-amyloidogenic pathway.

The non-amyloidogenic pathway is more common and avoids the formation and release of Aβ peptides. It consists in the cleavage by α-secretase inside the Aβ region and two fragments are released: a small carboxy-terminal fragment (C83) and a large ectodomain (sAPPα), the last one is secreted then in the extracellular medium, while C83 is further cleaved by γ-secretase and produces a short molecule called P3 (Kojro E. and Fahrenholz F. 2005). It was found that sAPPα has neuroprotective and neurotrophic characteristics (Zhu D., Bungart B. L., et al. 2015) and also it regulates, as a growth factor, the proliferation of adult embryonic neural stem cells (Caille I., et al. 2004).

A different pathway for APP cleavage is the amyloidogenic pathway, that is also called abnormal cleavage pathway, which leads to the production of Aβ. Firstly β-secretase mediated the proteolysis of APP at the level of the 99th amino acid from the C terminus and this process leads to the emission of sAPPβ in the ECM while the 99-amino-acid C-terminal (C99) remains into the phospholipid bilayer with the new N end that corresponds to the first amino acid of Aβ (Figure 2). Secondly γ-secretase cuts this last portion between the residuals 38 and 43 and releases an Aβ peptide (LaFerla F. M., Green K. N. and Oddo S. 2007).
Figure 2 – APP proteolysis: non-amyloidogenic and amyloidogenic pathways. Aβ peptides are derived from the proteolysis of APP by one of these known pathways. The non-amyloidogenic pathway precludes the formation of Aβ and it is the more common one. The amyloidogenic pathway is less common, it is characteristic of the AD pathology and leads to the production and release of toxic Aβ peptides (in the figure the top part in respect to the phospholipid bilayer represents ECM, while the bottom part represents intracellular space). Reprinted with permission of Nature Publishing Group [Appendix A] – LaFerla F.M., Green K.N., Oddo S.: Intracellular amyloid-β in Alzheimer’s disease. Neuroscience. 2007, volume 8, pp 499-509. (LaFerla F. M., Green K. N. and Oddo S. 2007)

The amyloidogenic processing of APP leads to the formation of Aβ monomers of different dimensions: amyloid-β (1-40) (Aβ40) is the more common one, while amyloid-β (1-42) (Aβ42) is approximately produced at 10% the rate of Aβ40 production. This last variant is more hydrophobic, due to the two extra C-terminal amino acid residues, and more fibrillogenic than Aβ40 and it is the form mostly found in the AD plaques (Jarrett J. T., Berger E. P. and Lansbury P. T. Jr. 1993).

Initially a monomeric form of Aβ is produced, but immediately it combines to constitute multimeric structures, that can vary from dimers and trimers to protofibrils and fibrils with a great molecular weight. It was found that is the oligomeric form of Aβ that is the more toxic

*In vitro* studies have shown that Aβ monomers exist in three major conformational states: α-helix, β-sheet and random coil (Liu D., et al. 2006).

The first step in the fibril formation is the conformational conversion of Aβ from α-helix to β-sheets; this change of state strictly depends on chemical and physical parameters of the environment (Zhu D., Bungart B. L., et al. 2015).

The second stage in Aβ aggregation process is the formation of hydrogen bonds between the amide and the carbonyl groups of β-sheets oriented in an anti-parallel way to obtain aggregation into higher order arrangements (Poduslo J. F. and Howell K. G. 2015).

The aggregation of Aβ into fibrils consists in a process that is made up by different phases and intermediate structural states (Figure 3):

I. monomers;  
II. misfolded monomers;  
III. soluble oligomers, so an assembly of peptide molecules but without a fibrillar form;  
IV. protofibrils, so aggregates of around 20 molecules with β-sheet structure;  
Figure 3 – Aβ intermediate structural states. In the monomeric state Aβ seems not to be toxic, but it can aggregate in order to form oligomers and high molecular structures, such as protofibrils and fibrils, characteristic of AD. Reprinted with permission of Nature Publishing Group [Appendix A] – LaFerla F.M., Green K.N., Oddo S.: Intracellular amyloid-β in Alzheimer’s disease. *Neuroscience*. 2007, volume 8, pp 499-509. (LaFerla F. M., Green K. N. and Oddo S. 2007)

This conversion from a peptide to a fibril consists of a lag phase, in which the thermodynamic barrier has to be overcome for the formation of a seed, that is followed by a growth phase (Figure 4), that is more rapid and involves the steps necessary for the formation of a structure composed by several layers of β-sheets (Zhu D., Bungart B. L., et al. 2015).
The transformation of Aβ into fibrils consists in a lag phase, which is slow and is needed for the formation of a seed, and a growth phase, which is more rapid, and lead to the formation of several layers of β-sheets. Reprinted with permission of Frontiers in Biotechnology and Bioengineering [Appendix B] – Zhu D., Bungart B.L., Yang X., Zhumadilov Z., Lee J.C-M., Askarova S.: Role of membrane biophysics in Alzheimer’s-related cell pathways. Frontiers in Neuroscience. 2015, volume 9, pp 1-13. (Zhu D., Bungart B. L., et al. 2015)

The aggregation of Aβ is influenced by: conformation of Aβ, electrical charge, pH, hydrophobicity or hydrophilicity of the environment, stiffness of the medium and so on. As a result, the regulation of physical and chemical parameters is fundamental and the identification of the driving force behind these interactions could help to find new therapeutic agents for the prevention of amyloid plaques (McLaren D. A., et al. 2000).

1.3 Microglial Cells

Microglia cells are considered the immune cells of the CNS and the spinal cord, and possess both neuroprotective and neurotoxic functions (Noda M. and Suzumura A. 2012).

The number of microglial cells vary in different regions of the brain, and these cells account approximately 5-12% in respect to the total number of cells of the cerebrum (Malm T. M., Jay
Microglia predominate in the grey matter and have a higher concentration in the hypothalamus, hippocampus, basal ganglia and substantia nigra (Block M. L., Zecca L. and Hong J. S. 2007).

Differently from other mononuclear phagocytes, such as monocytes and macrophages, microglia are not developed from ancestors in the bone marrow, instead are generated from primordial hematopoietic originators in the yolk sac (Malm T. M., Jay T. R. and Landreth G. E. 2015), and in adulthood are involved in the inflammation and immune response of the CNS.

The main function of microglia cells is to maintain the homeostasis in the CNS; these cells are regularly located inside the brain, at a density of around 6/mm$^3$ (Lee C. Y. D. and Landreth G. E. 2010). In resting state, microglia are characterized by long, thin and greatly movable filopodia-like processes, that act as detectors, and a small cell soma (Li Y., et al. 2014). Thanks to their processes, microglial cells constantly survey the brain parenchyma and for this reason are also called “surveillant” (Malm T. M., Jay T. R. and Landreth G. E. 2015).

*In vivo* two-photon imaging of neurons and microglia, marked with fluorescent dyes, have shown that the filopodia of microglia can interact with neighboring neurons more or less one time every hour (Wake H., et al. 2009); this continuous inspection is very useful in order to monitor the synaptic function, thanks to the contact between microglia and the other cells of the CNS, such as neurons, astrocytes and vascular endothelial cells, and also to react quickly to injury.

Microglial cells are very sensitive to the external environmental stimulation and when the brain tissue underlies to an injury or is in a pathological state, microglial cells are activated from their stationary state, assume an amoeba-like shape and migrate to the site of injury to begin their tissue repair functions, that include the phagocytosis of dead cells and debris and the pruning of the synapses (Li Y., et al. 2014).
In the development of AD, microglia cells exert two opposite functions: a nerve and neuron protective effect and a neurotoxic one. Their excessive activation can lead to immune damage and cause the death of neurons (Li Y., et al. 2014).

These cells are stimulated by alterations in the homeostasis of their neighborhood environment, and their activation leads to a cascade of phenotypic changes including morphology, transcription and cytokine and reactive oxygen species (ROS) production. There are different grades of microglial activation and two main opposed conditions are instituted:

- a pro-inflammatory state that is caused by the presentation to stimuli like lipopolysaccharide (LPS) and interferon-γ (IFNγ);
- an anti-inflammatory condition that encourage adjustment and resolution of the inflammation, promoted by elements such as interleukin-4 (IL-4) and interleukin-13 (IL-13) (Greter M. and Merad M. 2013).

Microglia cells can actively translate from an anti-inflammatory to a pro-inflammatory phenotype, so have the capacity to differentiate between a reactive and a repair state; however, microglia do not assume only these two roles, but can acquire a broad range of different phenotypes according to the microenvironment that surrounds them. The external and internal conditions, such as the nature and duration of insult, the force of the stimulation, the distance from the stimulus and the exposure to prior and existing stimuli can vary the magnitude of the microglial activation (Lue L-F., et al. 2010).

A dysregulation of the activation mode of microglia could be injurious to the CNS health; various neurodegenerative pathologies, such as Alzheimer’s and Parkinson’s diseases, are correlated to continuous stimulated pro-reactive microglial cells (McCarthy R. C., et al. 2016). Indeed, when microglia are activated from their resting state by a stimulus, they start to proliferate and produce cytokine to recruit more microglia to the site of damage and,
assuming and amoeboid-like shape, start the phagocytosis of debris and dead cells; on the other hand when microglia are over-activated they can translate in a dysfunctional phenotype with reduction of phagocytic activity and motility and an altered cytokine production, this could lead to senescent microglia, so an irreversible state of dysfunction which characterizes the disease. (Figure 5).

Figure 5 – Scheme of the different morphologies and states of microglial cells in healthy and AD brains. Resting microglia are the surveillant of the CNS and can be activated by different stimuli; activated microglia respond to stimuli and translate from their resting type to an amoeboid-like shape which possess phagocytic functions. In AD brains, microglial cells have some dysfunctions, like impaired phagocytic functions, that can lead to their transformation in senescent microglial cells, which characterized the disease. Reprinted with permission of Springer [Appendix C] – Prokop S., Miller K.R., Heppner F.L.: Microglia actions in Alzheimer’s disease. Acta Neuropathol. 2013, volume 126, pp 461-467. (Prokop S., Miller K. R. and Heppner F. L. 2013)
In the circumstance of AD brain, microglial cells are triggered by Aβ peptides, which are eliminated from the brain parenchyma by the mechanism of phagocytosis; when the disease persists the Aβ peptides chronically activate microglia cells and these cells produce an excessive amount of cytokines that, thanks to experimental evidence, is noticed to induce neighboring cells to synthesize more APP acting on the secretases activity (positive feedback loop), speeding the progression of the pathology (Malm T. M., Jay T. R. and Landreth G. E. 2015). In addition, microglia cells in AD brain have reduced motility and they are also unable to remove the fibrillar form of the Aβ, due to reduced phagocytic capacity, and so AD brain parenchyma is constituted by the extracellular deposition of amyloid in plaques.

Microglia cells are very important in the clearance of Aβ from the brain parenchyma to maintain the homeostasis of the environment, specially in the initial stages of amyloid deposition to avoid the plaque development. However, if over-activated by an excessive production of Aβ peptides, these cells can accelerate the disease progression, specially in the later stages of AD, releasing also different inflammatory factors, which includes tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), ROS and so on; the results are a chronic neuroinflammation, neurotoxic effects on the neighboring cells and an increase in the production of APP, which lead a long-repeated neurotoxicity cycle that is indicated as reactive cycle to microglia cells (Li Y., et al. 2014), that may also serve to promote the tau pathology (Prokop S., Miller K. R. and Heppner F. L. 2013).

There are two main hypotheses about the progression of AD due to microglia: the “neuroinflammation hypothesis” that states that the over-activated microglia are the origin of an uncontrolled inflammation that can lead to neuronal death and the chronic evolution of the disease, and the “microglial dysfunction hypothesis” that affirms the deficit of the microglial neuroprotective and phagocytic functions in the pathology (Solito E. and Sastre M. 2012).
Therefore, microglial cells are deeply implicated in the homeostasis of the brain and in the development of AD, for these reasons their study is relevant and imperative to find a possible therapeutic agent and target to eliminate or slow the progression of this pathology.

1.4 **Amyloid-β Uptake by Microglia Cells**

Microglia cells constantly inspect the brain parenchyma and, when encountering a pathogen or an injury of the tissue, these cells start to migrate toward those sites of damage. Microglia can be attracted by Aβ, and there is evidence of the accumulation of microglia cells around plaques (Mosher K. I. and Wyss-Coray T. 2014). The recruitment of those cells to the plaques, migration that is very rapid on the order of 1-2 days (McLarnon J. G. 2012), is believed to be mediated by the C-C-chemokine receptor type 2-chemokine C-C motif ligand 2 pathway (CCR2-CCL2) (Malm T. M., Jay T. R. and Landreth G. E. 2015). The levels of CCR2 are higher in the human AD brain (El Khoury J., Toft M. and Hickman S. E. et al. 2007).

After microglia are induced to migrate toward the plaques, microglia produce chemokines, such as chemokine C-X-C motif ligand 2, chemokine C-X-C motif ligand 8, CCL2 and CCL8, to recruit more microglia, and additional cytokines, such as IL-6, TNF and macrophage inflammatory peptide-1α also used as chemo-attractants (Guillot-Sestier M. V. and Town T. 2013).

According to the physical and biochemical properties of Aβ, microglia have different mechanisms to uptake these peptides, such as phagocytosis and pinocytosis (Malm T. M., Jay T. R. and Landreth G. E. 2015); in AD brain the reduced Aβ elimination is due to the impairments of these pathways, so understanding these processes is fundamental to comprehend the pathology.

One of the main mechanisms for oligomeric and fibrillar Aβ uptake by microglia cells is phagocytosis. Phagocytosis is the process by which immune cells recognize, engulf and
degrade extracellular materials; thanks to a variety of surface receptors, fibrillar Aβ (fAβ) can be internalized, and then phagosomes that contain Aβ are transported to lysosomal areas and broken down by proteases, such as cathepsin B (Yang C. N., Shiao Y. J. and Shie F. S. et al. 2011). Amyloid-β_{42} is degraded in the lysosome of the microglia within 5 minutes after internalization, but microglia cells are not able to degrade 100% of internalized Aβ, 40-50% accumulates within the lysosome and this Aβ may lead to amyloid seed that facilitate the deposition and aggregation in plaques, also called intracellular accumulation (Hu X., et al. 2009).

For the elimination of soluble molecules from the extracellular space the key mechanism is pinocytosis, that can occur via two pathways: micropinocytosis, a process mediated by cholesterol-rich lipid domains in the plasma membrane which do not need actin (Parton R. G. and Richards A. A. 2003), and macropinocytosis, a mechanism that need membrane ruffles formed by actin and tubulin (Conner S. D. and Schmid S. L. 2003). Macropinocytosis is crucial for the internalization of soluble Aβ (sAβ) that then is degraded through the endolytic pathway and through the production of several Aβ debasing catalysts, such as insulin-degrading enzyme (IDE) and neprilysin (NEP) (Miners J. S., et al. 2011); while the uptake of oligomeric and fAβ has as selective mechanism the micropinocytosis, which is mediated by several surface receptors, such as scavenger receptors A and B (SR-A and SR-B) (Mandrekar S., et al. 2009). Oligomeric and fAβ are bound by SR that mediate their endocytosis (Ries M. and Sastre M. 2016); in particular SR-As promote the fAβ uptake in microglia in combination with also other receptors, such as the complement receptor 3, also known as Mac-1/CD11b, (Fu H., et al. 2012), while SR-B, such as CD36/SRBI, influences the recruitment and the activation of the microglia cells as reaction to fAβ (El Khoury J. B., et al. 2003).

Another important receptor for the Aβ uptake by microglial cells is CD33, also called sialic acid binding Ig-like lectin 3 (SIGLEC3), a transmembrane protein which levels have been
shown to be increased in *in vitro* experiment in BV-2 microglia cells, diminishing the Aβ uptake and so highlighting a role in the AD pathology (Griciuc A., et al. 2013).

Also cytosolic phospholipase A$_2$ (cPLA$_2$), an enzyme able to hydrolyze phospholipids, seems to play a critical role in the internalization of Aβ in microglia cells. Indeed the alterations of the cellular membrane physical properties by cPLA$_2$ may play a role in Aβ uptake in microglia cells: the inhibition of cPLA$_2$ activation (phosphorylation) decreases the Aβ uptake in human and mouse immortalized microglia by up to 90%, but do not alter the degradation of the internalized Aβ present in the lysosomes of the microglia cells (Zhu D., Lai Y., et al. 2006).

Therefore, in AD brain, when microglia are activated by the uptake of Aβ, the constant activation of the phagocytic pathways could lead to an impairment of the phagocytic functions, due to an incorrect detection of the targets (downregulation of certain receptors) or to a dysfunction in the chemotactic functions and so a delayed migration to the site of damage. The consequence is the failure in the elimination of amyloid by microglia that can lead to an accumulation of Aβ in brain tissue, known as extracellular accumulation (Hickman S. E., Allison E. K. and El K. J. 2008). In addition microglia cells produce toxic compounds, such as free radicals, cytokine and glutamate agonists that in turn speed the neuronal death (McDonald D. R., Brunden K. R. and Landreth G. E. 1997). Furthermore, the generated cytokines can boost the generation of other inflammatory molecules by the other glial cells and neurons and forming a positive feedback loop that produce an amplification effect in the inflammation cascade and accelerate the neuronal degeneration (Block M. L. and Hong J. S. 2007). It has been noticed that the extent of inflammation in AD brain is dependent on the density of the activated microglia in an area-specific way (McLarnon J. G. 2012). Indeed, ROS and nitric oxide (NO) are overproduced (Li Y., et al. 2014); too much ROS can induce cell damage and apoptosis, due to an redundant aggregation of oxidation products of lipids,
protein and DNA (Li Y., et al. 2014), while NO, that is a special neurotransmitter that is implicated in synaptic plasticity, neuronal growth, learning, memory and attitude, if produced in excess can impair the membrane architecture, protein and DNA and so drive the neuronal necrosis and apoptosis (Law A., Gauthier S. and Quirion R. 2001).

Hence, microglial activation is very important and necessary for the defense of the CNS and for neuroprotection, but an increased or prolonged stimulation can have neurotoxic and adverse effects (Block M. L., Zecca L. and Hong J. S. 2007). For these reasons is very important to clearly understand the mechanisms by which microglial cells uptake Aβ and all the receptors involved in this process, and how these factors can vary and depend on the different parameters of the microenvironment surrounding these cells, knowing that the external conditions are fundamental in the development, differentiation and function of the cells and that the reaction of microglia to a pathological stimulation is deeply context-dependent (Prokop S., Miller K. R. and Heppner F. L. 2013), in order to better understand the course of the disease and find some possible therapeutic treatments.

1.5 **BRAIN ELASTICITY AND MAGNETIC RESONANCE ELASTOGRAPHY**

Another important characteristic in AD is that the global brain stiffness, measured thanks to an innovative technique called magnetic resonance elastography (MRE) in a study conducted by Murphy et al., is reduced in subject with AD (median age 85 years old), in respect to age-matched cognitively normal control subjects with (CN+) and without (CN-) a serious amount of brain amyloid (median age, respectively, 83 and 81.5), and compared also to control subjects without known neurological diseases (median age 29 years old) (Murphy M. C., Huston III J., et al. 2011). MRE is an emerging MRI-based method that is able to estimate the stiffness of a tissue in a noninvasive and quantitative way; it is a three step process:

1. shear waves are induced inside the considered tissue, applying an external mechanical vibration or stress;
2. The developed shear waves are represented by a phase contrast MR imaging pulse sequence by applying a motion encoding gradients which are coordinated with the motion of the vibration source; the shear waves induced have a wavelength that is shorter in soft tissue and longer in stiff tissues;

3. Ultimately, to obtain the tissue stiffness map, that is also called elastogram (Figure 6), the shear wave images are mathematically reversed (Murphy M. C., Jones D. T., et al. 2016).

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**Figure 6 – Example of MRE elastograms obtained with a spin-echo EPI pulse sequence.** The top row shows the results of a CN- control subject, while the bottom row shows the results from a subject with AD. The first column shows the magnitude images, the second column displays the MRE wave images and the resulting elastograms are shown in the third column. It is possible to notice that the decreased brain stiffness is correlated with the severity of the AD pathology, indeed control subject without a severe amyloid load has values of stiffness higher compared to AD subject. Reprinted with permission of John Wiley and Sons [Appendix D] – Murphy M.C., Huston J., Clifford R.J., Glaser K.J., Manduca A., Felmlee J.P., Ehman R.L.: Decreased brain stiffness in Alzheimer’s disease determined by Magnetic Resonance Elastography. *J Magn Reson Imaging*. 2011, volume 34, pp 494-498. (Murphy M. C., Huston III J., et al. 2011)
The vibration source is made by a soft “pillow-like” pneumatic actuator, composed by a generator of waves, an amplifying device and an acoustic speaker, that can produce intracranial shear waves when placed under the head of the subject. To allow fast-volumetric acquirements, that can entirely sample the region of interest, an accelerated single-shot spin-echo planar imaging sequence (EPI) is employed (Murphy M. C., Huston III J., et al. 2011).

The EPI pulse is made by a 90° pulse and a subsequent 180° pulse after a time interval T_E/2; the 90° pulse tilts the magnetization onto the y axis, where it decays with a time constant T_2*.

The result of the 180° pulse is the “refocus” of the magnetization such that at time τ after the 180° pulse, the individual vectors add constructively, and the signal reaches a peak; so in T_E spins are again in phase and an echo signal can be detected (Signorini M. G. 2015).

Thanks to a study conducted by Murphy et al. (Murphy M. C., Huston III J., et al. 2011) it has been shown that the average stiffness for the healthy subjects was 3.07 kPa, while the average stiffness of the CN- and CN+ groups respectively were 2.37 kPa and 2.32 kPa and the average stiffness of the AD category was 2.20 kPa (Figure 7). Hence, these results demonstrated that MRE technique can evaluate in a noninvasive and quantitative way the alterations in the mechanical features of the human brain and that the global stiffness in the brain parenchyma is reduced in subjects with AD (Murphy M. C., Huston III J., et al. 2011) and this decrease is correlated with AD severity; indeed AD subject have a great difference in the stiffness compared to healthy subject, and they have also a change in the stiffness compared to cognitively normal subjects with or without brain amyloid.
It is well established that the amyloid fibrils, characteristic of AD pathology, have a stiffness that is six orders of magnitude higher than the one of neurons and glia (Lu Y.B., Franze K. and Seifert G. et al. 2006) (Smith J. F., et al. 2006), thus it seems counterintuitive that the global stiffness is reduced due to AD. It has been hypothesized that the aggregation of Aβ, that contains β-sheets, induces an increment in the overall brain stiffness, but the results of Murphy et al. showed that the CN+ and CN- groups are not so different and, consequently, it could be that the brain amyloid is not the only responsible for the modification in the brain elasticity (Murphy M. C., Jones D. T., et al. 2016).

These findings suggest that the decreased parenchyma stiffness seen in AD could be caused by structural alterations associated with a loss of the integrity of the cytoarchitecture, such as destruction of the extracellular medium due to the deposition of Aβ, cytoskeletal degradation due to hyper-phosphorylation of tau proteins and loss in the interconnectivity of the synaptic
systems (Murphy M. C., Huston III J., et al. 2011). The deficit of the brain elasticity in AD compared to CN is not restricted only in the region of the cortex, so where the plaques composed by amyloid can be found, but also involves the white matter (Murphy M. C., Huston III J., et al. 2011).

The microenvironment is very important for the stem cells lineage specification; neural stem cells are extremely sensitive to tissue-level elasticity and can change their phenotypes according to the mechanical properties of the tissue and the environment surrounding them (Engler A. J., et al. 2006). Moreover, cells act differently on different substrates and the external conditions vary the functions of differentiated cells too. The microenvironment surrounding the cells determines their fate, specially the stiffness of the matrix has been shown to be fundamental for both undifferentiated and differentiated cell types of the CNS (Saha K., et al. 2008).

It is well known that stiffer substrates promote a higher focal adhesion and elongation, and that the cell adhesion plays many roles in cell functions, specially involving cell survival and cell differentiation (Leipzig N. D. and Shoichet M. S. 2009).

Stiffness is very important in the adhesion formation and, more rigid is the surface, more large adhesion of the cells is noticed. The stiffness can be used to tune the cell functions, indeed the cells translate a change in stiffness in a change in adhesive ligand presentation, thus in a change of their functions (Dalby M. J., Gadegaard N. and Oreffo R. O. C. 2014).

Furthermore, the alterations in the spreading, and so in the cytoskeletal structure and tension of the cell (integrin, that is the receptor involved in the cellular adhesion, is linked to actin filaments of the cytoskeleton), will alter also the shape of the nucleus and so the chromosomal arrangement and gene transcription, therefore this is another proof that the cell mechanics affect the functioning and the proliferation capabilities of the cells (Dalby M. J., Gadegaard N. and Oreffo R. O. C. 2014).
Consequently, according to the elasticity of the local microenvironment, cells can change their behavior, through a process known as mechanotransduction, in which transmembrane ECM receptors act as mechanotransducers and transfer the change in the mechanical adhesion force to the cytoskeleton; therefore, a dysregulation of the matrix stiffness could lead to an incorrect activation of the cells that can lead to neurodegeneration. For these reasons is very important to study the behavior of microglia cells on substrates with different elasticity, to better understand their activation states in different conditions of the microenvironment surrounding them and how the Aβ uptake function is modified.

1.6 **OBJECTIVE**

Nowadays, limited knowledge is available about the role of the local stiffness in the cell functions related to AD pathology, therefore this study wants to inspect how the mechanical properties of the cellular environment affect the microglial cell functions, in particular the oligomeric Aβ peptide uptake. The starting hypothesis is that the morphological changes in AD brain imply alterations also in the mechanical characteristics of the brain tissue and in the extracellular matrix, which provides an essential physical scaffolding for the cells and so affects also the functioning of these. Previous works have shown that the stiffness of the brain is decreased in patient with AD (Murphy M. C., Huston III J., et al. 2011), thus this change in the mechanical features of the tissue could lead to a change in the activation state and functions of microglia cells. The main hypothesis is that on softer substrates, that mimic the stiffness of the AD brain, microglia cells are over-activated and uptake more Aβ compared to harder substrates, that reproduce the healthy brain.
An excessive uptake of Aβ and an over-activation of microglia on the “diseased softer medium”, could lead to impaired functions of these cells, thus intracellular and extracellular accumulation of the peptides.

Therefore, the objective of this research is the quantitative analysis of the Aβ uptake by microglia BV-2 cell line seeded on substrate of different elasticity, to evaluate if the activation state of these immune cells, and so the Aβ uptake and the intracellular accumulation of Aβ, varies according to the stiffness of the medium surrounding them.

Furthermore, it is known that after 15 minutes the Aβ uptake should be saturated (Lee C. Y. D. and Landreth G. E. 2010): all the possible Aβ peptides are taken from the cells and only a small percentage remains in the solution, thus after 60 minutes the results should be more or less similar to the ones obtained after 15 minutes. This evidence justify the three different time points used in the experiments to verify if the Aβ internalization reaches a plateau after 15 minutes.

Six substrates of different Young’s Modulus were used: 0.2 kPa, 0.5 kPa, 1 kPa, 2 kPa, 4 kPa and hard surface, to simulate the typical stiffness of the healthy brain (4 kPa) and of the AD brain, with different values to mimic the different levels of AD severity (0.2 kPa, 0.5 kPa, 1 kPa and 2 kPa); the hard surface is considered as the control group.

BV-2 microglia cell line is seeded on the different substrates and then treated with oligomeric Aβ42 peptides (all the substrates are loaded with the same concentration of Aβ, equal to 1 µM), for three different time points (0 minute, without Aβ treatment, 15 minutes and 60 minutes, with Aβ loading).

For each sample, a count of the cells is performed and then a lysis buffer is used, to break down the cellular membrane and measure the concentration of intracellular Aβ.

To measure the concentration of Aβ inside the cells, so to quantify the Aβ uptake by microglia cells and the intracellular accumulation of these peptides, a specific assay for Aβ
quantification is employed (ELISA assay); 0-minute time point, without Aβ loading, is used to verify if the signal obtained is actually a measure of the intracellular Aβ concentration and it is not derived from other proteins present inside the cells, therefore to verify the specificity of ELISA kit to Aβ concentration.

In addition, to evaluate if the different substrate elasticity, so the local environment that surrounds the cells, affect the total protein concentration of these, a specific assay for quantification of total protein concentration is used (BCA assay).

Initially, the same seeding ratio is used on different substrates, to evaluate also the different proliferation capabilities of the cells on media of different stiffness, knowing that harder surfaces induce an higher proliferation of the cells (Dalby M. J., Gadegaard N. and Oreffo R. O. C. 2014).

After, on harder substrates, a lower dilution ratio is used compared to softer media to have a comparative analysis of the Aβ uptake, using approximately the same number of cells.
2 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

- Dulbecco’s Modified Eagle Medium with 4.5 g/L D-Glucose, L-Glutamine and without Sodium Pyruvate (1X) (DMEM) (from Life Technologies™, 11965-092, 500 mL);
- HyClone™ Fetal Bovine Serum Defined (Heat Inactivated) (FBS) (from GE Healthcare Life Sciences, SH30070.03, 500 mL);
- Penicilin-Streptomycin (10,000 units/mL) (P/S) (from Life Technologies™, 15140-122, 100 mL);
- Dulbecco’s Phosphate Buffered Saline without Calcium Chloride and without Magnesium Chloride (1X) (DPBS) (from Life Technologies™, 14190-144, 500 mL);
- Sodium Bicarbonate (from Fisher Chemical, S233-500, 500 g);
- Pierce® RIPA Buffer (from Thermo Scientific, 89900, 100 mL);
- Protease/Phosphatase Inhibitor Cocktail (100X) (P.I.) (from Cell Signaling Technology®, 5872, 1 mL);
- Phosphatase Inhibitor Cocktail Tablets (PhosSTOP) (from Roche, 04906837001);
- Halt™ Protease Inhibitor Cocktail (100X) (from Thermo Scientific, 1862209, 1 mL);
- Hybri-MAX Dimethyl Sulphoxide (DMSO) (from Sigma®-Aldrich, D2650, 10 mL);
- Ham’s F-12 without phenol Red (from Crystalgen, 226-057, 500 mL);
- 1,1,1,3,3,3-Hexafluoro-2-Propanol (HFIP) (from Fluka, 61657);
- Trypan Blue Stain 0.4% (from NanoEnTek, EBT-001, 1.5 mL);
• Amyloid-beta (1-42) (Aβ42) (from California Peptide, 641-15);
• Immortalized mouse microglia cell line BV-2 (from Dr. Grace Sun, Department of Biochemistry, University of Missouri).

2.2 CELL CULTURE

Immortalized mouse microglia BV-2 were seeded into a T75 cell culture flask (USA Scientific®, CC7682-4875) with complete cell culture medium (DMEM, 10% FBS, 1% P/S) at a dilution ratio 1:25, so in total 26 mL diluted cell solution was seeded into each T75 cell culture flask.

Cells were maintained in a humidified incubator at 37 °C and 5% CO₂, the medium was substituted every 2-3 days.

For sub-culture, when the confluence reached 80-90%, BV-2 cells were washed with pre-warmed DPBS (10 mL) and cells were detached from the culture flasks with a cell scraper (GeneMate, T-2443-1, 25 cm); cells were observed under the microscope (from Nikon, Eclipse TS100) to check whether they detached from the substrate of the flask and then they were sub-cultured into a new T75 flask.

BV-2 cells were used for experiments between passages 14 and 20 (P.14-P.20).

For experiments cells were also sub-cultured into 6-well plates (Customized 6-well Softwell®, from Matrigen, SW6-COL-C), each well is customized, using polyacrylamide hydrogel, with different stiffness of 0.2 kPa, 0.5 kPa, 1 kPa, 2 kPa, 4 kPa and hard surface, measured with Young’s modulus, and coated with Collagen I (bovine skin); cells were also sub-cultured into 6 well-plates with only hard surfaces (from Corning Incorporated, 3506).

2.3 PREPARATION OF AMYLOID-B42

Aβ42 aliquots were prepared following the protocol described by Dahlgren et al. (Dahlgren K. N., et al. 2002). Briefly, 1 mg of lyophilized Aβ42 was dissolved in HFIP to a concentration of
1 mM. The solution, composed of Aβ and HFIP, was incubated at room temperature for 60 minutes until it was clear and colorless. Then the solution was aliquoted, let sit out to evaporate overnight and speed vacuumed for 1 hour to evaporate any remaining HFIP and creating an Aβ42 film inside the tubes. The aliquots of Aβ42 were stored at -80 °C.

Before each experiment, Aβ oligomer preparation was performed: the Aβ42 film, reached the room temperature, was dissolved is 2 µL of DMSO, vortexed (vortex from Fisher Scientific, Mini Vortexer) and micro-centrifuged (micro-centrifuge from Corning), then it was dissolved in 98 µL of ice-cold Ham’s F-12 medium to a concentration of 100 µM, followed by vortex, microcentrifugation and sonication with a water bath sonicator (from VWR, B1500A-MT), using high mode (HI) and for 7 minutes. After it was aged at 4 °C for 24 hours to form oligomer aggregates.

2.4 CELL CULTURE ON DIFFERENT SUBSTRATES, CELL COUNTING AND OLIGOMERIC AMYLOID-β42 UPTAKE PROCEDURES

When confluent, BV-2 cells were cultured into nine 6-well plates (three trials for each time and three time points: 0 minutes, 15 minutes and 60 minutes).

In a preliminary experiment, no cells were seeded into each well of 6 well plate and only for 0-minute time point, thus only 2 mL/well of medium was added.

In a first experiment the same number of cells was seeded into each well, at a dilution ratio of 6:25, adding 2 mL of the mixture in each well.

In a second experiment the number of cells cultured into each well was increased, so it has been used a seeding ratio of 8:30 and 2 mL of the mixture was added into each well.

For the experiment with only hard surface wells (only 60 minute time point) two different dilution ratios were used: for three wells of the plate, 8 mL of BV-2 cells and 30 mL of cell culture medium were mixed and 2 mL/well were added (dilution ratio 8:30), and for other
three wells, 1 mL of BV-2 cells and 30 mL of cell culture medium were mixed and 2 mL/well were added (dilution ratio 1:30).

Finally, in a third experiment different seeding ratios were used into the hard surface wells in respect to the elastic surface wells: for elastic substrates 9 mL of BV-2 cells and 30 mL of cell culture medium were mixed into a 50 mL centrifuge tube for seeding (dilution ratio 9:30), while for hard surfaces 3 mL of BV-2 cells and 30 mL of cell culture medium were combined into a 50 mL centrifuge tube for seeding (dilution ratio 3:30), then for all the wells 2 mL were added.

For all the different experiments, except for the preliminary one and for the one with only hard surfaces, in which this passage was skipped, after 24 hours the cell culture medium was replaced without washing (2 mL of cell culture medium was added into each well).

For all the experiments, after other 24 hours BV-2 cells were starved for 4 hours, adding 1 mL/well of DMEM.

After 4 hours of serum deprivation, Aβ42 was added: 10 µL/well of Aβ42 (dilution 1:100, concentration 1 µM) were added for 60 minute and 15 minute plates and then the plates were maintained for 60 minutes and 15 minutes, respectively, in the incubator.

For 0 minute plates Aβ42 was not added, DMEM was removed and the wells were washed 2 times with 1 mL/well of DPBS, then 1 mL of cell culture medium was added in each well; the cells were scrapped with a cell scraper (SARSTEDT, 83.1832, 16 cm) and the samples were collected and put on ice.

Cell counting was performed using a cell counting machine (NanoEnTek EVE™, Automatic Cell Counter and Life Technologies, Countess II FL) and using a chip: 10 µL of cells and trypan blue stain 0.4% mixed together in each side of the chip, so two measure for each sample was performed (NanoEnTek EVE™, E1020 Cell Counting Slide).
Then the cells were pelleted by centrifugation (centrifuge from Fisher Scientific accuSpin Micro 17R and centrifuge from HERMLE Cabnet Z216MK) at 2500 × g for 5 minutes (centrifuge at 4°C) and the supernatant was discarded. The samples were washed two times in cold DPBS and then cells were pelleted by centrifugation at 2500 × g for 5 minutes (cold).

According to the cell number, RIPA buffer and P.I. were added to the cell pellet and the mixture was pipetted up and down to suspend the pellet. The mixture was shacked for 15 minutes in the refrigerator and then centrifuged at 14,000 × g for 15 minutes (cold) to pellet the cell debris. The supernatant was transferred into a new tube and the samples were stored at -20 °C for further experiments (ThermoScientific 2016).

The same procedure was done for the other two groups after the requested time.

2.5 **Amyloid-B_{42} Uptake ELISA Assay**

ELISA assay of human Aβ_{42} (from Life Technologies, KHB3441) was performed according to the manufacturer’s instructions. For the reconstitute human Aβ_{42} standard used in ELISA assay, the preparation was done following the instructions and using sodium bicarbonate.

Data was collected thanks to a plate reader (from BioTek, SYNERGY HI Microplate Reader). Data is reported as the total concentration of Aβ_{42} normalized by the total number of cells (pg Aβ_{42}/mL cells) and as a percentage of the control group (Hard Surface).

2.6 **Total Protein BCA Assay**

BCA assay (from Thermo Scientific, Pierce™ BCA Protein Assay Kit, 23225) was performed according to the manufacturer’s instructions. Data was collected with a plate reader (from BioTek, SYNERGY HI Microplate Reader).

Data is represented as the total protein concentration normalized by the total number of cells (µg protein/mL cells), and as a percentage of the control group (Hard Surface).
2.7 **IMAGING OF BV-2 CELLS**

Images of BV-2 cells on elastic and hard substrates were taken during the period of starving of the cells; images were acquired by a Nikon ECLIPSE Ti-S bright field microscope with both 4X and 40X objectives, so two images were acquired for each sample.

2.8 **STATISTICAL ANALYSIS**

Data is represented as the mean ± standard deviation (SD) from three independent trials. Statistical comparison between two groups was made with student’s t-test (unpaired data with unequal variance), with KaleidaGraph Software (Ver. 4.1.1).

P-values less than 0.05 (P < 0.05) are considered statistically significant.
3 RESULTS

3.1 Substrates Composition Do Not Influence Amyloid-b Concentration Results

To investigate if Aβ concentration quantification is influenced by the substrates composition, a 6 well plate with different substrates was used without seeding cells and without adding Aβ (0-minute group).

After waiting for 4 hours, to simulate the starving of the cells, the collection of the samples using a cell scraper was performed, simulating the conditions in which cells were present on the substrates, then 80 µL or RIPA Buffer + P.I. was added to each tube and samples were centrifuged and collected. ELISA assay was performed with a dilution of 1:2.5.

As it can be seen in Figure 8, the 0-minute group, without Aβ loading, has a very low ELISA signal, because no Aβ is present, and, as expected, it has no trend.

Therefore, it is possible to conclude that the composition of the substrates do not influence the Aβ concentration results, thus they can be used for further experiments.

In the following experiments the 0-minute control group, without Aβ, is used to verify the specificity of ELISA assay to intracellular Aβ concentration, so a flat and approximately null ELISA results should be obtained for this control.
3.2 Same Dilution Ratio Used on Different Substrates and Amyloid-β Concentration Quantification

A dilution ratio of 6:25 was used and 2 mL of the mixture were added to each well.

During the starving of the cells, images were taken with a bright field microscope, with a magnification of 4X and 40X, to see the different amounts of cells on different surfaces and the morphology of BV-2, as it is shown in Figure 9.

**Figure 8 - Effect of substrates on Aβ concentration results** - A 6 well plate with different substrate stiffness (0.2 – 0.5 – 1 – 2 – 4 kPa and Hard Surface, H.S.) for control (no cells and 0 min, so no Aβ loading) was used to verify if the substrates affect ELISA results (Aβ concentration results).
a) 0.2 kPa

b) 0.5 kPa
c) 1 kPa

d) 2 kPa
Figure 9 - Representative bright-field images of BV-2 cells on different elastic substrates (Exp. 1)
- BV-2 cells were imaged with bright-field microscope Nikon ECLIPSE Ti-S, with a magnification of 4X (first column) and 40X (second column), on substrate of different elasticity: (a) 0.2 kPa, (b) 0.5 kPa, (c) 1 kPa, (d) 2 kPa, (e) 4 kPa and (f) Hard Surface (H.S.)

As it is possible to see from the figures, the number of cells increases passing from the softer to the harder surfaces, and the higher number it is found on solid surface; these results are coherent with previous studies that found a higher proliferation capability of the cells on harder surface compared with softer ones (Dalby M. J., Gadegaard N. and Oreffo R. O. C. 2014).
In addition, in the images of hard surface, it is possible to recognize two different morphologies in BV-2 cells: one with a more extended shape to monitor the surrounding environment, and one with a round shape, amoeba-like, characteristic of activated microglia. The uptake of Aβ peptides was performed for the 15 and 60 minute trials, adding 10 µL/well of Aβ42 (dilution 1:100, concentration 1 µM) and then, the counting of the cells was achieved; the results are shown in Figure 10.
(b)

**BV2 - Cell number - 15 minutes**

Cell number $\times 10^6$

- 0.2 kPa
- 0.5 kPa
- 1 kPa
- 2 kPa
- 4 kPa
- H.S.

Substrate Stiffness (kPa)
Figure 10 - BV-2 cell number (Exp. 1) - Cell counting of BV-2 cells on different substrates and for the three different time points (0 min, 15 min, 60 min) was performed with a cell counting machine and the data is expressed as mean ± SD (cell number*10^5) from three independent trials (* P<0.05 compared with Hard Surface, H.S.; ^ P<0.05, ^^ P<0.001 compared with 1 kPa surface; ~ P<0.05 compared with 2 kPa surface; @@ P<0.01, @@@ P<0.001 compared with 4 kPa surface).

As noticed also in the representative bright-field images, the number of cells has a steep increase in the hard surfaces compared to the softer ones. In previous studies, it has been shown that the matrix elasticity is fundamental in the proliferation capabilities of the differentiated cell types of the CNS, and that stiffer surfaces promote a higher proliferation than softer surfaces (Dalby M. J., Gadegaard N. and Oreffo R. O. C. 2014), and this is also confirmed by the data.
After the lysis of the cells with 60 µL/tube of RIPA+P.I. and the collection of the samples, ELISA assay was used to measure the Aβ concentration, with a dilution of 1:2.5. The results of ELISA show few outliers in respect to the standard curve, since the dilution ratio was too low and consequently some values of concentration were excessively high. The data is represented in Figure 11.
(b) BV2 - Normalized intracellular Aβ - 15 minutes

(c) BV2 - Normalized intracellular Aβ - 60 minutes
Figure 11 – BV-2: normalized intracellular Aβ (Exp. 1) - ELISA assay was applied to the samples with a dilution of 1:2.5 to measure the Aβ concentration, and then the data was normalized by the cell number (pg/ml). Figures (a), (b) and (c) show the Aβ concentration normalized by cell number on different elastic substrate for each time point (0 min, 15 min and 60 min); data is expressed as percentage of control (Hard Surface, H.S.) and mean ± SD from three independent trials (* P<0.05, ** P<0.01 compared with Hard Surface, H.S.). Figure (d) show the total trend of Aβ uptake normalized cell number for the three different time points and for the different substrate stiffness; data is expressed as mean ± SD from three independent trials (* P<0.05, ** P<0.01 compared with 0-minute control; °° P<0.01 compared with 15 minutes)

From the obtained results, it is possible to notice that the control (0 minute) has no trend and low values, as expected, and that the general tendency of the other two groups (15 minutes and 60 minutes) is decreasing; thus, on softer substrates the Aβ uptake for each cell and the rate of intracellular Aβ accumulation are higher than on harder substrates. On hard substrate the values of the Aβ concentration are very low. These findings are consistent with the theory
that microglial cells are over-activated on softer AD brain tissue, in which also less cells are present, and so more Aβ is uptaken by the cells and more intracellular accumulation of these peptides is noticed; on the other hand, on stiff matrix the proliferation capabilities of the cells are higher and microglia experience a regular Aβ uptake and degradation of these peptides. However, on the hard surface the intracellular Aβ concentration should be low but more or less comparable to the values obtained on the 4 kPa substrate but it was not, therefore a higher number of cells was seeded in a following experiment to see if it is possible to obtain a correlation between the two harder surfaces or if the difference is still great.

3.3 Higher Dilution Ratio Used on Different Substrates and Amyloid-B Concentration Quantification

A mixture constituted by 8 mL of BV-2 cells and 30 mL of medium was made (seeding ratio 8:30) and 2 mL of the mixture were put into each well.

Cells were starved for 4 hours and, after this period, the Aβ uptake (10 µL/ well, dilution 1:100, concentration 1 µM) was achieved for 15 and 60 minute trials.

Cells were counted to verify a higher number compared to the previous experiment, and the data is shown in Figure 12.
(a) BV2 - Cell number - 0 minute

![Bar graph showing cell number at different substrate stiffness levels at 0 minutes.](image)

(b) BV2 - Cell number - 15 minutes

![Bar graph showing cell number at different substrate stiffness levels at 15 minutes.](image)
Figure 12 – BV-2 cell number (Exp. 2) - Cell counting of BV-2 cells on different substrates and for the three different time points (0 min, 15 min, 60 min) was performed with a cell counting machine and the data is expressed as mean ± SD (cell number*10^5) from three independent trials (* P<0.05 compared with Hard Surface, H.S.; @ P<0.05 compared with 4 kPa surface)

Compared to the previous results, shown in Figure 10, it is possible to notice an increase of the cell number in all time points, but the total trend remains the same with a higher number of cells on harder surfaces compared to more soft ones.

The cells were then lysed with 60 µL of RIPA+P.I. for each tube and then the samples were collected for further analysis.
ELISA assay was employed to measure the concentration of Aβ, using a higher dilution in respect to the previous experiment, to avoid outliers’ results compared to the standard curve, thus a dilution of 1:10 was used. The data is represented in Figure 13.
(b) BV2 - Normalized intracellular Aβ - 15 minutes

(c) BV2 - Normalized intracellular Aβ - 60 minutes
Figure 13 – BV-2: normalized intracellular Aβ (Exp. 2) - ELISA assay was applied to the samples with a dilution of 1:10 to measure the Aβ concentration, and then the data was normalized by the cell number (pg/ml). Figures (a), (b) and (c) show the Aβ concentration normalized by cell number on different elastic substrate for each time point (0 min, 15 min and 60 min); data is expressed as percentage of control (Hard Surface, H.S.) and mean ± SD from three independent trials (* P<0.05, compared with Hard Surface, H.S.; @ P<0.05 compared with 4 kPa surface; ~ P<0.05 compared with 2 kPa surface). Figure (d) show the total trend of Aβ uptake normalized by cell number for the three different time points and for the different substrate stiffness; data is expressed as mean ± SD from three independent trials (* P<0.05, compared with 0-minute control)

The total trend is comparable to the one obtained in the previous experiment, with a large Aβ uptake and intracellular accumulation in substrates with stiffness of 0.2 kPa and 1 kPa, that simulate the condition of a diseased brain, and with a flat and low data for the control group. However, also these findings highlight very low values of Aβ concentration in cells seeded on hard substrate, and this it may be imputable to an insufficient Aβ loading in respect to the vast
number of cells present on hard surface. Therefore, a following experiment with only hard surface and only 60-minute time point was performed, using different seeding ratios, to evaluate the consumption of Aβ on hard surface.

In addition, BCA assay was used to measure the total concentration of protein per cell (normalizing the results with the cell number data), to evaluate if the substrate stiffness influences the total protein amount in the cells; the data is shown in Figure 14.
(b)

**BV2 - Total protein/cell - 15 minutes**

![Bar graph showing total protein/cell (% H.S.) at different substrate stiffness (kPa).](image-url)
Figure 14 – BV-2: total protein concentration per cell (Exp. 2) - BCA assay was applied to the samples without dilution to measure the total protein concentration, and then the data was normalized by the cell number (µg/ml). Figures (a), (b) and (c) show the total protein concentration normalized by cell number on different elastic substrate for each time point (0 min, 15 min and 60 min); data is expressed as percentage of the control (Hard Surface, H.S.) and mean ± SD from three independent trials (* P<0.05, compared with Hard Surface, H.S.)

It is possible to deduce that the substrate stiffness do not play a critical role in the total protein concentration per cell; the values are roughly similar, so substrate stiffness plays a critical role in the Aβ uptake by BV-2 cells, but it seems not to influence the total protein concentration; indeed the amount of protein varies from cell to cell but the average total concentration remains approximately constant.
3.4 INSUFFICIENT AMYLOID-B CONCENTRATION FOR BV-2 CELLS ON HARD SUBSTRATE

One 6 well plate with only hard surfaces was used to determine if the Aβ concentration is not enough for the high number of BV-2 cells on hard substrate, and therefore if they would consume all Aβ peptides.

A seeding ratio of 8:30 was used for three wells of the plate (2 mL of the mixture per well), while in the remaining wells were added 2 mL of a mixture with a dilution ratio of 1:30, to have a different number of cells on the same hard surface.

BV-2 cells were starved for 4 hours and in this time period images, with a bright-field microscope, were taken (magnification 4X and 40X) to confront the quantity of cells and to examine the morphology of the microglia (Figure 15).
Figure 15 - Representative bright-field images of BV-2 cells on hard substrate (Exp. on hard surface only) - BV-2 cells, on hard surface only, were imaged with bright-field microscope Nikon ECLIPSE Ti-S, with a magnification of 4X (first column) and 40X (second column).

As expected, on hard surface on which more BV-2 cells (8:30 dilution ratio) were seeded a greater amount of microglia can be observed compared to the wells with a lower seeding ratio (1:30); additionally, both round and extended microglial cells are present for both seeding ratios.

To verify the different number of BV-2 cells, the cell counting was performed and the result is shown in Figure 16.
Figure 16 - BV-2 cell number (Exp. on hard surface only) - Cell counting of BV-2 cells on hard surfaces and for 60-minute time point was performed with a cell counting machine. The figure shows the average of the cell number of the two different seeding ratios (8:30 and 1:30), data is expressed as mean ± SD (cell number*10^5) from three independent trials.

These findings further confirm the difference in the cell number between the wells in which a dilution ratio of 8:30 was used and the wells with a seeding ratio of 1:30.

Aβ uptake was performed, using 10 µL/ well of Aβ42 (dilution 1:100, concentration 1 µM). After the lysis of the BV-2 cells with 70 µL/tube of RIPA+P.I. and the collection of the samples, the Aβ concentration was measure with ELISA assay with a dilution of 1:20 and the data was normalized by the cell number.
The purpose is to check if the concentration of Aβ peptides is too low for the high number of BV-2 cells on hard surfaces, and therefore justify the low values of Aβ inside the cells on hard substrates present in the results of the previous experiments. Data is shown in Figure 17.

![Figure 17 - BV-2: normalized intracellular Aβ (Exp. on hard surface only)](image)

*Figure 17 - BV-2: normalized intracellular Aβ (Exp. on hard surface only)* - ELISA assay was applied to the samples with a dilution of 1:20 to measure the Aβ concentration, and then the data was normalized by the cell number (pg/ml). Data is expressed as mean ± SD from three independent trials.

In the samples with less cells (1:30 dilution ratio) the intracellular concentration of Aβ peptides is higher compared to the samples with more BV-2 cells (8:30 seeding ratio), so a higher number of cells will consume all the Aβ peptides because the concentration of Aβ used
(1 µM) is too low. For this reason, in the previous experiments low values of intracellular Aβ concentration were obtained on hard surfaces.

Therefore, it is not possible to compare the intracellular concentration of Aβ on hard surface and on softer substrates, due to the big difference in the number of cells, therefore in a successive experiment less BV-2 cells were seeded on hard surface compared to the softer ones to have a quantitative comparison of the amyloid uptake by microglial cells between the different elastic substrates and approximately in the same condition of cell number.

3.5 **DIFFERENT DILUTION RATIO USED ON DIFFERENT SUBSTRATES AND AMYLOID-B CONCENTRATION QUANTIFICATION**

To have roughly the same amount of BV-2 cells on the different surfaces, to compare the results of the Aβ uptake with approximately the same number of cells and in different condition of stiffness, a lower dilution ratio was used on hard substrate compared to the softer ones: 9 mL of BV-2 cells with 30 mL of medium was prepared and then 2 mL/well were added in the elastic wells, while for hard surface 3 mL of BV-2 with 30 mL of medium (three times lower amount) was prepared and then 2 mL/well were added.

After 4 hours of starving, period of time in which also bright-field images were taken with a magnification of 4X and 40X, Aβ_{42} was added to the 15 minute and 60 minute plates (10 µL/well of Aβ_{42}, dilution 1:100, concentration 1 µM).

After, the counting of the cells was performed, the samples were collected in tubes and the lysis buffer composed by RIPA+P.I. was used (80 µL/tube).

From the bright-field images, shown in Figure 18, and from the cell counting data, shown in Figure 19, it is possible to notice the expected less amount of BV-2 on the hard surfaces in all times points, number that is now comparable with the data on the softer substrates, allowing a quantitative and comparative analysis between the softer (AD brain simulation) and harder (healthy brain simulation) surfaces.
a) 0.2 kPa

b) 0.5 kPa
c) 1 kPa

d) 2 kPa
Figure 18 - Representative bright-field images of BV-2 cells on different elastic substrates (Exp.3) - BV-2 cells were imaged with bright-field microscope Nikon ECLIPSE Ti-S, with a magnification of 4X (first column) and 40X (second column), on substrate of different stiffness: (a) 0.2 kPa, (b) 0.5 kPa, (c) 1 kPa, (d) 2 kPa, (e) 4 kPa and (f) Hard Surface (H.S.)
(a) BV2 - Cell number - 0 minute

Substrate Stiffness (kPa)

(b) BV2 - Cell number - 15 minutes

Substrate Stiffness (kPa)
Figure 19 - BV2 cell number (Exp. 3) - Cell counting of BV-2 cells on different substrates and for the three different time points (0 min, 15 min, 60 min) was performed with a cell counting machine and the data is expressed as mean ± SD (cell number*10^5) from three independent trials (@ P<0.05, @@ P<0.01 compared with 4 kPa surface).

As it is possible to notice, in the results of the cell counting there is not a significant difference between the number of BV-2 cells on hard surfaces and on the other elastic substrates.

ELISA assay was employed to measure the Aβ42 uptake, with a dilution ratio of 1:20, and the results are shown in Figure 20.
Experiment 3 (a)

**BV2 - Normalized intracellular Aβ - 0 minute**

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<th>Normalized intracellular Aβ (% H.S.)</th>
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<td>0.2 kPa</td>
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<tr>
<td>0.5 kPa</td>
<td>1.1</td>
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<tr>
<td>1 kPa</td>
<td>1.0</td>
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<tr>
<td>2 kPa</td>
<td>1.1</td>
</tr>
<tr>
<td>4 kPa</td>
<td>1.5</td>
</tr>
<tr>
<td>H.S.</td>
<td>1</td>
</tr>
</tbody>
</table>

(a)
(b) BV2 - Normalized intracellular Aβ - 15 minutes

(c) BV2 - Normalized intracellular Aβ - 60 minutes
Figure 20 - BV-2: normalized intracellular Aβ (Exp. 3) - ELISA assay was applied to the samples with a dilution of 1:20 to measure the Aβ concentration, and then the data was normalized by the cell number (pg/ml). Figures (a), (b) and (c) show the Aβ concentration normalized by cell number on different elastic substrate for each time point (0 min, 15 min and 60 min); data is expressed as percentage of control (Hard Surface, H.S.) and mean ± SD from three independent trials (*** P<0.001, compared with Hard Surface, H.S). Figure (d) show the total trend of Aβ uptake normalized by cell number for the three different time points and for the different substrate stiffness; data is expressed as mean ± SD from three independent trials (* P<0.05, ** P<0.01, compared with 0-minute control)

In these new findings, it is possible to notice that the values of intracellular Aβ concentration on the hard surface are higher in respect to the previous experiments (Figure 20, d) and so the concentration of Aβ peptides is enough also for the number of BV-2 cells present on hard surface, and a comparative analysis between the different substrates can be done.
Furthemore, the trend is always decreasing from the softer to the harder surfaces, so a higher Aβ_{42} uptake is performed by the BV-2 cells on soft substrates (the maximum is obtained for the 0.2 kPa) that simulate the AD brain with a possible over-activation of microglial cells, impairment of the phagocytic functions and an accumulation of Aβ inside the cell.

Moreover, the tendency of the intracellular Aβ concentration both for 15 minute and 60 minute trials is similar, proving that after 15 minutes the Aβ uptake by BV-2 microglial cells reaches a saturation.

Finally, the trend of the 0 minute trials is constant and has very low values, confirming the absence of the amyloid load in the microglial cells.

While the amyloid uptake is varying on different substrate, the total protein concentration per cell seems to be not influenced by the substrate stiffness. These findings highlight that the uptake by microglial cells, and so the intracellular accumulation, of these small and toxic peptides, but not the total protein concentration inside the cells, is the factor that is more influenced by the stiffness of the local environment surrounding the cells.

Data regarding the total protein concentration per cell is shown in Figure 21.
(a) BV2 - Total protein/cell - 0 minute

(b) BV2 - Total protein/cell - 15 minute
Figure 21 - BV-2: total protein concentration per cell (Exp. 3) - BCA assay was applied to the samples without dilution to measure the total protein concentration, and then the data was normalized by the cell number (µg/ml). Figures (a), (b) and (c) show the total protein concentration normalized by cell number on different elastic substrate for each time point (0 min, 15 min and 60 min); data is expressed as percentage of the control (Hard Surface, H.S.) and mean ± SD from three independent trials (@ P<0.05, compared with 4 kPa surface)
**4 DISCUSSION**

Aβ₄₂ peptides are oligomers derived from the endo-proteolysis of APP present on the membranes of the cells of the CNS; they can aggregate in fibrils and generate amyloid plaques, one of the principal hallmark of the AD brain.

The cells of the CNS, that are responsible for the maintenance of the homeostasis of this very important and vital system, are microglial cells; they can phagocyte Aβ and remove the risk of accumulation of these toxic peptides into plaques.

Unfortunately, when the disease progresses microglial cells could be over-activated by a redundant Aβ internalization, generating an excessive inflammation response, becoming unable to phagocyte in a correct way the toxic oligomers, thus drive an intracellular and extracellular accumulations of these peptides, leading to the formation of plaques and the progression of the pathology.

Differentiated cells, such as microglial cells, are highly sensitive to the environment surrounding them; recent findings, thanks to innovative technologies, such as MRE, have shown that one of the main factor that changes during the evolution of the Alzheimer’s disease pathology is the stiffness of the brain, passing from a harder healthy tissue to a softer diseased brain (Murphy M. C., Huston III J., et al. 2011).

This work wants to evaluate, in a quantitative way, whether the substrate stiffness influences the Aβ uptake by BV-2 microglial cell line, using substrates with a stiffness that varies from 0.2 kPa (soft tissue that simulate the diseased brain) to 4 kPa surface (harder tissue that simulate the healthy brain).

Initially it has been evaluated if the substrates composition influence the Aβ concentration results of the 0-minute group; the findings highlight no trend in different substrates and
low values of Aβ concentration (Figure 8), concluding that the composition of the substrates do not influence the Aβ concentration results.

Further experiments analyze the Aβ uptake by BV-2 microglia cell on different substrates and for three different time points; firstly, the same number of cells was seeded on different elastic substrate and the intracellular Aβ concentration was measured; the results have shown a flat tendency for the 0-minute group with very low values of intracellular Aβ concentration, and a decreasing trend for the 15 and 60 minute groups passing from softer to harder surfaces (Figure 11, Figure 13).

These findings highlight the fact that on harder substrate the number of cells is higher in respect to softer ones, due to the important role of the matrix stiffness in the proliferation capabilities of the cells, and additionally that on softer surfaces the Aβ uptake by BV-2 microglial cells is higher, which could lead to microglia impaired functions and so a higher intracellular accumulation of Aβ.

The values of Aβ concentration on harder surfaces were very low in these first experiments, and these results could be due to a too low concentration of Aβ compared to the high quantity of cells present on the matrix, leading so to the impossibility to compare the uptake between soft and hard surfaces.

Therefore, an analysis only on hard surface was performed in order to estimate if the concentration of Aβ was too low for the high number of BV-2 cells on hard surface, showing that less cells must be seeded on hard surface to have a comparative analysis of the Aβ uptake on different substrates (Figure 17).

For this reason, in a last experiment three times lower amount of BV-2 cells was seeded on hard surface compared to softer ones, to obtain values of cell number that can be comparable between the different substrates.
These findings show a reduction in the intracellular amyloid concentration passing from soft surface to harder ones, and the values correspondent to hard surface are higher and comparable to ones of the other surfaces (Figure 20).

Additionally, the 15 minute and 60 minute groups display a similar trend, since after 15 minutes the Aβ internalization arrives to equilibrium for BV-2 cells, so there is not an increase in the internal Aβ_{42} concentration with respect to time.

All this evidence shows that the uptake of toxic Aβ_{42} oligomers by BV-2 immortalized microglial cell line is influenced by the substrate stiffness: the internalization of Aβ_{42} is higher on softer substrates, specially the ones with a Young’s Modulus of 0.2 kPa and 1 kPa, that simulate the stiffness of AD brain, in respect to harder matrix of 4 kPa, that mimic healthy tissue.

These results support the hypothesis that the mechanical environment of AD brain causes microglia to have altered Aβ uptake, which could lead to an over-activation of the immune cells of the central nervous system, which in turn contribute to an excessive inflammation cascade, an intracellular and extracellular accumulation of amyloid peptides, that can lead ultimately to aggregation of amyloid oligomers into plaque and, at the end, to neural death and cognitive dysfunctions that characterize the disease.

Bright-field images and counting of BV-2 cells are also performed in order to demonstrate the different number of cells on different substrates and so the correlation between the matrix stiffness and the proliferation capabilities of the cells (Figure 10, Figure 12 and Figure 19).

The images show the different morphologies of microglia cells, from an extended shape, characteristic of surveillant microglia, able to inspect their local environment, to an amoeba-like shape, distinctive of activated microglia, able to uptake the toxic peptides (Figure 9, Figure 18).
The proliferation capability of microglial cells is deeply affected by the surrounding environment, specially by the stiffness of the matrix: hard surfaces influence the adhesion property of the cells. A higher adhesion force is exerted on the cells, consequently change the response of transmembrane ECM receptors, which act as mechanotransducers transferring the mechanical stimuli to the cytoskeleton and activating downstream pathways, which lead, ultimately, to an increased proliferation (Dalby M. J., Gadegaard N. and Oreffo R. O. C. 2014).

Studies regarding the total protein concentration per cell were performed, and the results exhibit a constant trend of the total protein amount per cell, highlighting the dependence of the Aβ uptake only on the substrate stiffness (Figure 14, Figure 21).

So, demonstrated that the mechanical features of the brain tissue surrounding the cells will impact directly on the functioning of the cells, including the oligomeric Aβ uptake, further studies could be directed to the different production of cytokines by microglial cells on different substrates, to highlight also the different inflammation condition on different medium. In addition, to overcome some limitations of this research, a broader range of stiffness could be used and also a wider number of samples could be analyzed to obtain more reliable results for further analysis.
5 CONCLUSION AND FUTURE DEVELOPMENTS

This work demonstrates that the internalization of Aβ_{42} oligomers by microglial BV-2 cells is influenced by the substrate stiffness of the local medium surrounding the cells. Softer substrates, that mimic the stiffness of the AD brain, induce a higher Aβ uptake by BV-2 cells that can lead to an over-activation of these immune cells, that in turn could provoke an uncontrollable inflammation response, impaired phagocytic function, and an intracellular or extracellular accumulation of Aβ peptides into plaques, which contribute to the quick progression of the pathology.

Future works could possibly focus on the connection between the Aβ uptake by microglial cells on different substrates and the excessive inflammation response, consequently concentrate on the different production of reactive oxygen species and cytokines on different elastic surfaces to find possible therapeutic agents able to act on oxidant and antioxidant pathways to restrict the over-production of inflammation factors by microglial cells.

Some recent works in our laboratory have shown an increase in the protein expression profile of cPLA₂, a key enzyme that facilitates the internalization of Aβ, on softer substrates, specially 1 kPa surface. The phosphorylation of higher level of cPLA₂, activation that is induced by Aβ, could consecutively lead to an increase in the Aβ uptake by microglial cells, while the inhibition of the phosphorylation of this protein could decrease the concentration of Aβ peptides inside the cells; those data are coherent with the theory that on softer substrate the rate of Aβ uptake is increased and the Aβ intracellular accumulation is higher. Therefore, future research should also explore new engineered treatments for microglia cells; one imperative study should be the comprehension of the role of cPLA₂ in the Aβ internalization,
in order to maximize the rate of the Aβ peptides uptake and minimize the rate of the intracellular and extracellular accumulation.

After determining the effects of the substrate stiffness on AD-related pathways of immortalized microglial cells (BV-2), including Aβ uptake, oxidant and antioxidant pathways, actin polymerization and membrane cytoskeleton adhesion, a future prospective is to investigate, with multidisciplinary approaches (from biochemical and biophysical methods to tissue and cell mechanics engineering), the influences of substrate stiffness also in primary mouse microglial cells.

These researches will provide new knowledge regarding the Aβ uptake, oxidant and antioxidant pathways in Aβ-stimulated microglial cells under the effects of different substrate stiffness in order to find a possible therapeutic agent able to limit the formation of amyloid plaques and slow or, in an hopeful prospective, defeat the advancement of this neurodegenerative disease.
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Permission to use an illustration in my thesis

Heppner, Frank <Frank.Heppner@charite.de>
To: Cecilia Teresa Brambilla Pisoni <cbramb2@uic.edu>

Dear Cecilia,

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Best wishes with your thesis.

Frank

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Appendix D (continued)

Department of Bioengineering – University of Illinois at Chicago (UIC)

1/4/2017


I am currently writing a thesis about the Amyloid-β uptake by microglial BV-2 cell in the Laboratory of Dr. James Lee at University of Illinois at Chicago, and in my introduction I am including a section about difference in the stiffness in healthy and Alzheimer’s disease brains, and I would like to include Figure 2 and Figure 3, page 10 and page 11, of the above article.

A copy of this letter is included for your records. Thank you for your kind consideration of this request.

Sincerely,

Cecilia Brambilla Pisoni

College of Medicine Research Building (COMRB)
909 S. Wolcott Street, University of Illinois at Chicago (UIC)
Chicago, IL 60612

The above request is approved.

Approved by: [Signature]  
Date: 4/3/2017
Department of Bioengineering – University of Illinois at Chicago (UIC)

1/4/2017


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I am currently writing a thesis about the Amyloid-β uptake by microglial BV-2 cell in the Laboratory of Dr. James Lee at University of Illinois at Chicago, and in my introduction I am including a section about difference in the stiffness in healthy and Alzheimer’s disease brains, and I would like to include Figure 2 and Figure 3, page 10 and page 11, of the above article.

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College of Medicine Research Building (COMRB)
909 S. Wolcott Street, University of Illinois at Chicago (UIC)
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Signorini M. G. *Biomedical Signal Processing and Medical Images.* Politecnico di Milano, Milano, 2015.


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University of Illinois at Chicago, Bioengineering Department, Chicago, IL, 2016/2017

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