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The Role of Adiponectin

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Tese no âmbito do Programa de Doutoramento em Ciências da Saúde - ramo de Medicina, orientada pela Professora Doutora Raquel Seiça e Professora Doutora Isabel Santana, e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Agosto de 2018

Faculdade de Medicina da Universidade de Coimbra

OBESITY AS A RISK FACTOR FOR ALZHEIMER´S DISEASE

THE ROLE OF ADIPONECTIN

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The clinical study was conducted at Centro Hospital e Universitário de Coimbra, Coimbra, Portugal. The experimental research was performed in the Institute of Physiology, Institute of Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal and also in Centro de Neurociências e Biologia Celular (CNC), Coimbra, Portugal.

Agradecimentos

Todo o entusiasmo, trabalho e dedicação envolvidos neste projecto (e que não consigo resumir nas páginas que se seguem) foram amplamente partilhados. Por isso agradeço…

… à Faculdade de Medicina da Universidade de Coimbra e Centro Hospitalar e Universitário de Coimbra que me forneceram as ferramentas necessárias à minha formação e me permitiram contactar com as pessoas que inspira(ra)m o meu espírito clínico e científico;

… aos doentes e seus familiares, porque são eles a minha maior motivação;

… a todos os que colaboraram directa ou indirectamente neste projecto ao longo dos anos, realçando a Drª Inês Baldeiras, o Técnico Rui Pascoal, a Enf.ª Alice Almeida, as neuropsicólogas Drª Diana Duro e Drª Raquel Lemos, a Drª Cristina Lemos, o Sr. Mário Simões, os meus colegas do Serviço de Neurologia do Centro Hospitalar e Universitário de Coimbra, e os meus colegas do Laboratório do Instituto de Fisiologia da Faculdade de Medicina da Universidade de Coimbra, especialmente ao Dr. Paulo Matafome, ao Dr. Tiago Rodrigues e à Drª Filipa Baptista pela sua motivação nos momentos de hesitação e sobretudo pela sincera amizade.

… às minha orientadoras, modelos de excelência nas suas áreas, por me ajudarem a construir uma *investigadora* dentro da *médica*. À Professora Doutora Isabel Santana, em especial pelo seu sentido crítico, construtivo e impulsionador, a quem reconheço uma inigualável capacidade de trabalho. À Professora Doutora Raquel Seiça, por constituir a minha maior referência na ciência e na docência, pelo apoio incondicional e confiança depositada ao longo destes anos;

… aos meus pais e irmã por constituirem as fundações nas quais se apoia este trabalho e a minha pessoa, em todas as suas dimensões. Aos meus segundos pais, os meus padrinhos, por partilharem comigo este sonho, mesmo antes de ser meu. Aos meus sogros, pelo precioso, genuíno e generoso apoio. Aos que já partiram, pela convicção de que se orgulhariam deste percurso;

… ao Tito, ao Tomé e à Clara, porque são o meu mundo e os meus *projectos* mais especiais.

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Abstract

Background: Recent studies have implicated adipose tissue dysfunction, particularly the modifications in its secretome, in the pathophysiology of Alzheimer's disease. Adipokines, such as leptin and adiponectin, are produced in adipose depots and cross the blood-brain barrier to influence brain's structure and function. Although leptin's role in neurodegeneration is already recognized, the involvement of adiponectin (the most abundant adipokine in circulation) and the clinical relevance of the leptin to adiponectin ratio (LAR) in Alzheimer's disease is not yet established.

Objectives: To determine serum and cerebrospinal fluid (CSF) levels of adiponectin and LAR in different stages of Alzheimer's disease and evaluate their correlation with the main risk factors and biomarkers of the disease. This study also aims to test adiponectin-mediated neuroprotective effects in the hippocampus of high-fat fed obese rats.

Methods: In the cross-sectional study, a total of 71 amnestic Mild Cognitive Impairment (MCI) and 53 mild to moderate Alzheimer's dementia (AD) subjects were included and underwent a thorough clinical, anthropometric and neuropsychological evaluation. Serum and CSF adiponectin and leptin, glycemia, insulinemia and insulin resistance

index (HOMA), as well as Alzheimer's disease biomarkers (CSF $A\beta_{42}$, t-tau and p-tau, and volumetry of medial temporal lobe structures) were determined. A comparative analysis between the two groups and between risk profiles (considering gender and *APOE* genotype) was performed, and correlations between disease biomarkers and adipokine concentrations were assessed. A subgroup analysis was also performed in female patients and in those with Alzheimer's disease CSF profile (low $A\beta_{42}$/p-tau). The longitudinal study included 67 MCI patients and studied the capacity of adipokines in predicting progression to AD as well as their correlation with the time elapsed until the diagnosis. In the animal model (Wistar rat, 12 months, fed with a hyperlipid diet in the last 4 months), globular adiponectin was administered through a peripheral continuous infusion pump (98 µg/day, 28 days), and its effects on insulin signaling and lipid storage were evaluated (in blood and adipose tissue). In the hippocampus, the expression of adipokines' receptors, insulin/PI3K/Akt/GSK3β/tau pathway, synaptic proteins levels, neuroinflammation and neuronal death were evaluated. Moreover, the performance of these animals in hippocampal-dependent cognitive tasks (Morris Water Maze) was also studied.

Results: Serum adiponectin was 33% higher in AD when compared to MCI patients. CSF adiponectin was positively correlated with $A\beta_{42}$ and with better cognitive performance, though only in women. In the

subgroup of patients with Alzheimer's disease CSF profile, higher CSF adiponectin levels were associated with higher hippocampal volume and lower systemic insulin resistance. In the follow-up of MCI patients (mean 38.2±18.83 months) 40.3% (n=27) progressed to AD. In this group, higher levels of serum adiponectin were associated with slower rate of progression, although neither adipokine concentration or their ratio were able to predict it. In the animal model, adiponectin administration decreased body weight and adipocyte size, leptinemia and LAR. On the other hand, it was able to prevent diet-induced insulin signaling defects, especially in epididymal/visceral adipose tissue, and partially restored lipolysis in this fat depot. Even though high-fat diet did not induce the expected deleterious cerebral effects which hindered the evaluation of adiponectin's neuroprotective actions, its peripheral administration improved insulin signaling in the hippocampus. This improvement was also significant when comparing with the control group.

Conclusions: The results arising from this study suggest that higher serum adiponectin levels in AD patients represent a metabolic strategy to compensate for central defects in adiponectin and/or insulin signaling. Besides, adiponectin might be especifically assigned to neuroprotective functions in women. Further studies are needed to establish the protective role of adiponectin in Alzheimer's disease as well as in other neurodegenerative diseases and assess its potential as therapeutic target.

Resumo

Introdução: Estudos recentes têm vindo a estabelecer uma relação entre a disfunção do tecido adiposo, em particular as modificações no seu secretoma, e a fisiopatologia da doença de Alzheimer. As adipocinas, tais como a leptina e a adiponectina, são produzidas neste tecido e conseguem atravessar a barreira hematoencefálica, influenciando a estrutura e função cerebrais. Embora o papel da leptina na neuro-degenerescência seja já reconhecido, o envolvimento da adiponectina (a adipocina mais abundante em circulação) e a relevância clínica da razão entre a leptina e a adiponectina (RLA) na doença de Alzheimer não estão ainda estabelecidas.

Objectivos: Determinar os níveis de adiponectina e RLA no soro e no líquido cefalorraquídeo (LCR) em diferentes estadios da doença de Alzheimer e avaliar a sua correlação com os principais factores de risco e biomarcadores da doença. Este estudo pretende ainda avaliar a capacidade neuroprotetora da adiponectina a nível do hipocampo num modelo animal de obesidade induzida por dieta gorda.

Métodos: No estudo clínico transversal foram incluídos 71 indivíduos com Défice Cognitivo Ligeiro amnésico (DCL) e 53 com demência de Alzheimer ligeira a moderada (DA), que foram submetidos a uma

rigorosa avaliação clínica, antropométrica e neuropsicológica. Determinaram-se as concentrações de adiponectina e leptina no soro e no LCR, a glicemia, a insulinemia e o índice de insulinorresistência (HOMA), assim como os biomarcadores da doença ($A\beta_{42}$, t-tau e p-tau no LCR e volumetria das estruturas do lobo temporal medial). Realizou-se uma análise comparativa entre os dois grupos e entre perfis de risco (tendo em conta o género e o genótipo *APOE*), e avaliaram-se as correlações entre os biomarcadores da doença e as concentrações das adipocinas. Foi ainda efectuada uma análise de correlação semelhante no subgrupo de doentes do género feminino e naqueles com LCR característico de doença de Alzheimer ($A\beta_{42}$/p-tau reduzida). No estudo longitudinal foram incluídos 67 doentes com DCL e avaliou-se a capacidade preditora das adipocinas no que respeita à progressão para DA e a sua correlação com o tempo decorrido até ao diagnóstico. No modelo animal (rato Wistar, 12 meses, alimentado com dieta hiperlipídica nos últimos 4 meses), foi administrada adiponectina globular a nível periférico através de bomba de infusão contínua (98 μg/dia, 28 dias) e avaliada, a nível sistémico e no tecido adiposo, a repercussão nas vias de sinalização da insulina e do armazenamento dos lípidos. No hipocampo foi avaliada a expressão dos receptores de ambas as adipocinas, a via da insulina/PI3K/Akt/GSK3β/tau, os níveis de proteínas sinápticas, a neuroinflamação e a morte neuronal. Foi ainda

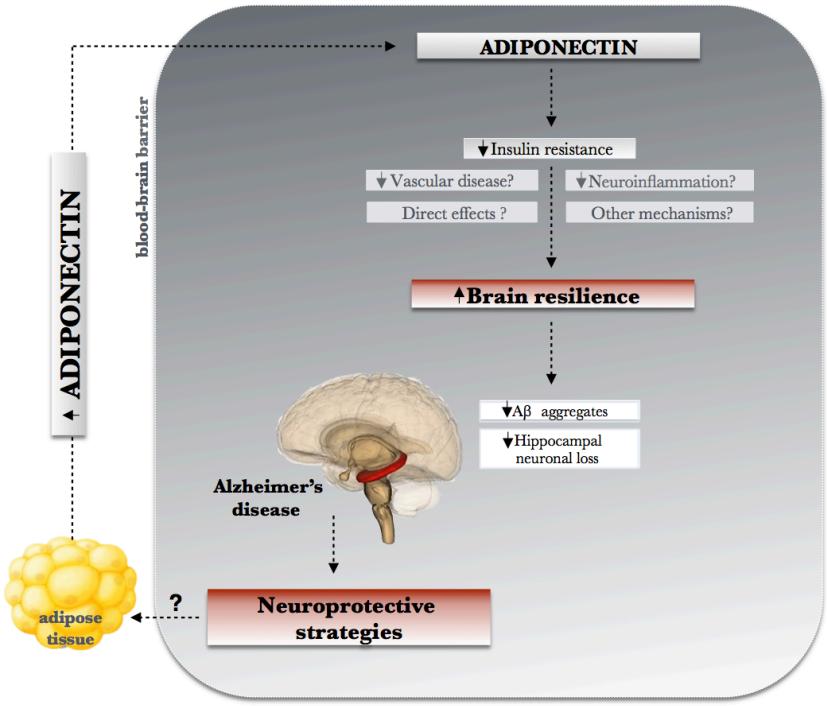
estudado o desempenho dos animais em tarefas cognitivas dependentes do hipocampo (Morris Water Maze).

Resultados: Em doentes com DA, a concentração sérica da adiponectina foi 33% superior à dos pacientes com DCL. A concentração de adiponectina no LCR correlacionou-se de forma positiva com a concentração de $A\beta_{42}$ e com melhor performance cognitiva, embora apenas em mulheres. Nos doentes com LCR característico de doença de Alzheimer, níveis mais elevados de adiponectina no LCR correlaciona-ram-se com maior volume do hipocampo e menor resistência sistémica à insulina. No grupo de doentes com DCL que mantiveram seguimento (média 38.2±18.83 meses) 40.3% (n=27) progrediram para DA. Nestes, níveis mais elevados de adiponectina sérica associaram-se a progressão mais lenta para a fase de demência, embora nem a concentração das adipocinas nem a sua razão tenham apresentado capacidade de a prever. No modelo animal, a administração de adiponectina diminuiu o peso corporal e o tamanho dos adipócitos, a leptinémia e a RLA. Por outro lado, foi capaz de prevenir as alterações na sinalização de insulina induzidas pela dieta, especialmente no tecido adiposo epididimal/visceral e restaurar parcialmente a disfunção da lipólise neste depósito de gordura. Embora a dieta gorda não tenha induzido as alterações esperadas a nível central comprometendo a avaliação dos efeitos neuroprotectores da

adiponectina, a administração periférica desta adipocina melhorou a sinalização da insulina no hipocampo. Esta melhoria foi igualmente significativa em relação ao grupo controlo.

Conclusões: Os resultados deste estudo sugerem que níveis séricos mais elevados de adiponectina em doentes com DA constituem uma estratégia metabólica que visa compensar defeitos centrais na sinalização da adiponectina e/ou da insulina. Por outro lado, esta adipocina parece desempenhar um papel neuroprotector especialmente relevante no género feminino. Mais estudos serão necessários para estabelecer o papel protector da adiponectina não só na doença de Alzheimer como também noutras doenças neurodegenerativas, e ainda para avaliar o seu potencial como alvo terapêutico.

Graphical Abstract



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(Q2; IF: 2.88)

Long-term globular adiponectin administration improves adipose tissue dysmetabolism in high-fat diet-fed Wistar rats

Matafome P, Rodrigues T, Pereira A, Letra L, Azevedo H, Paixão A, Silvério M, Almeida A, Sena C, Seiça R. (2014). *Arch Physiol Biochem.* 120(4):147-57.
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Adiponectin and sporadic Alzheimer's disease: Clinical and molecular links

Letra L, Rodrigues T, Matafome P, Santana I, Seiça R. (2017). *Front Neuroendocrinol.* pii: S0091-3022(17)30062-6.
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The effects of long-term globular adiponectin peripheral administration on hippocampal insulin signaling pathway

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Níveis elevados de adiponectina sérica em doentes com Demência de Alzheimer como possível estratégia sensibilizadora de insulina no hipocampo

Oral communication presented at the 13º Congresso Português de Diabetes 2017.

Prize for Best Clinical Oral Communication

Elevated serum adiponectin in Alzheimer's Disease as neuroprotective strategy

Poster communication at the 19th European Congress of Endocrinology 2017.

Adiponectina pode atrasar a progressão de defeito cognitivo ligeiro para demência de Alzheimer

Poster Communication presented at the 14º Congresso Português de Diabetes 2018.

Honorable mention for Best Clinical Investigation Poster

List of Abbreviations

Aβ	Amyloid beta
AD	Alzheimer's dementia
ADAS-Cog	Alzheimer's Disease Assessment Scale-Cognitive subscale
AdipoR	Adiponectin receptor
ADNI	Alzheimer's Disease Network Initiative
ADPN	Adiponectin
Akt	Protein kinase B
AMPc	Cyclic adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APPL	Adaptor protein containing the pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1
ATI	Amyloid tau index
AUC	Area under the curve
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CA	Cornus ammonis
CAA	Cerebral amyloid angiopathy
CDR	Clinical dementia rating
CNS	Central nervous system
CSF	Cerebrospinal fluid

CT	Computed tomography
CTL	Control
DG	Dentate gyrus
DLB	Dementia with Lewy bodies
DM	Diabetes *mellitus*
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
fAd	Full-length adiponectin
FDG	^{18}fluorodeoxyglucose
FFA	Free fatty acids
fMRI	Functional magnetic ressonance imaging
FOXO	Forkhead box O
FPLC	Fast protein liquid chromatography
FTD	Frontotemporal dementia
gAd	Globular adiponectin
GFAP	Glial fibrillary acidic protein
GLUT	Glucose transporter
HDL	High density lipoprotein
HFD	High-fat diet
HFDA	High-fat diet with adiponectin
HFDV	High-fat diet with vehicle
HMW	High-molecular weight
HOMA-IR	Homeostasis model assessment-insulin resistance
HSL	Hormone-sensitive lipase
Icv	Intracerebroventricular
IDE	Insulin-degrading enzyme
IkBα	NF-kB inhibitor alpha

IL	Interleukin
IPGTT	Intraperitoneal glucose tolerance test
IQ	Intelligence quoficient
IR	Insulin receptor
IRS	Insulin receptor substrate
IWG	International Working Group
LAR	Leptin to adiponectin ratio
LDL	Low density lipoprotein
LepR	Leptin receptor
LMW	Low molecular weight
LRP1	Low-density lipoprotein receptor-related protein 1
MAPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein tau
MCI	Mild cognitive impairment
MMP	Matrix metalloproteinases
MMSE	Mini-mental state examination
MoCA	Montreal cognitive assessment
MRI	Magnetic resonance imaging
MTL	Medial temporal lobe
NEP	Neprilysin
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NFT	Neurofibrillary tangles
NIA-AA	National Institute of Aging and Alzheimer's Association
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association
NMDA	N-methyl-D-aspartate

NO	Nitric oxide
p-tau	Phosphorylated tau
PET	Positron emission tomography
PH	Parahippocampus
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol triphosphate
PPAR	Peroxissome proliferation activated receptor
PSEN	Presenilin
ROC	Receiver operating characteristic
ROI	Regions-of-interest
S1p	Sphingosine-1-phosphate
SAT	Subcutaneous adipose tissue
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T-cad	T-cadherin
T2DM	Type 2 diabetes *mellitus*
TNF	Tumor necrosis factor
t-tau	Total tau
UCP-1	Uncoupled protein 1
VAT	Visceral adipose tissue
WAT	White adipose tissue
WC	Waist circumference
WHO	World Health Organization
WHR	Waist to hip ratio

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Thesis outline

This thesis is divided in four parts, which content is summarized below.

Part I includes a general introduction to the thesis, covering fundamental aspects of the clinical and physiopathologic features of Alzheimer's disease (Chapter I.1) and Obesity (Chapter I.2). It also includes a chapter addressing the life-long relationship between cognition and adipose tissue (Chapter I.3). The research purpose, which states for the motivation and scope of the thesis, concludes this Part (Chapter I.4).

Part II gathers the data from clinical investigation. It includes an extensive review of the current state of research (Chapter II.1), the main hypothesis and key research aims (Chapter II.2), a global overview of the research design and methodology (Chapter II.3), the main results (Chapter II.4) and a summary of the most relevant findings (Chapter II. 5). At the end of this Part, a detailed discussion of the results is presented (Chapter II.6).

Part III gathers the data from animal investigation and has a similar organization to that described for Part II.

Part IV includes an integrated conclusion arising from the main results and presents an outlook into possible future directions in this field of research.

PART I

INTRODUCTION

Chapter I.1

Alzheimer´s disease

"Nothing is ever really lost to us as long as we remember it."

L.M. Montgomery

Aloysius "Alois" Alzheimer described in 1906 the first documented (young) case of what would be assumed 100 years later as the most common cause of dementia named after him as Alzheimer's disease. Highlighting the histopathological hallmarks of the disease, still essential for a definitive diagnosis, he recognized that he had before him a "*peculiar disease process*" (Alzheimer, 1907). Later on, this pathology was also associated with the most prevalent senile dementia and Alzheimer's disease became one of the leading causes of death as well as a major cause of morbidity and economical burden worldwide (Alzheimer's Association, 2017). Surprisingly, after more than a century we are still not able to point direct causes though we recognize several risk factors, or point specific triggers though we can describe cascades of events that culminate in the typical pathologic findings of this disease. The following chapter will summarize the current knowledge on this neurodegenerative disease.

I.1.1 Epidemiology

Alzheimer's disease is the most prevalent cause of dementia accounting up to 75% of all cases (Lobo *et al*, 2000). Alzheimer's Disease International (ADI) estimated that 46.8 million people worldwide were living with dementia in 2015 and that this number could reach 131.5 million in 2050, driven by an increase in life expectancy. Indeed, between 2015 and 2050, the number of people aged 60 years or over living in high income countries is expected to increase by 56%, compared with 138-185% in middle income countries, and by 239% in low income countries (Prince *et al*, 2015). Each year over 9.9 million new cases of dementia are diagnosed although recent studies suggest a stabilization or reduction of its incidence in some developed countries probably due to improvements in education, lifestyle and healthcare (Livingston *et al*, 2017; Wu *et al*, 2017). According to the World Health Organization (WHO) deaths due to dementia more than doubled between 2000 and 2015, making it the 7th leading cause of death worldwide in 2015 (2.7%), reaching 3rd leading death cause in high-income countries (7%) (WHO, 2017).

In Portugal, epidemiological data is scarce and comprehensive Portuguese population-based studies on dementia are lacking. Nevertheless, Santana *et al* (2015) estimated the number of Portuguese people with dementia among those aged 60 years or above as being

160 287 in 2013, representing 5.9% of this population-stratum and a 1.3% increase in prevalence comparing to the one proposed in 1991 by Garcia *et al* (1994). Alzheimer Europe (AE) had estimated in 2012 that the number of people with dementia in Portugal was 182 526 (66% women) representing 1.71% of the total population, which was somewhat higher than the 2013 European Union (EU-28) average of 1.55%. According to the most recent data from Portuguese National Institute of Statistics (INE, 2018), in 2016 a total of 3 480 deaths (63% women) were caused by dementia (all causes), representing 3.1% of global Portuguese mortality (2.3% men, 4.0% women) with a 33.7 deaths per 100 000 people gross mortality rate and 122.7 per 100 000 standardized mortality rate in people aged 65 or above. It also reports that a total of 1 692 deaths (64% women) were caused by Alzheimer´s disease in the same year, representing 1.5% of global Portuguese mortality (1.1% men, 2.0% women) with a 16.4 deaths per 100 000 people gross mortality rate and 60.3 per 100 000 standardized mortality rate in people aged 65 or above.

I.1.2 Pathophysiology

Alzheimer's disease is a progressive neurodegenerative brain disease characterized by typical neuropathological and neurochemical

markers. These include amyloid (senile) plaques and neurofibrillary tangles (NFT) (Figure 1a) as well as selective neuronal death and synaptic loss leading to a decrease in specific neurotransmitters. In typical cases, amyloid plaques are first detected in the frontal and temporal lobes, hippocampus and other limbic system structures, while NFT deposition, apparently afterwards, begin in the medial temporal lobe and hippocampus and then slowly progress to other areas of the neocortex (Figure 1b) (Hyman *et al*, 2012; Masters *et al*, 2015).

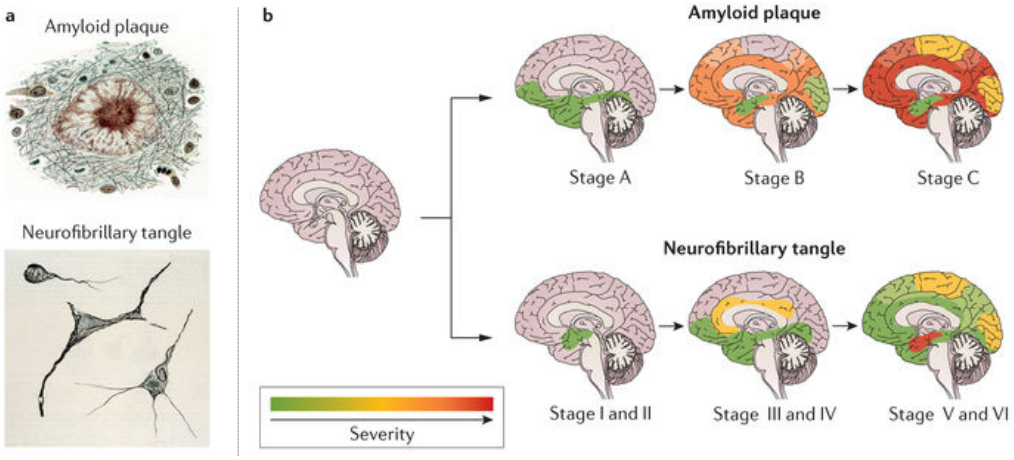


Figure 1. Pathological hallmarks and spatiotemporal evolution of Alzheimer's disease. (a) extracellular amyloid plaques and intracellular neurofibrillary tangle illustration using the Bielschowsky method of silver impregnation. (b) Amyloid plaque deposition stages (A, B and C) and neurofibrillary tangle deposition stages (I–VI), adapted from Braak and Braak classification (Masters *et al*, 2015).

Amyloid plaques are mainly constituted by insoluble forms of amyloid beta (Aβ) which originate from the proteolytic cleavage of a transmembrane glycoprotein named amyloid precursor protein (APP). Human APP is a large type I transmembrane spanning protein consisting of a large N-terminal extracellular domain, a hydrophobic transmembrane domain, and a short intracellular C-terminal domain. It is highly expressed in the brain and metabolized by a series of sequential proteases including the γ-secretase complex (O'Brien and Wong, 2011) which consists of several subunits that include Presenilin 1 (*PSEN1*, chromossome 14) and Presenilin 2 (*PSEN2*, chromossome 1). Brain APP is mainly produced by neurons and has been implicated in the regulation of synaptic formation and repair, anterograde neuronal transport and iron export, although its exact role is poorly understood (Chen *et al*, 2017).

There is strong evidence for Aβ as cause of Alzheimer's disease since familial cases, which account for less than 5% of all cases, are associated with mutations in genes encoding APP (*APP*, chromossome 21) or involved in its processing, as is the case of PSEN1 and PSEN2. APP can be processed by two main alternative proteolytic pathways: non-amyloidogenic and amyloidogenic pathways (Figure 2).

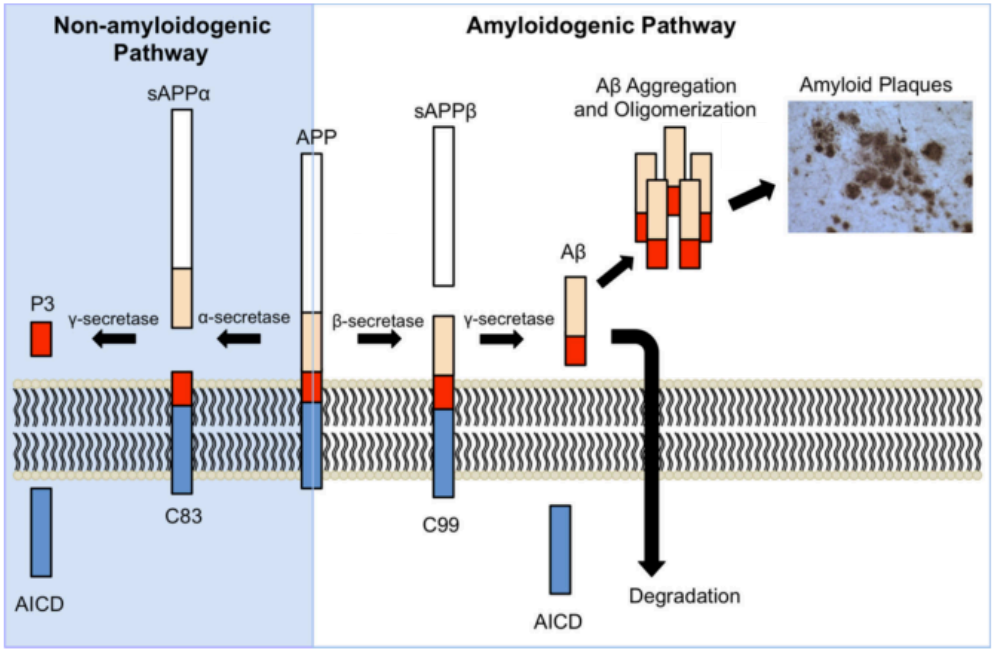


Figure 2. Non-amyloidogenic (blue) and amyloidogenic (white) amyloid precursor protein (APP) processing pathways. In the non-amyloidogenic pathway, *a*-secretase cleaves APP resulting in the generation of a soluble APP-fragment (sAPPα*);* the APP C-terminal fragment 83 (C83) is then cleaved by γ-secretase to release the APP intracellular domain (AICD) and P3 fragment. In the amyloidogenic pathway, β-secretase cleaves APP to produce the soluble fragment sAPP-β and the resultant C-terminal fragment 99 (C99) is then cleaved by γ-secretase to produce amyloid beta fragments (Aβ) and AICD (Adapted from McGuire and Ishii, 2016).

Although in familial Alzheimer's disease genetic alterations explain the increased production of Aβ, in the most common form of the disease (late-onset or sporadic Alzheimer's disease) Aβ dysregulation is far more controversial. Some acknowledged hypothesis are an increase activity of β-secretase and/or impairment of Aβ clearance in the central nervous system (CNS) (Figure 3), the last being pointed up as the preponderant mechanism (Mawuenyega *et al,* 2010).

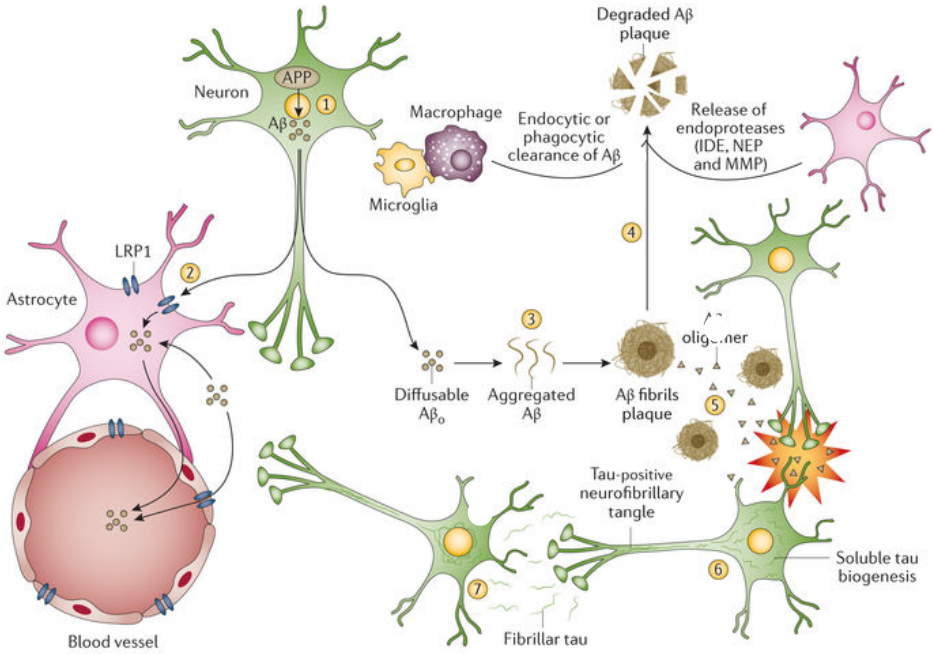


Figure 3. The amyloid-β (Aβ) theory of Alzheimer's disease. Aβ is cleaved from amyloid precursor protein (APP) and released as diffusible oligomers (Aβo) which can be cleared by different mechanisms or otherwise aggregate and assemble into plaques. These can be cleared or remain in the intercellular space and be toxic to adjacent synapses and promote tau phosphorylation. (Masters *et al*, 2015).
IDE: insulin-degrading enzyme; LRP1: low-density lipoprotein receptor-related protein 1; MMP: matrix metalloproteinase; NEP: neprilysin.

Aβ diffusible oligomers released in the extracellular space can be cleared by several pathways that involve transport across the blood-brain barrier (BBB) or in the cerebrospinal fluid (CSF), Apolipoprotein E (*APOE*) mediated transport (explained in more detail in section I.1.5.1) or they can be taken up by astrocytes via low-density lipoprotein receptor-related protein 1 (LRP1). On the other hand, these oligomers can otherwise aggregate in the intercellular space to form fibrillary

constructs, which in turn assemble into plaques. Aβ plaques can be degraded by endocytosis or phagocytosis (by macrophages and microglia), and by astrocytic endoproteases such as insulin-degrading enzyme (IDE), neprilysin (NEP) and matrix metalloproteinases (MMP). The failure of any of these mechanisms can aggravate Aβ burden and contribute to the development of the disease by promoting synaptotoxicity, proteasome dysfunction, inhibition of mitochondrial activity, dysregulation of intracellular Ca^{2+} levels and stimulation of inflammatory processes (Masters *et al*, 2015) (Figure 3).

Moreover, the impairment of perivascular glymphatic drainage of Aβ results in its accumulation not only in the brain parenchyma but also in the walls of cerebral arteries and capillaries (predominantly the more soluble form of Aβ - $Aβ_{40}$). This condition is known as cerebral amyloid angiopathy (CAA) which is also an important cause of lobar intracerebral hemorrhage in the elderly. CAA is highly prevalent in patients with Alzheimer's disease (90-96%) and may be revealed as multiple micro-bleedings in magnetic ressonance imaging (MRI). It contributes to Alzheimer's disease pathology once it further aggravates the failure in the perivascular drainage of Aβ and other metabolites from the brain, compromising neuronal homeostasis (Weller *et al*, 2009).

Additionally, Aβ interacts with the signaling pathways that regulate the phosphorylation of the microtubule-associated protein tau

(MAPT) and may inhibit its degradation by the proteasome. Hyperphosphorylation of tau disrupts its normal function in regulating axonal transport and leads to the intracellular accumulation of NFT and toxic species of soluble tau, which can be released and taken up by healthy neurons triggering tau damage in the uptaking cell (Bloom, 2014; Masters *et al*, 2015). Even though the Amyloid Cascade hypothesis, first described in 1992, still plays a prominent role in explaining the etiology and pathogenesis of this disease (Hardy and Higgins, 1992) it presents some limitations. For instance, mutations in the gene encoding for tau (*MAPT*, chromosome 17) cause frontotemporal dementia (FTD), which is histologically distinct from Alzheimer's disease since it does not present amyloid plaques (Small and Duff, 2008), suggesting that tau dysfunction can cause neurodegeneration independently of the presence of Aβ. In this context, and though pathological alterations of tau were classically described as downstream events of Aβ deposition on the Amyloid Cascade, it is plausible that tau and Aβ act in parallel enhancing each others toxic effects (Scheltens *et al*, 2017). By different but synergistic mechanisms, Aβ and tau dysfunction and accumulation in the brain trigger neurodegenerative processes characterized by early damage to the synapses, progressive impairment in neuronal function and loss of neurons or their processes (axons and dendrites). Both neuronal and synapse loss in the limbic system, neocortex, and basal

forebrain contribute to the typical cortical atrophy pattern seen in the Alzheimer's disease brain. Interestingly, synaptic dysfunction is one of the strongest correlates of cognitive decline in this disease, and although synaptic loss strongly correlates with neuronal loss (in space and time), the former seems to precede the later (Serrano-Pozo *et al*, 2011; Aisen *et al*, 2017).

After some decades of research, however, the assumption that Alzheimer's disease has a linear causal source has been turned away and it is increasingly recognized that its pathophysiology is highly complex and permeable to multiple local and systemic factors. A great amount of evidence have questioned if Aβ alone is sufficient to cause dementia in Alzheimer's disease once Aβ is normally present in the brain and seems to have a physiological role, but also because individuals with fully penetrant causative mutations take several decades to develop symptoms. Besides, the progression rate in sporadic Alzheimer's disease varies considerably between patients and some individuals, despite having abundant Alzheimer's disease pathology, are resilient to its neurotoxic effects and remain asymptomatic (Struble *et al*, 2010).

I.1.3 Diagnosis

The diagnosis of all-cause dementia is based on classical clinical criteria which comprise a set of, at least, two cognitive domains including memory with impact on functionality. These cognitive symptoms include impaired ability to acquire and remember new information, impaired reasoning and handling of complex tasks as well as poor judgment, impaired visuospatial abilities and language in such degree that it must interfere with the ability to function in profession or usual daily activities. Moreover, the impairment must not be explained by acute or psychiatric disease and should represent a decline from previous level of cognition. Neuropsychiatric symptoms such as changes in personality and behavior are also frequent and considered in classical ICD10 and DSM-IV criteria (WHO, 1992; American Psychiatric Association, 1994). The terminology of these major classifications is now changing and the new DSM-V proposes the banishment of the designation of *dementia* and its substitution by *major cognitive deficit* (American Psychiatric Association, 2013).

Considering the criteria described above, an accurate neuro-psychological evaluation is required to identify the existence of cognitive impairment and draw profiles based on the impact of the disease on distinct neural networks involved in different cognitive domains. This allows the distinction between neurodegenerative and non-neuro-

degenerative disorders as well as between different forms of dementia, being most informative in the early stages of the disease. Alzheimer's disease patients present typically with episodic memory impairment, due in large part to ineffective consolidation or storage of new information, semantic memory deterioration which is reflected in object naming, verbal fluency and semantic categorization deficits, visuospatial impairment, and also variable although typically less pronounced deficits in executive functions such as attention and problem-solving (Weintraub *et al*, 2012). Although the most common presentation of Alzheimer's disease-like dementia includes amnesic impairment, it may also have non-amnesic presentations including language, visuospatial or executive predominant dysfunction (variants).

The diagnosis of Alzheimer's disease has been largely refined since the first publication of the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA) diagnostic criteria in 1984 (McKhann *et al*, 1984), which merely relied on clinical symptoms. Over the years, converging evidence from neuropathological studies suggested that the pathophysiological process of the disease begins decades before the emergence of clinical symptoms, meaning that Alzheimer's disease has a rather extensive and variable preclinical stage (Sperling *et al*, 2011 and 2013). This finding boosted research to find suitable biomarkers in

order to improve diagnostic accuracy and identify candidates for disease-modifying therapies, when these become available.

Meanwhile, with the advances on imaging and CSF biomarkers, the International Working Group (IWG) as well as the National Institute of Aging and Alzheimer's Association (NIA-AA) have proposed new criteria (McKhann *et al*, 2011; Albert *et al*, 2011; Sperling *et al*, 2011; Dubois *et al*, 2014). Overall, they recognized Alzheimer's disease as a continuum from pathophysiological, biomarker, and clinical perspectives, rather than standardized stages (Figure 4).

Among the main refinements stands the acceptance of a "Preclinical Alzheimer's disease" (according to NIA–AA) or "pre-symptomatic at risk for Alzheimer's disease" (according to IWG) in cognitively healthy individuals with evidence of Alzheimer's disease pathological changes, and the assignment of different degrees of likelihood of diagnosis to clinical syndromes of cognitive impairment based on biomarker evidence.

Between the preclinical and dementia stages of Alzheimer's disease stands an intermediate stage of cognitive impairment that is often, but not always, a transitional phase designated by Mild Cognitive Impairment (MCI). This concept was introduced in the late 1980s by Reisberg (Reisberg *et al,* 1988) and further developed by Petersen (Petersen *et al,* 1999) to designate individuals with lower cognitive

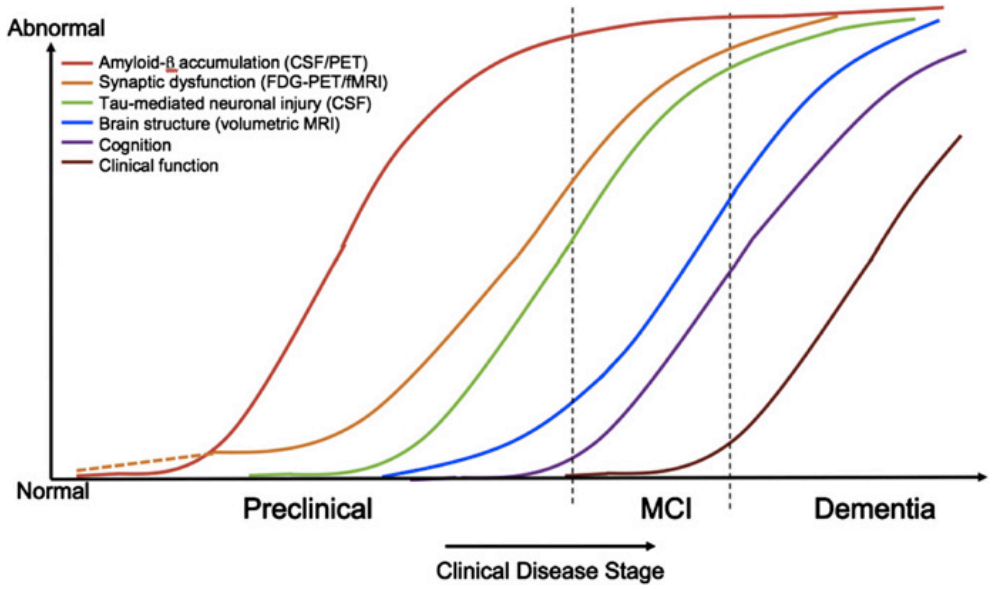


Figure 4. The Alzheimer's disease continuum - hypothetical model of the clinical and biomarker trajectory of the disease. Aβ accumulation as an "upstream" event in the neuropathological cascade (identified by CSF $A\beta_{42}$ assay or PET amyloid imaging) that is associated with "downstream" synaptic dysfunction (evidenced by FDG-PET or fMRI; dashed line indicates that synaptic dysfunction may be detectable in carriers of the *ApoEε4* allele before detectable Aβ deposition), neuronal injury (evidenced by CSF tau), and neuronal loss with consequent atrophy (evidenced by volumetric MRI), all beginning in the preclinical stage of the disease (Sperling *et al*, 2011).

Aβ: amyloid beta; CSF: cerebrospinal fluid; FDG: ^{18}fluorodeoxyglucose; fMRI: functional magnetic resonance imaging; MCI: mild cognitive impairment; MRI: magnetic resonance imaging; PET: positron emission tomography.

performance than expected for age and education (typically 1 to 1.5 standard deviations below the mean) that elicits complaints but does not cause significant impairment in social or occupational functioning. This entity is characterized by a cognitive spectrum that may include cognitive impairment in memory and non-memory domains. Both type

and number of affected domains have important implications for understanding the underlying etiology and pathology as well as the likelihood of progression to dementia and which type. In accordance, if memory is affected, MCI is classified as amnestic (aMCI), whereas the absence of memory impairment with presence of executive function/ attention, language, and/or visuospatial skills is classified as non-amnestic (naMCI). In addition, MCI may consist of impairment in a single cognitive domain or multiple cognitive domains, the later denoting, according with some authors, a greater extent of disease. Globally, individuals with MCI progress to dementia at a rate of approximately 12–16% per year. Single or multiple domain aMCI patients progress in most cases to Alzheimer's dementia, while single domain and multiple domain naMCI patients more frequently progress to FTD and Dementia with Lewy Bodies (DLB), respectively. In theory, any MCI subtype could precede vascular dementia (VaD) (Roberts and Knopman, 2013). However, long-term studies show that a significant proportion of MCI patients do not progress to dementia and a subset may even revert to normal cognition (Visser *et al*, 2006; Malek-Ahmadi, 2016). In fact, cognitive impairment on MCI patients may have several causes such as degenerative, vascular, neoplastic, depressive, traumatic, inflammatory and metabolic disorders, or have instead a multifactorial etiology. Most of these can be diagnosed or ruled out by performing a

detailed medical history alongside with neuropsychological assessment, neuroimaging and laboratory assessments. Nevertheless, and similarly to what was above described for Alzheimer's dementia, it became especially important to improve the identification of MCI individuals with Alzheimer's disease pathophysiology as they represent the majority of cases. This concept was also introduced by the most recent NIA–AA criteria for "MCI due to Alzheimer's disease" which incorporates biomarker data as an (low, intermediate and high) evidence of pathology (Albert *et al*, 2011).

I.1.4 Biomarkers

Biomarkers for chronic neurodegenerative diseases such as Alzheimer's disease are of particular importance since cognitive symptoms frequently overlap in different disorders and the brain is not a tissue easily sampled. Recent advances in CSF assays and neuroimaging techniques have provided the ability to detect Alzheimer's disease pathophysiology process *in vivo*. The most important and commonly used biomarkers in clinical trials and in clinical practice include those reflecting brain Aβ accumulation (CSF $A\beta_{42}$ and amyloid positron emission tomography: PET), neuronal injury (CSF t-tau and p-tau), Alzheimer's disease-related synaptic dysfunction (^{18}fluorodeoxyglucose-

PET: FDG-PET and functional MRI: fMRI) and also biomarkers of neurodegeneration and brain atrophy (volumetric MRI) (Figure 4). Apart from its usefulness in the diagnostic process, once they reflect Alzheimer's disease pathophysiology, they are also able to predict progression, monitor effects of future disease-modifying therapies and allow a more deep understanding of the pathogenesis of the disease (Zetterberg and Schott, 2016; Blennow, 2017). The IWG for New Research Criteria for the Diagnosis of Alzheimer's disease has further suggested the distribution of these biomarkers in two groups: diagnostic markers and progression markers. In the first group are included those reflecting *in vivo* pathology (targeting Aβ and tau), present at all stages of the disease including in the asymptomatic state and thus suitable for inclusion in protocols of clinical trials, though may not correlate with clinical severity. Progression markers (structural or metabolic) have poor disease specificity and may not be present in the early stages of disease, but are good indicators of clinical severity (staging markers) (Dubois *et al*, 2014).

I.1.4.1 Core cerebrospinal fluid biomarkers

Cerebrospinal fluid freely communicates with the brain's interstitial fluid and thus reflects biochemical changes related to brain

pathology. Its collection by lumbar puncture is routinely performed in clinical practice, and although invasive, it is a procedure quite devoided of complications and does not require advanced equipment. During the past two and a half decades, three core CSF biomarkers for Alzheimer's disease have been identified in hundreds of studies. $A\beta_{42}$, which is the most amyloidogenic Aβ isoform and found at low concentrations in patients with Alzheimer's disease due to its deposition in the brain tissue; total tau (t-tau), at high concentrations due to neuronal loss; and phosphorylated tau (p-tau, tau with phosphorylation at residues 181 or 231) at high concentrations, reflecting NFT formation (Olsson *et al*, 2016) (Figure 5). CSF levels of $A\beta_{42}$ have been consistently correlated with post-mortem plaque burden and with amyloid ligand retention on Aβ PET imaging. On the other hand, CSF levels of t-tau increase not only in Alzheimer's disease but also in other neurodegenerative disorders suggesting that t-tau is an unspecific marker of neuronal and axonal degeneration. CSF levels of p-tau are, otherwise, more specific and correlate with post-mortem measures of NFT pathology, rate of hippocampal atrophy and clinical progression. The combination of low CSF $A\beta_{42}$ levels and high t-tau and p-tau levels is often referred as "Alzheimer's disease CSF profile" and helps to differentiate Alzheimer's disease from other dementia subtypes or non-degenerative disorders. This combination is 85–90% sensitive and specific for Alzheimer's

disease, providing better diagnostic performance than any of the CSF biomarkers alone (Masters *et al*, 2015), explaining the use of ratios such t-tau/$A\beta_{42}$ and $A\beta_{42}$/p-tau or the amyloid-tau index (ATI=$A\beta_{42}$/ [240+1.18tau]). In our opinion, the second constitutes probably the most relevant ratio because it includes the two most specific markers for the disease, while t-tau may be elevated in several other conditions (Figure 5).

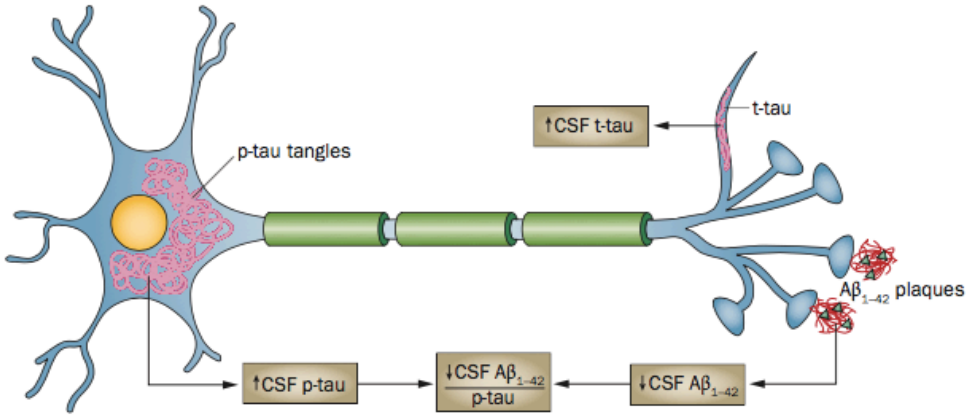


Figure 5. Cerebrospinal fluid biomarkers for Alzheimer's disease. Low levels of Aβ and high levels of t-tau and p-tau reflect the underlying pathology, which is characterized by extracellular Aβ plaques and accumulation of intracellular t-tau and p-tau tangles (De Deyn, 2015).

Aβ: amyloid beta; CSF: cerebrospinal fluid; p-tau: phosphorylated tau; t-tau: total tau.

Other CSF biomarkers have been proposed and a vast list of candidates include synaptic proteins, inflammatory proteins, endosomal-autophagy-lysosomal system proteins, oxidative stress markers, axonal

neuron-specific proteins (e.g. neurofilament light protein - NFL) and dendritic proteins (e.g. neurogranin - Ng), among others (Counts *et al*, 2016; Blennow, 2017). However, and although some of these recent CSF markers have already proven their usefulness, none is currently approved to be used routinely.

I.1.4.2 Core imaging biomarkers

Imaging has a key role in the clinical assessment of patients with suspected Alzheimer's disease. Either computed tomography (CT) or MRI are mandatory in the initial diagnostic work-up to rule out non-degenerative causes of cognitive impairment, although the clinical utility of these techniques goes far beyond differential diagnosis.

MRI-based evidence of brain atrophy, especially in the medial temporal lobe (MTL), is regarded as a valuable diagnostic and prognostic information. The MTL plays a central role in memory circuits and encompasses hippocampal formation (Cornus Ammonis - CA areas 1 to 4 and the dentate gyrus) and the parahippocampal gyrus (PH) that can be divided into anterior and posterior portions. The anterior portion of PH, sometimes referred as the rhinal cortex, consists on entorhinal (medial) and perirhinal (lateral) cortices, and the posterior

portion of PH consists on the parahippocampal cortex (Figure 6) (Raslau *et al*, 2015).

Volumetric measurements of MTL structures by a visual scale rating is commonly used in the clinical practice. However, MRI volumetry is much more accurate and currently manual hippocampal segmentation is considered the gold standard. Because this requires a high level of training and is time and resource consuming, automated methods using specialized softwares are frequently preferred (Sheikh-Bahaeia *et al*, 2017). Atrophy of hippocampal and entorhinal cortices, which result from synaptic and neuronal loss, are strongly correlated with the severity of cognitive impairment along the continuum of Alzheimer's disease.

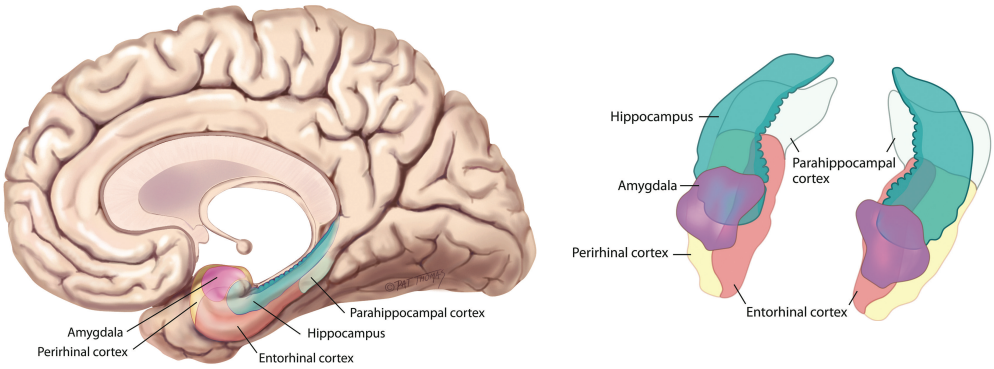


Figure 6. Medial temporal lobe (MTL) major components. MTL includes the hippocampus (CA1 to 4 and dentate gyrus) and parahippocampal gyrus (entorhinal, perirhinal and parahippocampal cortices) (Adapted from Raslau *et al*, 2015).

In addition, the degree of MTL atrophy correlates with Braak staging (Figure 1) at autopsy (Jack *et al*, 2010). The entorhinal cortex rather than the hippocampus appears to be the earliest site of Alzheimer's disease pathology and volume loss, thus atrophy of both and also the decrease in total parahippocampal volume seem to be good diagnostic predictors and reliable markers of disease progression (Echávarri *et al,* 2011). However, when the results of clinical examination and structural imaging are inconclusive, functional imaging can be used as a more advanced option. PET with metabolic ligands such as FDG or PET imaging for *in vivo* detection of Aβ or tau aggregates have provided significant sensitivity and specificity improvements for Alzheimer's disease diagnosis, particularly at the early stages of the disease. FDG-PET is used to measure brain glucose metabolism, and in the context of Alzheimer's disease, decreased glucose uptake is an indicator of impaired synaptic function. It is also highly correlated with post-mortem measures of synaptophysin, a synaptic structural protein, though it also measures glucose uptake by neurons and glia. Ultimately, FDG-PET studies in Alzheimer's disease patients frequently show a specific topographic pattern of glucose hypometabolism in temporoparietal association areas including the precuneus and posterior cingulate cortex (classic default mode network pattern), whereas some patients with focal deficits (language, praxis, visuospatial) may present with more localized

neocortical hypometabolic areas (Jack *et al*, 2010; Dubois *et al*, 2014; Scheltens *et al*, 2016). More recently, Aβ selective radio tracers, such as Pittsburgh compound B (PiB) and Florbetapir (^{18}F), have substantially contributed to the acknowledgement that Aβ deposition begins several decades before structural alterations and cognitive decline. Amyloid-PET imaging correlates with CSF $A\beta_{42}$ levels and, more importantly, has enabled the inclusion of patients in the preclinical phase of the disease in clinical trials. PET ligands for imaging fibrillary tau aggregates have also been developed and are already being applied in clinical trials. Interestingly, it shows a better correlation with hypometabolism and atrophy than does amyloid-PET, but its usefulness for diagnosis purposes remains unclear (Masters *et al*, 2015; Scheltens *et al*, 2016).

I.1.4.3 Blood-based biomarkers

The costs and technical specificities of CSF and imaging biomarkers make them difficult to be incorporated in the routine clinical set of most neurological centers. Therefore, measurement of biomarkers in peripheral blood through a minimally invasive and inexpensive procedure would present a significant breakthrough. Although several blood-based biomarkers have been suggested, their inclusion in biomarker panels has been precluded due to several drawbacks. One of

the biggest challenges is the fact that brain-specific proteins that reflect Alzheimer's disease pathology are at much lower concentrations in the blood than in the CSF and in some, plasma and CSF levels are poorly correlated (e.g. tau). On the other hand, many of these protein serum/plasmatic levels overlap between Alzheimer's disease and controls or other neurodegenerative diseases. Some strategies to overcome these difficulties have been suggested by studies using neurally derived blood exosomes or combined analysis of proteins (biomarker panels) (Counts *et al*, 2016). Unfortunately, the field of blood-based biomarkers is still far behind that of CSF, and their high variability and low reproducibility across centers have precluded its implementation in clinical practice. In this regard, a set of guidelines were recently developed in order to standardize pre-analytical procedures, which will allow to more accurately assess and cross-validate potential candidates (O'Bryant *et al*, 2015).

I.1.5 Risk factors

I.1.5.1 Non-modifiable risk factors

Apolipoprotein E and other genetic risk factors

Apolipoprotein E (*APOE*, chromosome 19) polymorphic alleles are the main genetic determinants of Alzheimer's disease risk. Individuals carrying the ε4 allele are at increased risk of Alzheimer's disease compared with those carrying the more common ε3 allele, whereas the ε2 allele decreases the risk. These 3 isoforms are based on differences of two single aminoacids (112 and 158) that affect *APOE* structure and function (Liu *et al*, 2013). In caucasians, *APOE*ε4 homozygotes correspond to nearly 2% of the population and at the age of 85, their lifetime risk for Alzheimer's disease ranges from 51% (male) to 60% (female). On the other hand, in heterozygotes *APOE*ε4/ε3 the risk ranges from 23% (male) to 30% (female), consistent with semi-dominant inheritance of a moderately penetrant gene (Genin *et al*, 2011).

APOE is a protein component of triglyceride-rich lipoproteins with an important role in cholesterol metabolism in peripheral tissues and also in the CNS. Most *APOE* in the brain is produced and expressed

by astrocytes and is essential for cholesterol transport through *APOE*-containing high density lipoprotein (HDL)-like particles, which play an important role in synaptic development and maintenance. *APOE*ε4 confers a gain of toxic function and/or a loss of protective strategies in the brain that predispose to the development and progression of Alzheimer's disease by several mechanisms: (1) impairment of Aβ turnover, once it is the less efficient isoform in Aβ clearance; (2) weakening of the ability of reelin and *APOE* receptor signaling to protect against Aβ-induced synaptotoxicity; (3) increased tau hyperphosphorylation, cytoskeletal disruption and mitochondrial dysfunction by neurotoxic proteolitic fragments; (4) aberrant CNS cholesterol homeostasis which is essential for axonal and synaptic growth and remodeling; (5) inhibition of hippocampal neurogenesis by impairing maturation of hilar γ-aminobutyric acid (GABA)-containing interneurons; and (6) increased inflammatory response (glial activation) (Liu *et al*, 2013; Lane-Donovan and Herz, 2017). Finally, *APOE*ε4 genotype acts synergistically with diabetes *mellitus* (DM) to increase the risk of Alzheimer's disease and also with atherosclerosis, once it is also a risk factor for vascular disease (Irie *et al*, 2008; Gottesman *et al*, 2017). Although the presence of *APOE*ε4 does not necessarily entail disease development (up to 75% of heterozygotes do not develop the disease),

this genetic isoform significantly increases the risk of Alzheimer's disease and may anticipate the age of onset (Loy *et al*, 2014).

Other less frequent and less relevant genetic loci have been recently identified by genome-wide association studies and include genes implicated in immune and inflammatory responses (eg. Triggering Receptor Expressed on Myeloid cells 2 - TREM2, Complement Receptor 1 - CR1), lipid metabolism (eg. ATP-binding cassette sub-family A member 7 - ABCA7, $Na^+/K^+/Ca^{2+}$-exchange protein 4 - SCL24A4), and endosomal-vesicle recycling (eg. Sortilin Related Receptor 1 - SORL1, Phosphatidylinositol binding clathrin assembly protein - PICALM) (Karch and Goate, 2015). Conversely, rare PSEN1 and PSEN2 mutations as well as APP mutations and duplications (chromosome 21 extra copy in Down Syndrome) are highly or fully penetrant (PSEN2: 95%; PSEN1 and APP: 100%) and cause early-onset Alzheimer's disease (<65 years), though only explain about 86% of these cases (Loy *et al*, 2014). Mutations in one of these genes result in increased APP amyloidogenic processing and thus in Aβ overproduction (Masters *et al*, 2015).

Age

Although dementia is not a normal feature of aging neither older age is by itself sufficient to cause dementia, it is unquestionable that

advancing age is the single most significant non-genetic risk factor for Alzheimer's disease.

The incidence of dementia increases exponentially with aging, doubling for every 6.3 year increase in age (Alzheimer's Association, 2017). Aging is associated not only with global accumulation of damage but also with the failure of nutrient-sensing pathways (insulin/insulin-like growth factor/Target of Rapamycin - IIS/TOR; adenosine mono-phosphate-activated protein kinase - AMPK), impaired mitochondrial activity with consequent metabolic and energetic imbalance, and impaired DNA damage response, telomere activity and autophagic processes. However, the relevance of each mechanism to the age-associated enhanced risk of Alzheimer's disease is not clear (Niccoli and Partridge, 2012).

Gender

It is already recognized the existence of a disproportional higher risk for dementia in women (mainly in the oldest-age categories), that is not fully explained by their increased life expectancy (Fratiglioni *et al*, 1997; Andersen *et al*, 1999; Chêne *et al*, 2014). Furthermore, women with MCI have higher rates of progression (Lin *et al*, 2015), and those with Alzheimer's disease have more severe neuropathological alterations

(Barnes *et al*, 2005), experience faster progression of hippocampal atrophy (Ardekani *et al*, 2016) and faster cognitive and functional decline after the diagnosis (Sinforiani *et al*, 2010; Tschanz *et al*, 2011). Several factors seem to contribute to this gender bias: (1) lower educational level; (2) differences in brain development, adult brain structure, function, and biochemistry (Tunç *et al*, 2016; Köglsberger *et al*, 2016; Djordjevic *et al*, 2017); (3) levels of sex steroid hormones such as progesterone, estradiol, and testosterone, and also gonadotropins such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Barron *et al*, 2006; Koyama *et al*, 2016; Pike, 2017; Lee *et al*, 2017); and (4) higher susceptibility to inflammation (either systemic or microglial) (Podcasy and Epperson, 2016; Mangold *et al*, 2017). In addition, genetic and environmental risk factors seem to have different penetrance according to gender (Pike, 2017). Based on this evidence, some studies and clinical trials already consider a deliberate stratification by gender and analyze results and outcomes in men and women separately.

Family history

Most people with a family history of Alzheimer's disease does not have a mendelian form of dementia. In fact, the probability of finding a pathogenic mutation in one of the recognized causative genes in a patient with two or more affected first-degree relatives with late

symptom onset (>65 years) is inferior to 1% (Loy *et al*, 2014). However, individuals who have a first-degree relative with Alzheimer's disease are more likely to develop the disease and those who have two or more first-degree relatives with the disease are at even higher risk. According to the results of MIRAGE study, the lifetime risk of Alzheimer's disease in first-degree relatives is approximately 39% by the age of 96 and individuals with both parents affected have a cumulative risk of 54% by the age of 80 (5 times greater than the general risk) (Lautenschlager *et al*, 1996). This is most probably the reflection of familial clustering of genetic factors such as *APOE*ε4 or other unknown or rarely studied susceptibility genes that may, in turn, interact with shared environmental and lifestyle factors to increase the risk of developing the disease (Donix *et al*, 2012; Alzheimer's Association, 2017).

I.1.5.2 Modifiable risk factors

Facing the discouraging results in the search for an effective treatment for Alzheimer's disease, preventive strategies are being explored and a growing interest in modifiable risk factors have arisen. Even a future disease-modifying treatment for Alzheimer's disease will not remove the need for its effective prevention.

Several clinical and experimental studies have supported the hypothesis that neurodegenerative disorders often coexist with metabolic dysfunction, which can exacerbate or even trigger central harmful signaling pathways (Cai *et al*, 2012). It is estimated that at least 35% of dementia risk factors are potentially modifiable and include obesity, hypertension, DM and physical inactivity, among others (Livingston *et al*, 2017). The interplay between obesity, DM and Alzheimer's disease is a good example of the complex interface between brain and adipose tissue and will be discussed in more detail in the next sections.

Chapter I.2

Obesity and adipose tissue dysfunction

Obesity is currently recognized as a multifaceted disease determined by a highly complex interaction between genetic, environmental and neuropsychological factors that leads to an imbalance between caloric intake and energy expenditure. It is defined by an excessive accumulation of fat that, in most cases, is associated with pathophysiological adipose tissue remodeling that induces systemic metabolic stress and consequent adverse health effects (González-Muniesa *et al*, 2017).

In the last decade, a surprising amount of evidence have unveiled adipose tissue's metabolism and its influence on other tissues and organs across lifespan, in physiological or pathophysiological conditions, that will be discussed along this chapter.

I.2.1 Epidemiology and classification of obesity

Obesity is one of the greatest public health challenges of the 21st century, being associated with an increased risk of disease and death alongside with major socioeconomic impact. According to the WHO, its prevalence has tripled in many European countries since the 1980s, and the numbers of those affected continue to rise at an alarming rate. In

2014, more than 1.9 billion adults (>18 years) were overweight and over 600 million of these were obese. In children and adolescents the prevalence of obesity is also increasing and almost doubled since 1980.

WHO obesity classification using body mass index (BMI), defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2), is described in Table 1.

Table 1. Obesity-related anthropometric measures cut-off points and risk of metabolic complications*

BMI (kg/m^2)		**WC (cm)**		**WHR (cm)**	
Underweight	< 18.5	**Increased risk of metabolic complications**	>94 (M) >80 (W)	**Substantially increased risk of metabolic complications**	≥0.90 (M) ≥0.85 (W)
Normal weight	18.5 - 24.9	**Substantially increased risk of metabolic complications**	>102 (M) >88 (W)		
Overweight	25 - 29.9				
Obesity	30 - 34.9 (Class I) 35 - 39.9 (Class II)				
Extreme obesity	> 40 (Class III)				

* according to World Health Organization (WHO).
BMI: Body mass index; M: men (European); W: women (European); WC: Waist circumference; WHR: Waist-to-hip ratio.

Though BMI constitutes a strong and the most widely used indicator of adiposity, its correlation with the total amount of body fat tissue decreases with age (Luchsinger, 2008) and does not provide

information on body composition neither distinguishes between visceral and subcutaneous deposits (Cereda *et al*, 2007). To refine risk assessment, other surrogate indicators, such as waist circumference (WC) and waist-to-hip ratio (WHR) whose cut-off values are listed in Table 1, can be useful and are more accurate in predicting adverse metabolic outcomes (Luchsinger, 2008; Scicali *et al*, 2018).

However, more precise methods have been designed to assess visceral and total body fat, though not easily available for routine clinical use. These include indirect measurement methods such as bioelectric impedance analysis or dual-energy X-ray absorptiometry (DEXA), and direct measurement methods such as CT and MRI. Though only used for this purpose in research settings, these two imaging techniques are considered the most accurate methods for measuring tissue, organ, and whole-body fat mass and allow for measurement of specific fat compartments (abdominal and subcutaneous) (Fosbøl and Zerahn, 2015).

Unfortunately, no standardized plasmatic biomarker that accurately correlate with adipose tissue dysfunction and its metabolic outcomes is currently available.

I.2.2 Adipose tissue structure and distribution

Adipose tissue is composed by cells with lipid storage functions - adipocytes, and a stromovascular fraction composed by endothelial and mesenchymal stem cells, preadipocytes, fibroblasts and resident cells from the immune system (Rajala and Scherer, 2003). During embryonic development, the vascular network develops before adipocytes, and the extracellular matrix which supports blood vessels is the first to be deposited (Neels *et al*, 2004; Christiaens and Lijnen, 2010). In fact, during embryonic development, a close communication between the stromovascular fraction and the adipocytes results in a mutual control between angiogenesis and adipogenesis. Recent data show that adipocytes may develop from capillary networks as the progenitor cells respond to pro-angiogenic stimuli in association with the expanding capillaries (Min *et al*, 2016). In adult life, a well-developed vascular network is observable, very dynamic and continuously adapting to changing nutritional fluxes, and each adipocyte is surrounded by at least one capillary. The capillaries of the adipose tissue are fenestrated and rich in trans-endothelial channels, which allow a close communication with adipocytes (Christiaens and Lijnen, 2010).

There are two major types of adipose tissue: brown adipose tissue (BAT) and white adipose tissue (WAT), both involved in sensing and responding to changes in systemic energy balance. BAT is a key site of

heat production (thermogenesis) in mammals via the action of uncoupled protein 1 (UCP-1) located in the inner membrane of the mitochondria. Its name derives from its brown appearance due to high mitochondria content and dense vascularization. It is mainly located in supraclavicular/cervical and paravertebral/interescapular deposits in humans and rodents (Figure 7), being abundant in newborns but decrease with age. WAT is the most abundant type of adipose tissue and has a crucial role in energy-storing and thus is the most implicated in the pathogenesis of obesity and its complications. WAT has also the unique capacity to change its body distribution, size, secretoma and inflammatory status according to nutritional status. A third type of adipocytes known as "beige" or "brite" can develop within WAT and present thermogenic capacity in response to various stimuli ("browning"), though its precise role in physiological and patho-physiological conditions is currently under debate (Harms and Seale, 2013; Kwok *et al*, 2016; Kusminski *et al*, 2016; González-Muniesa *et al*, 2017).

Adipose tissue can also be classified according to its location, which is a determining factor to its metabolic identity and function. In both humans and rodents, adipose tissue is a multi-depot organ and there are anatomical differences that must be taken into consideration (Figure 7).

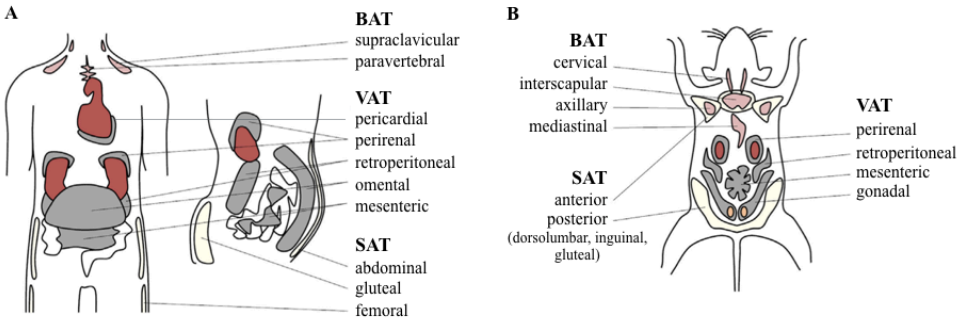


Figure 7. Brown and white adipose tissue distribution in human (A) and rodent (B). (Adapted from Choe *et al*, 2016).

BAT: brown adipose tissue; SAT: subcutaneous adipose tissue; VAT: visceral adipose tissue.

In humans, visceral adipose tissue (VAT) includes pericardial, retroperitoneal and perirenal, omental and mesenteric adipose tissue and its accumulation has been closely correlated with metabolic disease risk. Subcutaneous adipose tissue (SAT), in contrast, can play a protective role in energy homeostasis, as seen in mouse models of SAT expansion, the so-called "metabolically healthy obesity", which have shown to be resistant to the detrimental effects of high-fat diets (HFD) or mutations in obesity-associated genes. Human upper-body SAT (mainly abdominal) can otherwise be categorized as deep or superficial depending on its location in relation to the *fascia superficialis*, while the lower-body SAT includes the fat localized on the gluteofemoral region (Choe *et al*, 2016). Interestingly, fat distribution is influenced by aging, being redistributed throughout life from subcutaneous to intra-

abdominal deposits (Raguso *et al*, 2006), but also by gender. In gynoid obesity, the most common form in women, the excess of fat accumulates in the hip and thigh subcutaneous areas, contrarily to what is observed in android or visceral obesity, the most common form in men, that is characterized by excessive accumulation of fat in the abdominal region (González-Muniesa *et al*, 2017).

In rodents, WAT is also distributed by different locations and, like in humans, can be divided in VAT and SAT. VAT includes retroperitoneal and perirenal, mesenteric and gonadal/perigonadal depots (named epididymal in males and ovarian in females). Gonadal VAT is typically the largest and most accessible fat pad and, thus, the most frequently used in experimental research. Although mesenteric VAT is the most similar to human intra-abdominal adipose tissue (due to its location and biology, once it has access to the portal vein), it has been precluded due to limitations in surgical manipulation and separation from contaminating vessels. Similarly to humans, rodent SAT has two main depots but they are located in the anterior region, descending from the neck to the axillae, and in the posterior region, that spreads from the dorsolumbar to the gluteal region, comparable to human gluteofemoral SAT (Chusyd *et al*, 2016).

Regardless of the specie, a clear heterogeneity is present among these different anatomical WAT depots concerning its development origin, adipogenesis, vascularization and metabolic properties.

I.2.3 Adipose tissue physiology

In addition to its role in providing insulation and mechanical support, adipose tissue is a central and critical metabolic regulator of energy homeostasis by different synergetic mechanisms. WAT is primarily a specialized organ in the storage of extra energy in the form of lipids, mainly triglycerides, within intracellular large unilocular droplets (Rajala and Scherer, 2003). Triglycerides synthesis, a process called esterification, occurs from one molecule of glucose-derived glycerol and three fatty-acyl chains. Adipocytes have a limited ability to store glycogen and thus all the glucose that is not consumed in the adipocyte metabolism is transformed into glycerol and stored in the triglycerides pool (Tilg and Moschen, 2006; Goossens, 2008). Esterification is mainly stimulated by insulin, which induces tyrosine kinase activity in its receptor and, in turn, leads to the tyrosine phosphorylation and activation of the insulin receptor substrate-1 (IRS-1), initiating a signaling pathway which involves phosphoinositol 3-

kinase (PI3K) and protein kinase B (Akt) activation. Among other actions, the activation of this signaling cascade leads to the translocation of glucose transporter type 4 (GLUT4)-containing vesicles to the membrane, allowing glucose uptake (Wellen and Hotamisligil, 2005). Besides inducing glucose uptake, insulin is also responsible for lipolysis inhibition, through the inhibition of adenylate cyclase, the main enzyme involved in cyclic adenosine monophosphate (AMPc) synthesis. AMPc activates protein kinase A (PKA), which in turn phosphorylates and activates hormone-sensitive lipase (HSL), the main enzyme involved in triglycerides hydrolyzation. On the other hand, in times of nutrient demand, contra-regulatory hormones like glucagon, cortisol, growth hormone, and adrenaline, increase AMPc levels, leading to the activation of HSL (lipolysis) and thus the release of fatty acids into the circulation which are released and transported in the blood to the liver, muscle, and BAT to be used in fatty acid oxidation (Tilg and Moschen 2006; Goossens, 2008). Alternatively to esterification, fatty acids may be metabolized by several mediators in intracellular signaling pathways, namely eicosanoids, which are important activators of the peroxissome proliferation activated receptor-gamma (PPARγ). PPARγ is expressed in adipocytes and macrophages, controlling genes involved fatty acid uptake (fatty acid transport protein - FATP, lipoprotein lipase - LPL and CD36), metabolism (phosphoenolpyruvate carboxykinase - PEPCK and

stearoyl-CoA desaturase 1 - SCD-1), storage (perilipin A) and oxidation (adiponectin and UCP-1). It also stimulates preadipocyte differentiation into mature adipocytes and inhibits cellular inflammatory pathways (Lee JY *et al*, 2011). Moreover, adipose tissue regulates cholesterol metabolism, as adipocytes are involved in the biogenesis and lipidation of HDL, essential to send excessive cholesterol to the liver (McGillicuddy and Reilly, 2010; McGillicuddy *et al*, 2011).

Besides its historically well defined storage function, it is currently recognized that adipose tissue is a very complex and metabolically active endocrine organ since it is capable to communicate with other organs by secreting dozens of adipokines. The term *adipokines* (*adipo-kinos*: adipose tissue-movement) refers collectively to cell-signalling polypeptides such as cytokines, acute phase reactants, inflammatory mediators, hormones and other chemical messengers, that are synthetized and secreted by the adipose tissue and participate in a complex network of autocrine, paracrine and endocrine signaling pathways (Pardo *et al*, 2012). The most abundant and well studied adipokines, leptin and adiponectin, will be discussed in more detail.

I.2.3.1 Leptin

Leptin was the first hormone originally identified as an adipokine and its discovery in 1994 changed the traditional view of adipose tissue

(Zhang *et al*, 1994). Leptin (from the greek *leptos,* meaning "thin") is a 167 amino acid peptide, highly homologous amongst different species, that is encoded by the *ob* (obese) gene (chromosome 6 in mice; chromosome 7 in humans) and mainly produced by WAT, being the subcutaneous depot the most active (Nielsen *et al*, 2009), although also expressed by the placenta, ovaries, mammary epithelium, bone marrow, lymphoid tissues, skeletal muscle and gastric mucosa (Rajala and Scherer, 2003). Accordingly, circulating leptin levels are directly in proportion to the amount of body fat, thereby reflecting the amount of energy stored. Leptin presents a pulsatile pattern of secretion according to the time of the day and acute changes in calorie intake: highest levels at midnight and after high calorie meal ingestion. In addition, leptin levels are also regulated by many other factors including sex steroids (higher levels in women), glucocorticoids, catecholamines, cytokines and not surprisingly, by insulin (Kelesidis *et al*, 2010; Park and Ahima, 2015). Its biological activity strongly depends on the interaction with specific receptors (LepRs), encoded by the diabetes (*db*) gene (also known as *ObR* or *LepR*), first described in the choroid plexus by Tartaglia *et al* (1995). LepRs are class I cytosine receptors expressed in six isoforms (LepRa-f): the long isoform LepRb, which has been identified as the main signaling form, four short isoforms (LepRa, c, d, f) that regulate the internalisation and degradation of leptin and are involved in its receptor-mediated

transcytosis to the brain (LepRa), and finally LepRe, which represents the major binding site of circulating leptin (Gorska *et al*, 2010; McGregor and Harvey, 2017). Leptin is primarily involved in the regulation of energy homeostasis by acting mainly on neuronal targets, although there is no evidence that leptin is produced in the brain. Instead, it has been demonstrated that leptin is actively transported across the blood–brain barrier by a saturable transport system (Banks *et al*, 1996; Kurrimbux *et al*, 2004). According to leptin's physiological role, LepRs are highly expressed in hypothalamic nuclei involved in the regulation of food intake and energy expenditure (Spiegelman and Flier, 2001). This includes the arcuate (ARC), ventromedial (VMH), dorsomedial (DMN), paraventricular (PVN), and the lateral hypothalamic (LH) nuclei. In leptin-sensitive neurons, leptin signaling decreases expression of orexigenic peptides such as neuropeptide Y (NPY) and agouti-related peptide (AgRP), and increases the expression of anorexigenic peptides such as α-melanocyte-stimulating hormone (αMSH) (Park and Ahima, 2015) (Figure 8).

In addition, LepR-expressing neurons are also found in the nucleus of the solitary tract (NTS, in medulla oblongata), where leptin may act synergistically with glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK) to promote saciety, and also in the ventral tegmental area (VTA in mesencephalon) where leptin is able to influence

the hedonic control of feeding mediated by the dopaminergic mesolimbic (reward) pathway, suppressing the drive to feed. Central leptin signaling also increases energy expenditure through the modulation of sympathetic activity, once it stimulates BAT thermogenesis (Figure 8).

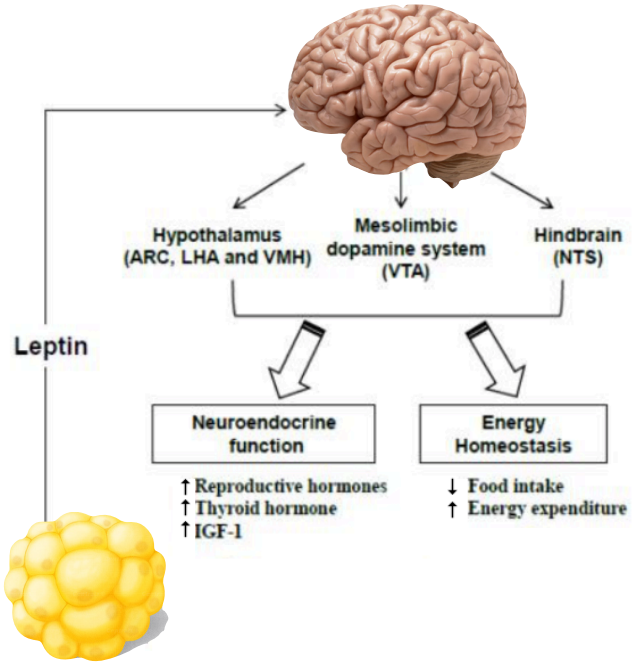


Figure 8. Leptin's physiology: regulation of energy homeostasis and neuroendocrine effects. In states of leptin deficiency or leptin resistance (obesity), the homeostatic and neuroendocrine actions described become impaired. (Adapted from Park and Ahima, 2015).

ARC: arcuate nucleus; CNS: central nervous system; IGF: insulin-like growth factor; LHA: lateral hypothalamic area; NTS: nucleus of the solitary tract; VMH: ventromedial hypothalamus; VTA: ventral tegmental area.

Many extra-hypothalamic brain regions, including the hippocampus, thalamus, cerebellum and brainstem, also express LepRs (Park and Ahima, 2015; McGregor and Harvey, 2017) as well as peripheral tissues. When leptin binds to LepRb, several signaling pathways are activated, including Janus kinase 2/Signal transducer and activator of transcription (JAK2/STAT). When JAK2 is activated, it induces the phosphorylation of tyrosine residues within the LepRb intracellular domain (Tyr985, Tyr1077 and Tyr1138) and downstream signaling cascades are activated, including extracellular signal-regulated kinase (ERK), IRS-PI3K and target rapamycin complex 1 (mTORC1) (Frühbeck, 2006).

Much of the knowledge on leptin's physiology results from the study of leptin deficiency syndromes in humans and has been explored at the molecular level in rodent models of leptin or LepR deficiency (for example *ob/ob* and *db/db* mice, respectively). Mutations of the leptin or LepR genes are known to induce obesity due to marked hyperphagia, but are also associated with neuroendocrine alterations that pretend to counterbalance a pseudo-energy deprivation state, and include decreased levels of reproductive hormones, thyroid hormones and insulin-like growth factor 1 (IGF-1), as well as increased growth hormone (GH) levels (Figure 8).

Metabolic abnormalities including hyperglycemia, hyperinsulinemia and dyslipidemia are also associated with impaired leptin signaling (Amitani *et al*, 2013). Glucose lowering properties of leptin are partly mediated by its direct effects on peripheral tissues such as leptin-mediated glucose uptake in muscle and BAT, lipolysis stimulation and reduced hepatic glucose production. In addition, leptin suppresses the release of insulin and glucagon secreted from pancreatic β-cells and α-cells, respectively (Figure 9). This adipoinsular axis is complex and a dual hormonal feedback loop involving insulin and leptin may function to maintain nutrient balance (D'souza *et al*, 2017).

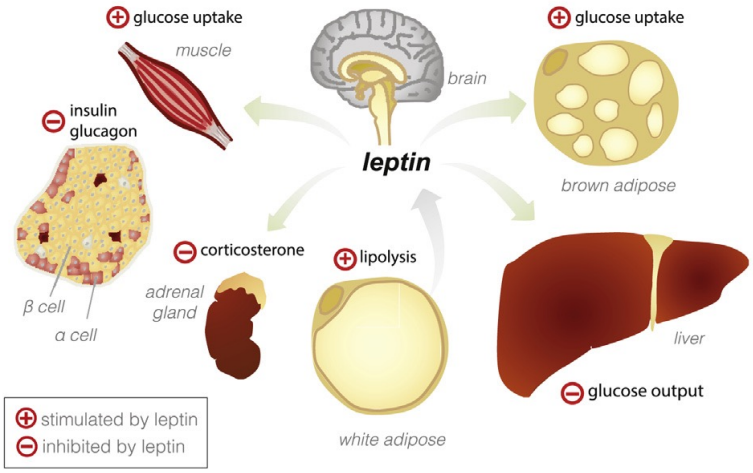


Figure 9. Glucoregulatory effects of leptin. (Adapted from D'souza *et al*, 2017).

I.2.3.2 Adiponectin

Adiponectin was originally cloned as an adipocyte-enriched protein highly induced upon 3T3-L1 adipocyte differentiation (Scherer *et al*, 1995) and almost simultaneously identified by four independent groups (Scherer *et al*, 1995; Hu *et al*, 1996; Maeda *et al*, 1996; Nakano *et al*, 1996). It is a 30 kDa 244-amino acid protein (247 amino acids in mouse) encoded by the *ADIPOQ* gene on chromosome 3 whose primary structure includes four domains: an amino-terminal signal sequence, a nonconserved variable region, a collagenous-like domain, and a carboxyterminal globular domain (Figure 10) similar to that found in complement factor C1q, tumor necrosis factor-α (TNF-α), and collagens VIII and X (Panno *et al*, 2016).

To date, three adiponectin receptors have been identified: AdipoR1, AdipoR2 and T-cadherin (T-cad). The first two, which homology between human and mice is superior to 95% (Yamauchi *et al*, 2014), are highly structurally related and ubiquitously expressed, though with variable affinity to different isoforms and variable predominance in some tissues. T-cad, which lacks an intracellular domain, is mainly involved in the binding of the high-molecular weight (HMW) isoform and may play a critical role as tissue anchoring molecule for adiponectin, though its regulation and relation with AdipoRs remain to be characterized (Matsuda *et al*, 2015).

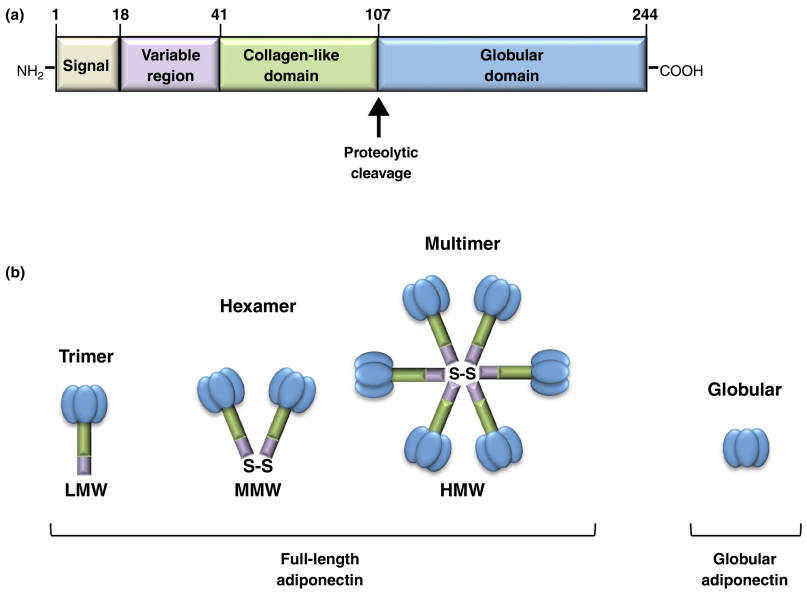


Figure 10. Adiponectin molecular structure and isoforms. Schematic molecular structure of adiponectin monomer (a), which undergoes post-translational modifications, resulting in the formation of different isoforms present in plasma: full-length proteins - trimers (Low Molecular Weight, LMW), hexamers (Medium Molecular Weight, MMW) or multimers (High Molecular Weight, HMW), and globular adiponectin, a proteolytic cleavage fragment generated by elastase digestion (b). (Panno *et al*, 2016)

In the brain, AdipoRs are widely expressed with AdipoR1 expression being more pronounced. In humans, they have been localized in the hypothalamus, pituitary gland and also in the nucleus basalis of Meynert and in the hippocampus (Thundyil *et al*, 2012). Despite its much lower CSF concentration (1000x), there is a good correlation between CSF and serum adiponectin levels (Kusminski *et al*, 2007; Hietaharju *et al*, 2010; Une *et al*, 2011), which favors BBB crossing as the

major source of cerebral adiponectin, although intrathecal synthesis of this adipokine has not been ruled out.

In peripheral tissues, adiponectin binding to AdipoR1, mainly found in the skeletal muscle, and AdipoR2, most abundant in the liver, activates a series of downstream signaling pathways including AMPK, PPARα and p38-mitogen-activated protein kinases (MAPK), leading to increased glucose uptake and insulin sensitivity, fatty acid oxidation and energy expenditure. In addition, adiponectin binding to both receptors has been associated with potent ceramidase activity, with consequent reduction on ceramide levels and lipotoxicity and increased sphingosine-1-phosphate (S1p), thereby reducing inflammation, and improving insulin sensitivity and cell survival (Ruan and Dong, 2016) (Figure 11).

Several studies have demonstrated the potent insulin-sensitizing and antiapoptotic roles of adiponectin in different cell types (Scherer, 2016) although its anti-inflammatory role may depend on the isoform, specific tissue it acts on and the presence or absence of disease (Thundyil *et al*, 2012). Furthermore, adiponectin is proangiogenic and pro-adipogenic, favoring a metabolically healthy expansion of the adipose tissue (Scherer, 2016), and also presents antiatherogenic properties. It decreases endothelial cell apoptosis and migration, angiotensin-induced endothelial permeability and atherosclerotic plaque size, at least in part

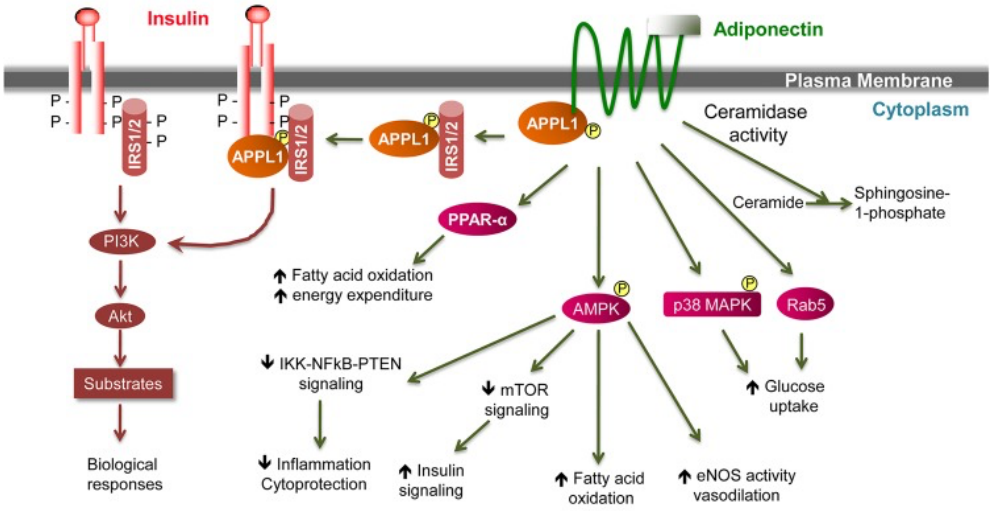


Figure 11. Adiponectin signal transduction pathway and crosstalk with the insulin signaling pathway. AdipoR1 or AdipoR2 interact with intracellular APPL1 which mediates the effects of adiponectin on the activation of multiple pathways and the crosstalk with insulin signaling transduction. Most of the metabolic effects of insulin are mediated by the PI3K/Akt pathway, leading to biological responses that include increased protein synthesis, lipogenesis, glucose uptake and utilization, and glycogen synthesis, as well as reduced lipolysis and gluconeogenesis. (Ruan and Dong, 2016).

APPL1: adaptor protein containing the pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1; IRS: insulin receptor substrate; PI3K: phosphatidylinositol 3-kinase; Akt: protein kinase B; IKK-NFkB-PTEN: IκB kinase-nuclear factor-κB-Phosphatase and tensin homolog; mTOR: mammalian target of rapamycin; PPAR: Peroxisome proliferator-activated receptor; AMPK: 5' adenosine monophosphate- activated protein kinase; MAPK: mitogen-activated protein kinase; Rab5: Ras-related protein Rab-5A.

due to increased cholesterol efflux from monocytes, decreased formation of foam cells and increased M2 macrophage polarization (Ouchi *et al.*, 2001; Kobayashi *et al*, 2004; Li *et al*, 2010; Mahadev *et al*, 2008; van Stijn *et al*, 2014; Wang *et al*, 2013). It also has the capacity to activate endothelial nitric oxide synthase (eNOS), implicated in the association of higher adiponectin levels with a reduced risk of vascular ischemic

diseases (Nishimura *et al*, 2008). Accordingly, lower adiponectin levels have been closely related to endothelial dysfunction (Barseghian *et al*, 2011), increased carotid intima-media thickness (Lo *et al*, 2006; Gardener *et al*, 2012) and may predict cerebro- and cardiovascular disease (Yang *et al*, 2015; Horáková *et al*, 2015). In addition, there is also evidence that adiponectin may protect BBB integrity by reducing its permeability, microvascular metalloproteinase expression and parenchymal leukocyte accumulation (Chen *et al*, 2009; Vachharajani *et al*, 2012).

Besides the pleiotropic role of both the adipokines described above, there is evidence of their effect on brain development and cognition, namely in memory circuits, but this subject will be addressed in more detail in the following chapters.

I.2.4 Adipose tissue dysfunction

The systemic ability to adjust to dynamic changes in nutritional status, namely to positive energy balance due to excessive calorie intake and/or diminished energy expenditure (metabolic flexibility) is mainly dependent on the ability of the adipocyte and adipose tissue to expand. The effectiveness of this expansion process will determine the degree of metabolic dysfunction associated with obesity.

An adequate angiogenesis is crucial for adipose tissue physiology, once it ensures the delivery of oxygen and nutrients, growth factors, hormones, stem cells and immune cells, as well as the removal of waste products and transportation of fatty acids and adipokines into the circulation (Cao, 2010). It is a process regulated by angiogenic factors and inhibitors, including vascular endothelial growth factor A (VEGFA), fibroblast growth factor (FGF), adiponectin and thrombospondin 1. Similarly to other tissues, a rapidly expanding fat mass may determine a relative impairment of angiogenesis, which can lead to local hypoxia with consequent release of hypoxic response mediators (eg. hypoxia-inducible factor 1 alpha - HIF1α). This will stimulate fibrosis with disproportionate accumulation of extracellular matrix constituents (especially collagen VI), activation of inflammatory pathways, dysregulation of secretome and consequent impairment of insulin-sensitivity and lipid storage. In this context, not only adipogenic potential is decreased but also the rate of adipocyte death increases, which attracts macrophages and other immune cells, that are further recruited by the increased expression and secretion of pro-inflammatory adipokines and citokines (TNF-α, interleukins 6 and 8 - IL-6 and IL-8, monocyte chemoattractant protein 1 - MCP-1, leptin, resistin). Meanwhile, anti-inflammatory mediators (adiponectin, IL-10) are diminished along the macrophage polarization from M2 (anti-

inflammatory) to M1 (pro-inflammatory) phenotype, contribuiting to a mild but chronic systemic inflammatory state (Rodrigues *et al*, 2013; Rutkowski *et a*l, 2015).

Although obesity is associated with elevated circulating levels of leptin, this adipokine is incapable of decreasing food intake and body weight. Several mechanisms contribute to this leptin resistance phenomenon: (1) chronically elevated levels of free leptin in the brain desensitize LepRb although obese individuals present reduced leptin passage across the BBB; (2) impaired trafficking of LepRb to the membrane of hypothalamic nuclei neurons; and (3) overexpression of molecules involved in the inhibitory negative feedback systems (suppressor of cytokine signaling 3 - SOCS3, protein tyrosine phosphatases - PTPs, pro-inflammatory cytokines). Additionally, chronic overnutrition and increased free fatty acids (FFA) may induce lipotoxicity and endoplasmatic reticulum stress that trigger inflammation and further contribute to leptin resistance in obesity (Cui *et a*l, 2017).

In most cases, obesity is associated with a metabolically unhealthy state that occurs when adipose tissue expansion is based on increased adipocyte size, i.e., hypertrophy. In these hypertrophic and saturated adipocytes basal lipolysis is elevated, leading to the leakage of FFA that will be taken by other tissues such as the liver, skeletal muscle, heart, kidneys and pancreas, causing ectopic lipid accumulation. Overall,

insulin sensitivity becomes impaired in insulin-sensitive tissues and chronic insulin resistance develops, a frequent phenomenon in patients with metabolic syndrome. Nevertheless, adipose tissue can also expand through the increase of adipocyte number, a process named hyperplasia, which has been associated to "metabolically healthy obesity", since it is related to normal systemic glucose, lipid and inflammatory parameters (González-Muniesa *et al*, 2017) (Figure 12).

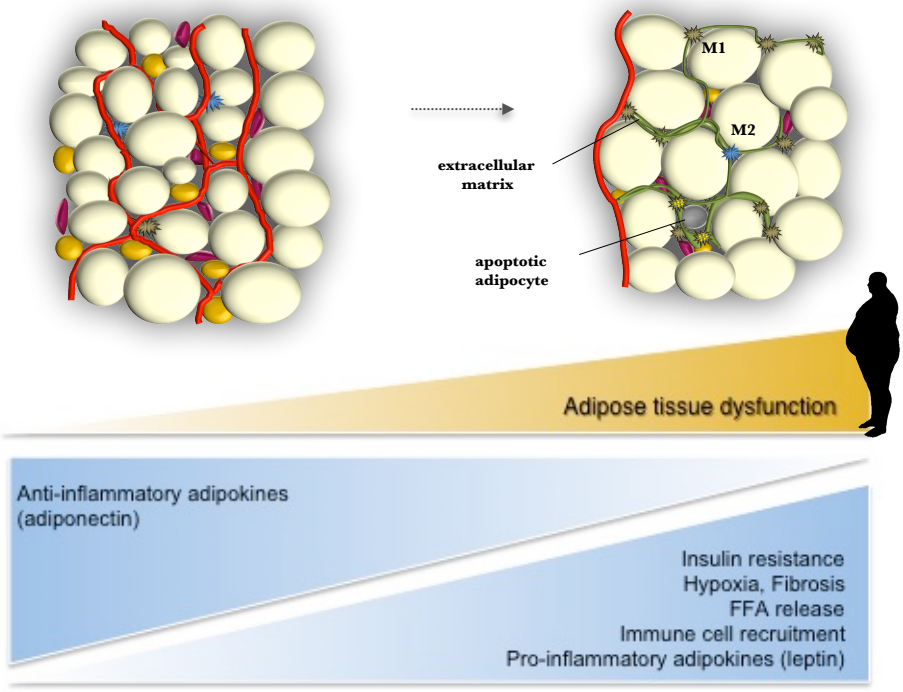


Figure 12. Obesity-associated adipose tissue dysfunction. Recruitment of M1 macrophages, with elevated M1 (in green)/M2 (in blue) ratio, impaired adipogenesis (yellow and small adipocytes), enhanced adipocyte hypertrophy and apoptosis (black and small adipocyte), impaired vascularization with consequent hypoxia, and excess deposition of extracellular matrix proteins (in green) will progressively occur. These alterations in immune, vascular, and structural adipose tissue composition will, in turn, result in an altered secretoma including decreased adiponectin and increased leptin production, increased levels of free fatty acids (FFA) and induce a chronic inflammatory state, contributing to the development of insulin resistance. (Adapted from Letra *et al*, 2017).

Therapeutic strategies that privilege adipose tissue hyperplasia over hypertrophy or promote its adequate angiogenesis are currently under investigation.

Chapter I.3

Obesity as risk factor for Alzheimer´s disease

Obesity represents one of the most relevant risk factors for the development of type 2 diabetes *mellitus* (T2DM) and other metabolic, cardiovascular and chronic inflammatory diseases, but also influences the risk of neurodegenerative disorders such is the case of Alzheimer's disease (González-Muniesa *et al*, 2017). Several studies have pointed mid-life obesity as an important contributor for the development of this type of dementia, independently of the co-ocurrence of other pathologies, namely T2DM (Gorospe and Dave, 2007; Beydoun *et al,* 2008; Whitmer *et al,* 2008). Life-long relation between adiposity and cognition has indeed triggered some questions about the mechanisms by which adipose tissue might influence cerebral structures related to learning and memory, which will be further discussed in this chapter.

I.3.1 The influence of adipose tissue on cognition across lifespan

Large epidemiological studies have described lower cognitive abilities, developmental delay, and increased incidence of autism spectrum and attention deficit hyperactivity disorders in the offspring of obese individuals. Maternal obesity seems to be particularly determinant and is associated with a 1.3 to 3.6-fold increase in the risk of cognitive

impairment in the offspring (Edlow *et al*, 2014; Edlow, 2017). It is, therefore, highly suggestive that *in utero* environment plays a substantial role in neurodevelopment. Animal models of maternal diet-induced obesity have provided important mechanistic insights that improved our comprehension on this association. They describe impaired neurogenesis and neuronal arborization (mainly, though not exclusively, in the hippocampus) with subsequent cognitive impairment and learning, behavioral abnormalities and eating disorders. These alterations may arise as a consequence of disrupted neuronal fetal programming, which in turn, results from an association between altered placental permeability and increased oxidative and inflammatory burden, impaired metabolic signaling (characterized by insulin and leptin resistance), impaired serotoninergic and mesolimbic dopaminergic signaling and brain-derived neurotrophic factor (BDNF)-mediated synaptic plasticity (Edlow, 2017). Moreover, women that in addition have elevated gestational weight gain have a threefold increased risk of Intelligence Quoficient (IQ) deficit in offspring (Huang *et al*, 2014) (Figure 13).

When considering infancy and adolescence, the existing literature supports that obesity is negatively linked to neurocognitive performance in several domains such as motor skills, attention, visuo-spatial abilities and executive functions (Liang *et al*, 2014). Notoriously, in this broad

area of executive performance, a more consistent inverse relationship is found with mental flexibility and verbal fluency (Cserjesi *et al*, 2007; Verdejo-Garcia *et al*, 2010; Delgado-Rico *et al*, 2012) relatively to impulsivity, planning and decision-making. In addition, overweight children display less effective inhibition strategies (Lokken et al, 2009; Nederkoorn *et al*, 2012) and more difficulties in dealing with delayed gratification (Bruce *et al*, 2011) (Figure 13).

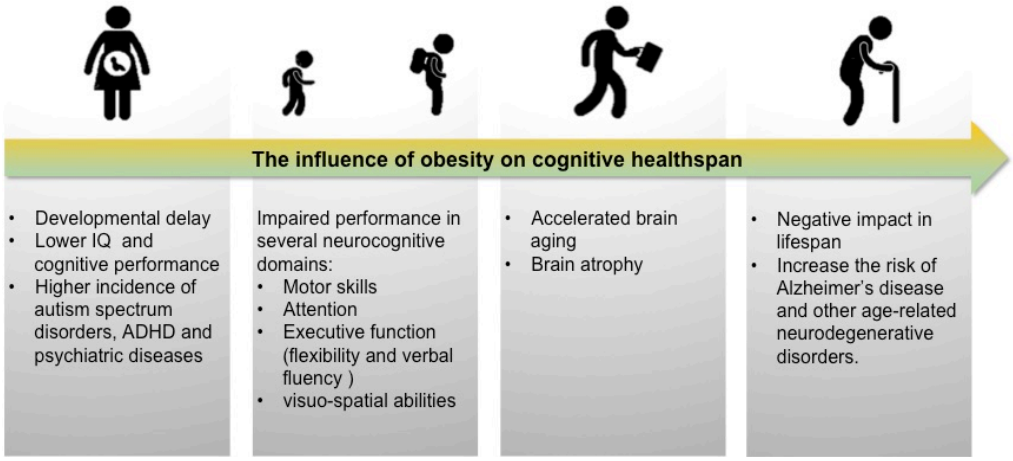


Figure 13. The influence of obesity on cognition across lifespan. Cognitive consequences of adipose tissue dysfunction in different lifespan development stages: *in utero*, infancy and adolescence, middle-age, and older age. (Letra and Santana, 2017).
ADHD: attention deficit and hyperactivity disorder; IQ: intelligence quoficient.

Results from studies on the association between obesity and general cognition (verbal and non-verbal domains), language, learning and memory are, otherwise, less congruent (Liang *et al*, 2014). Findings from research in this area also demonstrate a two-way relationship

between early-life obesity and cognition once mental deficits also influence decision making, impulsivity and overall behaviors (food-related, physical activity) that increase, by themselves, the risk of future obesity (Seeyave *et al*, 2009). However, socio-economic factors, which determine the amount and quality of care and stimulation in key developmental stages should not be overlooked (Barrigas and Fragoso, 2012).

With aging, adipose tissue becomes dysfunctional with consequent dysregulation of adipose-derived hormone production and signaling. Within the context of overweight, these changes are accelerated and may play a critical role in the etiology of obesity-related complications, including in the increased risk of age-related neurological diseases (Figure 13). In addition, it is recognized that caloric restriction, which causes profound changes in the adipose tissue, increases lifespan in humans and other species and seems to prevent or delay age-related pathologies (Van Cauwenberghe *et al*, 2016). Multiple interactive pathways and molecular mechanisms seem to be involved in neuronal beneficial effects of caloric restriction, including insulin-dependent pathways, forkhead box (FOXO) transcription factors, sirtuins and PPARs (Martin *et al*, 2006). Research that focus specifically on the impact of reducing visceral fat deposits (either through diet or using surgery),

indicate a positive effect mediated by an improvement of age-related insulin resistance related to adipokine secretion (Gabriely *et al*, 2002).

I.3.2 Diabesity and Alzheimer's disease

Similarly to what was previously described for Alzheimer's disease pathology, glucose dysmetabolism is considered a continuum from normal glucose tolerance to T2DM, in which progressive loss of pancreatic β-cells function determines the level of glycemia on a background of increasing insulin resistance. The causes of insulin resistance are complex and not yet completely clarified although there are well-established risk factors such as obesity (Biessels and Reagan, 2015). The occurrence of obesity and T2DM in the same individual has been termed *diabesity*, reflecting the intricate relationship between both metabolic conditions (Verma and Hussain, 2017).

Growing evidence supports the concept that Alzheimer's disease represents a metabolic disease in which brain glucose utilization and energy production are impaired (Cai *et al*, 2012; de la Monte, 2012) and epidemiological studies show that the overall relative risk of dementia in T2DM patients can be up to 73% higher than non-diabetic patients (Biessels *et al*, 2014). In fact, insulin resistance and T2DM have been consistently correlated with brain structural and functional changes

including brain atrophy particularly in temporal and frontal lobes, microvascular damage, and abnormal functional connectivity, although some studies do not support a clear correlation between T2DM and the burden of Alzheimer's disease neuropathology (Lee *et al*, 2014; Moheet *et al*, 2015; Arnold *et al*, 2018). Moreover, post-mortem brain studies in non-diabetic Alzheimer's disease patients have provided important evidence of disturbed insulin signaling consistent with a cerebral insulin resistance state (Biessels and Reagan, 2015).

In view of the limitations associated with clinical studies, animal models have been used to identify the mechanisms underlying cognitive deficits associated with insulin resistance and T2DM. These include rodents with mutations in the gene encoding leptin or its receptor (*ob/ob* mice and *db/db* mice or Zucker diabetic fatty rats), rodents with downregulated brain insulin receptor expression, as well as rodents fed with high-fat diets. These are also referred to as Western diets or cafeteria diets and are typically composed of a higher proportion of fat than the proportion found in normal laboratory diets, either alone or in combination with glucose or fructose. These animal models of glucose dysmetabolism share common metabolic abnormalities including hyperleptinemia, hyperglycemia, hyperinsulinemia, glucose intolerance, both peripheral and central insulin resistance, and display impaired learning and memory. Furthermore, it has been observed in these

models, an Alzheimer's disease-like pathology, especially affecting the hippocampus (Heni *et al*, 2015). The hippocampus, a crucial structure in memory formation and highly targeted in this disease, is one of the main insulin-sensitive brain areas. Likewise, the intracerebroventricular (icv) administration of streptozotocin (STZ), a diabetogenic drug associated with the development of central insulin resistance, has emerged as a suitable experimental approach for studying sporadic Alzheimer's disease (Grieb, 2016 for review).

After crossing the BBB via a carrier-facilitated process, insulin binds to the α-subunits of its receptors and, similarly to what is observed in the periphery, activates two canonical signaling cascades following autophosphorylation of the insulin receptor β-subunits, including the PI3K–Akt and the MAPK/ERK kinase (MEK)-ERK pathways. Dysregulation of insulin signaling in the hippocampus has been shown to occur at several points in the signaling cascade and to contribute to the development of hippocampal insulin resistance which has been directly involved in neurocognitive deficits (Biessels and Reagan, 2015) (Figure 14). It should be noted that insulin transport into the CSF is attenuated in individuals with systemic insulin resistance and has also been described in Alzheimer's disease patients (Heni *et al*, 2015).

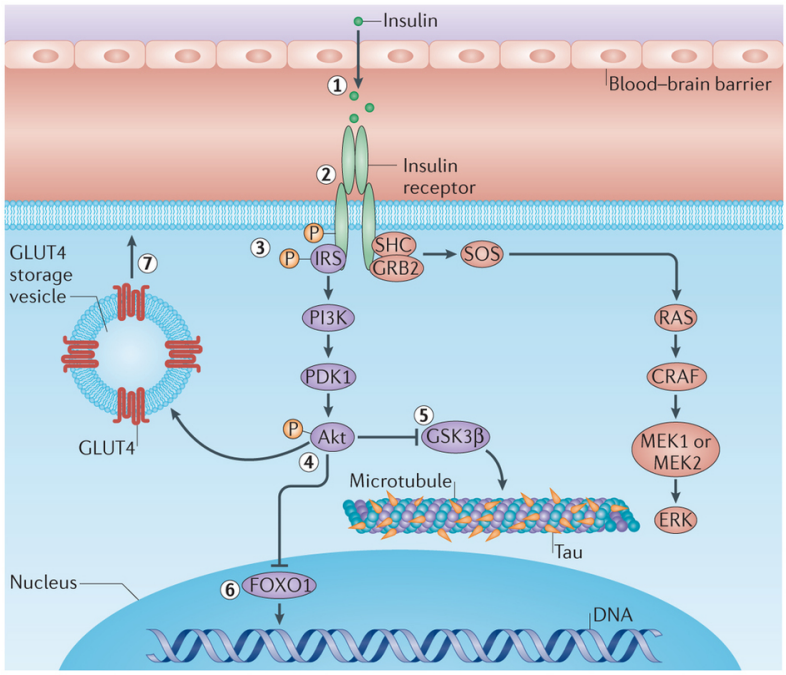


Figure 14. Insulin receptor signalling in the hippocampus and possible mechanisms involved in hippocampal insulin resistance development.

(1) Impaired insulin blood-brain barrier transport; (2) Decreased expression and/or activity of the insulin receptor; (3) Altered phosphorylation state of IRS proteins; (4) Decreased insulin receptor-stimulated phosphorylation of Akt; (5) Altered phosphorylation state of GSK3β; (6) Upregulated FOXO1 transcriptional factor; (7) Decreased traffic of the insulin-sensitive GLUT4 to the plasma membrane (Biessels and Reagan, 2015).

BBB: blood-brain barrier; CRAF: proto-oncogene serine/threonine-protein kinase; ERK: extracellular-signal regulated protein kinase; FOXO1: forkhead box O 1; GLUT4: glucose transporter type 4; GRB2: growth factor receptor-bound 2; GSK3β: glycogen synthase kinase 3β; IRS: insulin receptor substrate; MEK: mitogen and extracellular-signal regulated protein kinase; PDK1: 3-phosphoinositide-dependent protein kinase 1.

Thereby, for many years, the impact of obesity on Alzheimer's disease incidence has been almost exclusively related to direct detrimental effects of insulin resistance. It seems though that this may not be the only mechanism by which adipose tissue dysfunction contributes to cognitive impairment. Some studies have demonstrated

that cognitive deficits observed in diet-induced-obese animals were not prevented by insulin sensitizers (McNeilly *et al*, 2012), and that spatial learning deficits and hippocampal tau pathology could be potentiated by obesity independently of systemic insulin sensitivity (Leboucher *et al*, 2013). Given the high complexity of both diseases is not difficult to accept that other mechanisms may also be involved in the increased risk of dementia associated to dibesity, and most likely also involve adipokine-mediated actions on the CNS.

Chapter I.4

Purpose of research

Why are middle-aged obese individuals at higher risk of developing Alzheimer's disease?

Based on undeniable epidemiological evidence that middle-age obesity contributes to the development of Alzheimer's disease (Beydoun *et al*, 2008), the hypothesis that adiponectin, the most abundant circulating adipokine, could be involved in adipose tissue-brain crosstalks in this disease context has emerged.

After coming across with several gaps in the body of knowledge on adiponectin's physiology and its influence on brain and cognition, we have designed a research project which aimed to contribute to the clarification of whether adiponectin levels are altered in different stages of Alzheimer's disease and related to its pathophysiology and progression. For that purpose, we have enrolled patients in the spectrum of Alzheimer's disease but also felt the need of exploring some of the clinical questions raised in an animal model.

Ultimately, this study proposes to help to understand adipose tissue dysfunction as modifiable risk factor for dementia. This will hopefully draw attention to the need of prioritizing strategies aimed at reducing the incidence and burden of this devastating disease.

PART II

CLINICAL

INVESTIGATION

Chapter II.1

Current state of research

II.1.1 Adiponectin and Alzheimer's disease

Strong evidence support the view of sporadic Alzheimer's disease as a metabolic brain disorder whose natural history may intrinsically be linked to adipose tissue physiology. However, and contrarily to cerebrovascular disease, the role of adipokines, and particularly of adiponectin, in neurodegenerative disorders is still an open-field (Yang *et al*, 2015; Ishii and Iadecola, 2016).

The existent results from clinical studies addressing the contribution of adiponectin to the risk of developing Alzheimer's disease or to its progression was gathered after extensive and careful review of the literature and is listed in Table 2.

Table 2. Population-based studies aiming to assess the association between adiponectin and Alzheimer's disease

Study, country of origin First author, year	n	Age	Gender	Follow-up	*APOE*	Cognitive Tests	Sample (method)	Results
Rochester Epidemiology Project, USA Roberts, 2009	747 NC 143 MCI	79.8 (75.0, 83.7)[a] 82.0 (78.2, 85.7)[a]	333 ♂ /414 ♀ 76 ♂ /67 ♀	0	yes	MOANS	Plasma (RIA)	Total adiponectin is **similar** in MCI and NC [MCI=14.4 (9.8, 19.6) vs NC=14.5 (9.8, 19.6) mg/L; p=0.97] [a], though OR of naMCI was 2.5 in the lowest compared to the highest quartile of adiponectin (95%CI: 0.85-7.73). Adjustments: age, gender, education, *APOE*, comorbidities.
J-SHIPP Study, Japan Kamogawa, 2010	397 NC 120 MCI	67 (62, 72)[a] 72 (68, 77)[a]	123 ♂ /274 ♀ 56 ♂ /64 ♀	0	no	MCI Screen HDSR	Plasma (ELISA)	A 10 mg/l increase in total adiponectin was associated with a 54% **reduction in OR** for MCI in men (OR: 0.46; 95%CI: 0.20-0.97; p=0.041). Adjustments: age, subcutaneous abdominal fat area.
WHICAP II, USA Gu, 2010	1219 NC	76.7±6.4[b]	407 ♂ /812 ♀	3.8±1.3y	yes	SRT, BNT, VFT, BVRT, RDT, Similarities test (WAIS-R), Identities& Oddities (MDRS), Repetition& Comprehension (BDAE)	Serum (RIA)	Total adiponectin was **not associated** with baseline cognitive score nor associated with AD risk [incident AD (n=118)=10.2 (6.8-18.5)[a]; dementia-free (n=1101)=11.3 (7.0-18.0)[a] µg/ml, p=0.75)]. Adjustments: age, gender, race, education and cohort.

Clinical series, Germany Bigalke, 2011	37 NC 41 AD	67.3±10.2[b] 74.3±9.1 [b]	19 ♂ /18 ♀ 19 ♂ /22 ♀	0	no	MMSE	Plasma (ELISA)	Total adiponectin levels did not show **any significant difference** between groups (AD=18.5±18.1 vs. NC=16.7±8.9 µg/mL; *p*=0.641).
Clinical series, Japan Une, 2011	28 NC 18 aMCI 27 AD	72.5±2.82[b] 74.2±2.16[b] 77.4±0.95[b]	12 ♂ /16 ♀ 9 ♂ /9 ♀ 8 ♂ /19 ♀	0	yes	MMSE	Plasma + CSF (ELISA)	Total adiponectin was significantly **higher** in MCI and AD compared to NC (*p*< 0.05); CSF ADPN was significantly **higher** in MCI compared to NC (*p*<0.05); when adiponectin levels were normalized to body weight only the difference between MCIvsNC and ADvsNC remained significant. Plasma and CSF adiponectin levels showed a positive correlation (r=0.406, *p*=0.005). Adjustments: age, gender.
TARCC, USA Warren, 2012	197 NC 150 AD	70 (38)[c] 79.5 (37)[c]	61 ♂ /136 ♀ 45 ♂ /105 ♀	0	yes	MMSE, DS, TMT, BNT, LMVR VFT, CDT, AMNART	Serum (NI)	Total adiponectin levels were **similar** between the two groups (NC=5.1±18 vs AD=5.75±32 µg/ml; *p*=0.101). Adjustments: age, education.
Clinical series, Brazil Diniz, 2012	51 NC 47 LLD (32CI; 15noCI)	68.7±5.6[b] 70.2±4.7[b]	11 ♂ /40 ♀ 10 ♂ /37 ♀	0	no	MMSE, CAMCOG	Serum (ELISA)	**No significant difference** was observed in adiponectin levels between LLD subgroups (LLD-CI: 55005.26±3616.78; LLD-noCI: 54422.06±4469.64 pg/mL; *p*=0,6) though reduced in LLD when compared with NC; adiponectin levels were positively correlated with language (r=0.44; *p*<0.001) and calculation (r=0.23, *p*=0.02), as well as abstraction, attention and praxis, but *not* with memory or orientation.
Framingham Heart Study, USA van Himbergen, 2012	826 NC	72±4[b] (♂) 73±4[b] (♀)	299 ♂ /541 ♀	13 y	yes	MMSE	Plasma (ITA)	Total adiponectin was associated with an **increased risk** of all-cause dementia (HR 1.29; 95%CI: 1.00–1.66; *p*=0.054) and AD (HR=1.33; 95%CI: 1.00–1.76; *p*=0.050) only in women.(by 1-SD increase in adiponectin concentration). Adjustments: age, BMI, weight change, ApoE, education, plasma DHA.
Clinical series, Brazil Teixeira, 2013	51 NC 65 aMCI 41 AD	68.7±5.6[b] 71.1±13.4[b] 75.4±7.9[b]	10 ♂ /41 ♀ 17 ♂ / 48 ♀ 14 ♂ /27 ♀	34.6±13 .2m 26.4±17 .4m	yes	CAMCOG MMSE, SCT, RBMT, FOME, TMT, CDT, VFT	Serum (ELISA)	Total adiponectin levels were significantly **lower** in MCI and AD when compared to NC (MCI=52898.69±7651.59; AD=52145.53±7431.62; NC=63337.38±4233.84 pg/ml; *p*<0.001) and **did not predict progression** from NC to MCI or from MCI to AD (F=2.8; df=1, *p*=0.1). adiponectin inversely correlated with TMT B (r=-0.325, *p*=0.002); the correlation between CAMCOG, RBMT and FOME total scores showed a trend toward significance (r=0.195, *p*=0.06; r=0.198, *p*=0.06; r=0.199, *p*=0.06, respectively). Adjustments: age, education, *APOE*, BMI, comorbidities.
Clinical series, India Khemka, 2014	60 NC 60 AD	64.75±4.40[b] 66.97±6.36[b]	34 ♂ /26 ♀ 33 ♂ /27 ♀	0	no	MMSE	Serum (ELISA)	Total adiponectin levels were significantly **elevated** in AD compared to NC (33.33±17.28 vs 8.27±3.0 µg/ml; *p*<0.001) and showed a negative correlation with MMSE score in AD sujects (r=-0.5236; *p*<0.0001).
Clinical series, Croatia Dukic, 2015	50 NC 48 MCI 70 AD	66 (63-73)[c] 72 (68-76)[c] 74 (69-79)[c]	20 ♂ /30 ♀ 17 ♂ /31 ♀ 20 ♂ /30 ♀	0	no	MMSE MoCA	Serum (ITA)	Concentration of total adiponectin **did not differ** between groups [AD: 10.6 (8.4–13.8); MCI: 10.4 (7.7–14.8); NC: 8.8 (5.8–13.3) µg/ml; *p*= 0.268]**.**
OSACA2, Japan Kitagawa, 2015	466 NC	67.8±8.3[b]	275 ♂ /191 ♀	6.9 y (NI)[b]	yes	MMSE	Serum (CLEIA)	Risk of AD was **identical** in patients with high vs low HMW adiponectin levels (*p*=0.740; cutt-off: 3.47 µg/ml); no association with medial temporal lobe atrophy. Adjustments: age, gender, education, *APOE*, baseline MMSE, vascular risk factors and cerebrovascular events.
Clinical Series, Poland Gorska-Ciebiada, 2016	132 NC 62 MCI (all DM2)	72.5±4.9[b] 74.7±3.9[b]	82 ♂ /50 ♀ 30 ♂ /32 ♀	0	no	MoCA BADL IADL	Serum (ELISA)	Concentration of total adiponectin was **lower** in diabetic MCI vs diabetic controls (BMI 26–27.9: 9.37±3.33 vs 11.76±3.05, p=0.009; BMI 28–29.9: 6.14±2.85 vs10.38±2.77, p<0.001; BMI≥30: 2.89±1.59 vs 5.44±3.78 µg/ml p<0.001). Adiponectin negatively correlated with MoCA score (r=-0.3, *p*=0.016). Adjustments: BMI
Clinical series, China Ma, 2016	91 NC 91 AD	80.03±8.32[b] 82.96±9.33[b]	37 ♂ /54 ♀ 36 ♂ /55 ♀	0	no	MMSE	Serum (ELISA)	**Higher level** of total adiponectin were found in AD (NC: 8.21±0.42 vs AD: 14.51±1.00 µg/ml; *p*<0.0001). Adiponectin levels showed significant inverse correlation with MMSE score in AD subjects (r =− 0.518, *p*< 0.01) Adjustments: MMSE (cut-off 15), gender Meta-analysis result: pooled WMD of adiponectin levels in AD vs NC=9.42 µg/mL (95%CI: 4.21, 14.62)

Clinical series, Japan Waragai, 2016	62 NC 64 MCI 63 AD	76.0±7.9[b] 76.0±6.5[b] 78.8±6.1[b]	34 ♂ /28 ♀ 24 ♂ /40 ♀ 21 ♂ /42 ♀	0	yes	MMSE	Serum and CSF (ELISA) Brain (IH)	Total serum adiponectin was significantly **higher** in patients with MCI and AD compared to NC (MCI: p<0.05, AD: p<0.001), whereas CSF adiponectin was significantly lower in patients with AD compared to those with MCI and NC (p<0.001). CSF adiponectin levels were positively correlated with MMSE scores (r=0.4247, p<0.001) and CSF $A\beta_{42}$ levels (r=0.1917, p<0.01) and negatively correlated with severity of medial temporal lobe atrophy (r=−0.2910, p<0.001) and CSF p-tau levels (r=−0.2979, p<0.001). There were no correlation between serum adiponectin and AD biomarkers.
MCSA Study, USA Wennberg, 2016	535 NC	80 (77, 84)[a]	328 ♂ /207 ♀	0	yes	MMSE AVLT, WMS-R (LMVR), BNT, Category Fluency, TMT B, WAIS-R (Digit Symbol, Picture Completion, Block Design)	Plasma (RIA)	In men, total adiponectin was associated with reduced cerebral glucose uptake (FDG-PET; B=−0.087; 95%CI: −0.166, −0.008) and was **not associated** with odds of MCI. In women, higher adiponectin levels were associated with smaller hippocampal volume (B=-0.595; 95%CI: -1.19, -0.005) poorer language and global cognitive performance (B=-0.777; 95%CI: -1.42, -0.138 and B=-0.729; 95%CI: -1.40, -0.053, respectively) and with **greater odds** of MCI (OR=6.23; 95%CI: 1.20, 32.43). Adjustments: age, education, WHR, DM, HT, *APOE*, amyloid status

Aβ: amyloid beta; AD: Alzheimer's dementia; aMCI: amnesic Mild Cognitive Impairment; AMNART: American National Adult Reading Test; ApoE: Apolipoprotein E; AVLT: Auditory Verbal Learning Test; BADL: Basic Activities Of Daily Living (Katz); BDAE: Boston Diagnostic Aphasia Evaluation; BMI: Body Mass Index; BNT: Boston Naming Test; BVRT: Benton Visual Retention Test; CAMCOG: Cambridge Cognitive Test; CDT: Clock Drawing Test; CI: Cognitive Imparment; CLEIA: chemiluminescence enzyme immunoassayCSF: Cerebrospinal Fluid; DHA: Docosahexaenoic Acid; DM: Diabetes Mellitus; DS: Digit Span; ELISA: Enzyme-Linked Immunosorbent Assay; FDG-PET: Fludeoxyglucose-Positron Emission Tomography; FOME: Fuld Object Memory Evaluation; HDSR: Hasegawa Dementia Scale Revised; HMW: high molecular weight; HT: hypertension; IADL: Instrumental Activities Of Daily Living (Lawton); IH: Immunohistochemistry; ITA: Immunoturbidimetric Assay; J-SHIPP: Japanese-Shimanami Health Promoting Program; LLD: Late-Life Depression; LMVR: Logical Memory And Visual Reproduction; MCI: Mild Cognitive Impairment; MCSA: Mayo Clinic Study Of Aging; MDRS: Mattis Dementia Rating Scale; MMSE: Mini-Mental State Examination; mo: months; MOANS: Mayo Older Americans Normative Studies; MoCA: Montreal Cognitive Assessment; naMCI: non amnesic-Mild Cognitive Impairment; NC: Normal Cognition/Control; NI: No Information; noCI: No Cognitive Impairment; OR: Odds Ratio; OSACA2: Osaka Follow-Up Study For Carotid Atherosclerosis, Part 2; RBMT: Rivermead Behavioral Memory Test; RDT: Rosen Drawing Test; RIA: Radioimmunoassay; SCT: Short Cognitive Test; SRT: Selective Reminding Test; TARCC: Texas Alzheimer's Disease Research And Care Consortium; TMT: Trail Making Test; VFT: Verbal Fluency Test; WAIS-R: Wechsler Adult Intelligence Scale-Revised; WHICAP: Washington Heights Inwood Columbia Aging Project ; WHR: Waist-Hip Ratio; WMD: Weighted Mean Difference; WMS-R: Wechsler Memory Scale-Revised; y=years. ♂ : male, ♀ : female.

[a]Median (1st, 3rd Quartiles); [b]Mean ± SD; [c] Median (Range).

(Letra *et al*, 2017)

Overall, the involvement of adiponectin in Alzheimer's disease natural history is poorly understood, with divergent results showing a decrease (Kamogawa *et al*, 2010; Teixeira *et al*, 2013; Gorska-Ciebiada *et al*, 2016), increase (Une *et al*, 2011, van Himbergen *et al*, 2012; Khemka *et al*, 2014; Waragai *et al*, 2016; Wennberg *et al*, 2016), or no significant changes (Roberts *et al*, 2009; Gu *et al*, 2010; Bigalke *et al*, 2011; Diniz *et al*, 2012; Warren *et al*, 2012; Dukic *et al*, 2015; Kitagawa *et al*, 2015) in the concentration of this adipokine in different stages of the disease or when patients are compared to cognitively normal controls. At present,

the only meta-analysis assessing the correlation between adiponectin and Alzheimer's disease, which includes 5 studies comprising a total of 727 subjects (254 patients and 473 controls), shows higher peripheral levels of adiponectin in Alzheimer's disease patients when compared to controls (Ma *et al*, 2016).

Detailed analysis of the observational studies presented in Table 2 unveils substantial caveats that contribute to the incongruences afore-mentioned. One of the most important is patient selection, or more specifically, diagnostic accuracy. The inclusion of non-Alzheimer's dementias and the etiological diversity of MCI groups definitely contribute to the heterogeneity of the cohorts. In fact, MCI is a clinical entity and not all cases translate a prodromal phase of Alzheimer's disease. The incorporation of biomarker information in the diagnostic process is therefore essential to distinguish those related to Alzheimer's disease from non-Alzheimer's disease or nondegenerative cases but, surprisingly, few studies include them in the classification of patients. On the other hand, insufficient tools are used in neuropsychological characterization and workout of the patients enrolled. For instance, it is well established that amnesic and non-amnesic MCI carry a different risk of progression to Alzheimer's dementia but in some of these studies there is no clear distinction between these two conditions. Besides, a great number of studies rely exclusively on Mini Mental State

Examination (MMSE) to confirm the diagnosis of cognitive decline (Bigalke *et al*, 2011; Une *et al*, 2011; van Himbergen *et al*, 2012; Diniz *et al*, 2012; Khemka *et al*, 2014; Kitagawa *et al*, 2015; Ma *et al*, 2016; Waragai *et al*, 2016) despite the recognized limitations of this instrument to establish the diagnosis (Chapman *et al*, 2016).

Analysis of CSF adiponectin levels could, in theory, help to overcome some of the difficulties described and better mirror the influence of this adipokine on Alzheimer's disease pathology. Nevertheless, the only two clinical studies comparing CSF adiponectin levels in cognitively normal controls and in MCI or Alzheimer's dementia patients reached contradictory conclusions (Une *et al*, 2011; Waragai *et al*, 2016). Though most of the studies measured adiponectin irrespectively of its conformation, this protein has a wide range of multimers that may act differently according to its structure and target cell/tissue, though the pathophysiological relevance of each of these forms remains undetermined. On the other hand, neuroinflammatory changes associated to neurodegeneration can affect BBB integrity and alter its function and permeability to peripheral proteins, influencing CSF adiponectin concentration in these patients (Carvey *et al*, 2009).

Meanwhile, there are three important prospective longitudinal studies in community cognitively healthy subjects concerning the relation between peripheral adiponectin and the future development of

dementia: the Washington Heights Inwood Columbia Aging Project (WHICAP II) (Gu *et al*, 2010), the Framingham Heart (van Himbergen *et al*, 2012), and the Osaka Follow-up Study for Carotid Atherosclerosis (OSACA2) (Kitagawa *et al*, 2015). They are the largest longitudinal studies that have assessed the correlation between baseline adiponectinemia and the risk of progression to MCI or dementia. Despite the large amount of patients included (1219, 826 and 466, respectively) and prolonged periods of follow-up (approximately 4, 13 and 7 years, respectively), only the second study described an increased risk of Alzheimer's disease in women with higher plasmatic adiponectin concentration (HR=1.33), while the others reported no association. Considering the results presented by the Framingham Heart Study two opposing hypothesis can be postulated: (1) adiponectin has detrimental effects and increases the risk of developing the disease in the future; or (2) adiponectin increase is already signaling asymptomatic patients with neuropathological Alzheimer's disease alterations, and eventually adiponectin is required to counterbalance neurodegeneration. In our opinion, supported by the evidence gathered so far, the last hypothesis seems to be more consistent and is targeted in our investigation. Another unaccounted issue is the evidence of differences in gender-based subgroups analysis, which in our opinion also deserves further clarification.

II.1.2 Leptin and Alzheimer's disease

Leptin is probably the most well-studied adipokine concerning its signaling and function in the brain. When directly administered on the dentate gyrus or CA1 hippocampal region, this adipokine is able to promote beneficial effects on memory, including enhancement of long-term potentiation with consequent cognitive improvement (Shanley *et al*, 2001; Wayner *et al*, 2004), and influences hippocampal synaptic plasticity by enhancing N-methyl-D-aspartate (NMDA) receptors (Durakoglugil *et al*, 2005). Data from cell cultures and Alzheimer's disease animal models demonstrate the role of leptin in APP metabolism, namely in a reduction of Aβ peptide production by blocking β-secretase activity and increasing *APOE*-dependent Aβ uptake (Fewlass *et al*, 2004). Moreover, leptin seems to prevent aberrant hyperphosphorylation of protein tau by deactivation of the glycogen synthase kinase beta (GSK-3β), the main responsible for abnormal tau phosphorylation (Greco *et al*, 2009). Additional studies support these mechanisms, like the results of Li *et al* (2012) showing that obese leptin-resistant mice (*db/db*) had multiple Alzheimer's disease-like brain changes including increased Aβ and phosphorylated tau as well as decreased synaptic proteins with consequent impairment of their performance in cognitive tasks.

Despite extensive animal research, the association between leptin and Alzheimer's disease in epidemiological and clinical studies is limited.

Results from large longitudinal studies like the Framingham and the Health ABC pointed out to an association between higher serum leptin levels in late-life at baseline and higher brain volume and lower dementia risk at 4 and 7.7 years of follow-up, respectively (Lieb *et al*, 2009; Holden *et al*, 2009). These results were replicated by Zeki *et al* (2013) and Littlejohns *et al* (2015), although only in women with BMI<25kg/m2. However these results are not supported by the Prospective Population Study of Women in Gothenburg, Sweden in which mid-life serum leptin levels were not related to late-life dementia, suggesting that the changes in circulating leptin levels associated with Alzheimer's disease may rather be a late-life biological event (Gustafson *et al*, 2012). Furthermore, cross-sectional studies are also somehow incongruent concerning the association between circulating leptin and Alzheimer's disease. Some found lower leptin levels in MCI or Alzheimer's dementia subjects compared to controls (Powers *et al*, 2001; Bigalke *et al*, 2011; Johnston *et al*, 2013; Khemka *et al*, 2014; Baranowska-Bik *et al*, 2015) and a negative correlation with the severity of dementia (Kamogawa *et al*, 2010; Bigalke *et al*, 2011; Khemka *et al*, 2014), while others did not find any association (Warren *et al*, 2012; Oania and McEvoy 2015; Teunissen *et al*, 2015). By analyzing these discrepancies one can admit a set of confounders similar to those previously pointed to adiponectin studies. Nevertheless, we must take

into consideration that obese individuals develop brain resistance to leptin action despite its peripheral hyperproduction. In fact, Bonda *et al* (2014) demonstrated an up-regulation of CSF and hippocampal leptin in Alzheimer's disease patients, as well as decreased levels of leptin receptor mRNA in brain tissue and co-localization of ObR+ cells with NFT, unveiling a possible mechanism of central leptin resistance associated to this disease. Interestingly, CSF leptin levels showed a positive relation with the Braak staging independently of age and BMI. These results confirm those from Rajagopalan *et al* (2013) showing that elderly with higher leptin levels showed greater global brain atrophy. Another study using a larger cohort (from the Alzheimer's Disease Network Initiative - ADNI) reported otherwise similar CSF leptin levels in controls, MCI and Alzheimer's dementia patients. (Maioli *et al*, 2015).

II.1.3 Leptin to adiponectin ratio and Alzheimer's disease

Although it has been proposed that the equation between circulating leptin and adiponectin (leptin to adiponectin ratio: LAR) is a more reliable marker of adipose tissue dysfunction and consequently of its metabolic effects than adipokine levels separately (Satoh *et al*, 2004; Bravo *et al*, 2017), there is to the best of our knowledge, only one study describing a correlation between serum LAR and CSF Alzheimer's

disease biomarkers in male participants (age adjusted only) (Kim S *et al*, 2014). It is therefore almost inexistent the information on the association between serum and CSF LAR and Alzheimer's disease pathophysiological hallmarks or its biomarkers, which are further explored in the study presented.

Chapter II.2

Hypothesis and Aims

II.2.1 Hypothesis

The main hypothesis proposed in this thesis are the following:

1. Adiponectin contributes to brain resilience strategies against Alzheimer's disease-associated neurodegeneration;
2. Decreased levels of adiponectin in middle-age increases the risk of late-onset Alzheimer's disease.

II.2.2 Objectives

Main objectives

- To determine if serum and CSF adiponectin and LAR are significantly different in MCI and Alzheimer's dementia patients;
- To determine if serum and CSF adiponectin and LAR are correlated with the main Alzheimer's disease risk factors and biomarkers, as well as with cognitive scores;
- To explore if serum and CSF adiponectin and LAR influence the time of disease progression and can constitute predictors of MCI-to-Alzheimer's dementia progression.

Secondary objectives

- To explore to which extent is serum and CSF adiponectin and LAR correlated with systemic insulin resistance indices;
- To analyze the association of anthropometric measures with the levels of serum and CSF adiponectin and LAR.

Chapter II.3

Research design and Methods

II.3.1 Participants and clinical evaluation

The study was conducted in a group of 124 subjects prospectively followed at the Dementia outpatient Clinic at Centro Hospitalar e Universitário de Coimbra (CHUC) between 2009 and 2012, which were extensively characterized until a final probable diagnosis of an Alzheimer's disease spectrum disorder. At baseline we included 53 patients with a probable diagnosis of dementia due to Alzheimer's disease, that we further refer as AD (mild to moderate) and 71 patients with the diagnosis of MCI (amnestic: 54; amnestic multidomain: 17). In the longitudinal study, a total of 67 MCI patients were available for re-evaluation, while 4 lost follow-up. A group of healthy or assymptomatic/ pre-clinical individuals was not considered once performing a lumbar puncture in these individuals would not be ethically acceptable.

At baseline, patients and an informant underwent a structured clinical interview conducted by a neurologist that included a detailed description of complaints with special focus on time course and leading symptoms. Handedness, past medical and family history as well as drug history were also registered. A complete neurological examination was performed to rule out non-Alzheimer´s disease causes of cognitive decline. It included cranial nerve, sensory and motor examination, reflex and coordination assessment as well as gait testing. Patients were also

submitted to biochemical evaluation and brain imaging (CT and/or MRI) to exclude other forms of dementia and most of them were also studied with CSF biomarkers, functional imaging (SPECT, FDG-PET or amyloid-PET) and *APOE* genotying.

Probable Alzheimer's dementia was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders criteria (APA, 2000), the NINCDS–ADRDA (McKhann *et al*, 1984) and more recently to NIAAS revised criteria (McKhann *et al*, 2011). MCI patients were selected according to Petersen's criteria (Petersen *et al*, 1999). Clinical Dementia Rating (CDR) (Morris, 1993; Garrett *et al*, 2008) of 0.5, 1, and 2 as well as neuropsychological battery tests scores (described beneath) were indicative of MCI, mild and moderate dementia, respectively. According to our follow-up protocol, all patients had regular clinical consultations at intervals of 6 months. All participants or respective caregivers gave informed consent to participate and all the procedures were conducted in accordance with the Ethical Committee of CHUC.

II.3.2 Neuropsychological evaluation

The neuropsychological protocol included a battery of tests composed by the Mini-Mental State Examination (MMSE) Portuguese

version (Folstein *et al*, 1975; Guerreiro *et al*, 2003), the Montreal Cognitive Assessment (MoCA) Portuguese version (Nasreddine *et al*, 2005; Freitas *et al*, 2010), and the Alzheimer's Disease Assessment Scale-Cognitive subscale (ADAS-Cog) Portuguese version (Mohs *et al*, 1983; Guerreiro *et al*, 2008). Assessment was performed by experienced neuropsychologists that also administered the Portuguese versions of CDR for global staging and Geriatric Depression Scale (GDS-30) (Yesavage *et al*, 1983; Barreto *et al*, 2003) to exclude major depression. MCI patients had neuropsychological and staging reassessments at intervals of approximately 12 months in order to screen a progression to dementia stage and those that progressed to non-Alzheimer's type of dementia were excluded from the cross-sectional and longitudinal studies.

II.3.3 Anthropometric measurements

Weight, height and waist and hip circumferences were measured according to the recommendations of World Health Organization Expert Consultation report (WHO, 2008) although a flexible tape rather than a stretch-resistant tape was used. Subjects were weighted and heighted after overnight fast using the same instrument (a double ruler body scale), wearing thin clothing and with no shoes, and BMI was calculated using the following formula: BMI=body weight (kilograms)/

height^2 (meters). For waist and hip measurements subjects were standing with arms at the sides, with feet positioned close together and in a relaxed posture. Waist circumference was measured at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest and hip circumference was measured around the widest portion of the buttocks.

II.3.4 Magnetic Resonance Imaging data aquisition and imaging analysis

A sub-group of participants (36 MCI and 31 AD) underwent MRI scanning on a 3T Siemens Magnetom Trio scanner (Erlangen, Germany), using a 12-channel birdcage head coil. High-resolution 3D T1-weighted magnetization-prepared rapid gradient echo (MP-RAGE) scans were collected per participant, with parameters defined on the basis of guidelines from ADNI (Jack *et al*, 2008). Structural MRI scans were processed by the FreeSurfer 5.0.0 software package (http://surfer.nmr.mgh.harvard.edu) using fully automated methods which are elsewhere described in detail (Dale *et al*, 1999; Fischl *et al*, 1999; Han *et al*, 2006). Briefly, for each participant, cortical thickness was estimated at each point of the cortical mantle by finding the shortest distance from the white matter surface to the grey matter surface (and vice-versa) and

averaging those two values (Fischl and Dale, 2000). The neocortex was parcellated into 32 gyral-based regions-of-interest (ROI) (Fischl *et al*, 2002; Fischl *et al*, 2004) in each hemisphere, and in addition non-neocortical ROIs, such as hippocampus, were defined on the basis of automated procedures (Desikan *et al*, 2006). Total hippocampal volume was calculated by summing up right and left hippocampal volumes. For each participant, the accuracy of the grey and white matter surfaces and of each individual ROI was carefully inspected by a trained neuroradiologist.

II.3.5 Sample collection and processing

Peripheral blood and CSF samples were collected as part of patient routine diagnostic investigation. In the majority of cases, samples were collected simultaneously, although at enrollment some of the patients had already undergone lumbar puncture (in the last 12 months) and thus did not repeat this procedure.

Peripheral blood

Peripheral blood samples were collected after overnight fast (8-12 hours) between 9 and 11 am, in the median cubital vein, with the patient in a sitting or lying position.

General metabolic profile, including fasting glycemia, renal and hepatic parameters, and also serum cholesterol (total and HDL) and triglycerides (TG), were determined using commercial kits (Olympus-Diagnóstica, Produtos de Diagnóstico SA, Portugal). Low density lipoprotein (LDL) cholesterol was calculated according to the Friedewald equation (LDL-cholesterol = Total Cholesterol − HDL cholesterol - [TG/5] in mg/dl). Adiponectin, leptin and insulin concentrations were determined using Human Adiponectin and Leptin ELISA (Enzyme-Linked Immunosorbent Assay) Kit (DuoSet, R&D Systems) and Human Insulin ELISA kit (Mercodia, Sweden), respectively. The homeostatic model assessment of insulin-resistance index (HOMA-IR) was calculated as [(If)*(Gf)]/ 22.5, where (If) is the fasting insulin level (mU/ml) and (Gf) is the fasting glucose level (mmol/L).

APOE genotype was determined by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) assay after DNA isolation from whole EDTA-blood using a commercial kit (Roche Diagnostics GmbH, Manheim, Germany), as previously described (Crook *et al*, 1994).

Cerebrospinal fluid

Pre-analytical and analytical procedures were done in accordance with previously proposed protocols (del Campo *et al*, 2012). Briefly,

lumbar puncture was performed after overnight fast (8-12 hours), between 9 and 11 am, with the patient in a flexed lateral decubitus position. A 20 gauge needle was inserted in the L3/L4 or L4/L5 interspace and CSF was collected into sterile polypropylene tubes (approximately 10 ml) after discarding the first 0.5 ml. The samples were posteriorly centrifuged at 1800xg for 10 minutes at 4°C, aliquoted into polypropylene tubes and stored at −80°C until analysis at the Laboratory of Neurochemistry (CHUC, Coimbra, Portugal). Here, CSF $A\beta_{42}$, t-tau and p-tau_{181} were measured separately by commercially available sandwich ELISA kits (Innotest, Innogenetics, Ghent, Belgium) as previously described (Baldeiras *et al*, 2009). External quality control of the assays was performed under the scope of the Alzheimer's Association Quality Control Program for CSF Biomarkers (Mattsson *et al*, 2011). CSF concentrations of leptin and adiponectin were measured by commercially available sandwich ELISA (DuoSet, R&D Systems) at the Institute of Physiology (Faculty of Medicine, University of Coimbra, Coimbra, Portugal).

II.3.6 Statistical analysis

A power analysis was performed in Gpower 3.1.9.2, concerning the comparison between the serum adiponectin levels measured for both groups. The resulting power was 85.6%.

Each quantitative variable was assessed for normality resorting to Shapiro-Wilk tests and graphical analysis. When found to be normally distributed, it was described by its mean and standard deviation. Otherwise, the median, 25^{th} and 75^{th} percentiles were used instead. Categorical variables were described in terms of absolute and relative frequencies. Comparisons of quantitative variables between categories were performed with t-Student tests whenever the normality assumptions held and with Mann-Whitney U tests otherwise. Fisher or Chi-square tests were used to evaluate the association between the groups and categorical variables, whereas correlations between pairs of quantitative variables were assessed by computing Spearman's correlation coefficients. Partial Spearman correlation coefficients were also computed to account for the influence of covariables. The predictive value of progression to dementia for different quantitative variables was assessed resorting to Receiver Operating Characteristic (ROC) analysis. For each such variable, the area under the curve (AUC) and a 95% confidence interval for the AUC were computed. Additionally, the sensitivity and specificity were computed for the

threshold value of the variable that maximizes Youden's index. The analysis was performed in R version 3.3.2 and the plots in IBM SPSS Statistics 24. The level of significance adopted was 0.05.

Chapter II.4

Results

II.4.1 Elevated serum adiponectin in Alzheimer's disease as neuroprotective strategy

II.4.1.1 Baseline characteristics and adipokine profile of the study population

Demographic, clinical and genetic data

Demographic, clinical and genetic characteristics of the study population are summarized in Table 3. There were no significant differences between MCI and AD patients regarding age, gender, years of education and percentage of *APOEε4* carriers. Anthropometric measurements as well as typical vascular risk factors and co-morbid metabolic diseases were also similar in both groups. As expected, MMSE and MoCA scores were lower and ADAS-Cog scores higher in the AD group ($p<0.001$).

Table 3. Demographic, clinical and genetic data of the study population according to baseline diagnosis

	MCI n=71	AD n=53	*p*
Age (years)	73 (67; 76)	75 (68; 80)	0.060
Age onset (years)	67.33±8.30	68.53±9.08	0.507
Female gender n (%)	48 (67.6%)	37 (69.8%)	0.794
Education (years)	4 (4; 6.75)	4 (4; 9)	0.620
***APOEε4* carrier n (%)**	41 (61.2%)	23 (45.1%)	0.082
BMI	26.36±3.71	25.24±4.22	0.143
WC (cm)	96.39±10.70	92.45±11.93	0.083
HC (cm)	102 (96.25; 107)	100 (96; 103)	0.202
WHR	0.94 (0.89; 1.00)	0.91 (0.87; 0.96)	0.074
Hypertension n (%)	38 (54.3%)	19 (38%)	0.078
Type 2 DM n (%)	13 (18.6%)	13 (26%)	0.330
Dyslipidemia n (%)	45 (64.3%)	24 (48%)	0.075
Heart Failure n (%)	8 (11.4%)	2 (4%)	0.191
MetS[1] n (%)	27 (38.6%)	13 (24.1%)	0.242
MMSE	27 (25; 29)	19 (15.75; 23.25)	<0.001
MoCA	19.04±4.94	10.38±4.89	<0.001
ADAS-Cog	11 (7; 13)	21 (16; 27.25)	<0.001

Data presented as mean±standard deviation, median (1st quartile, 3rd quartile) or as number (percentage) of patients, as applicable. The p-values (*p*) included in the table were obtained with independent samples t-Student tests or Mann-Whitney U tests for quantitative variables and with chi-square tests for qualitative variables.

[1]MetS defined according to the International Diabetes Federation (IDF).

AD: Alzheimer's dementia; ADAS-Cog: Alzheimer's Disease Assessment Scale-Cognitive subscale; *APOEε4*: Apolipoprotein E allele 4; BMI: Body Mass Index; DM: Diabetes Mellitus; HC: hip circumference; MCI: Mild cognitive impairment; MetS: Metabolic Syndrome; MMSE: Mini Mental State Examination; MoCA: Montreal Cognitive Assessment. WC: waist circumference; WHR: waist-to-hip ratio.

Biochemical and biomarker data

Additional serum and CSF biochemical and biomarker data is presented in Tables 4 and 5, respectively.

Table 4. Serum biochemical data of the study population according to baseline diagnosis

	MCI	n	AD	n	*p*
Fasting glicemia (mg/dl)	89 (83; 97.25)	67	92 (86; 105)	52	0.140
Fasting insulinemia (mU/L)	4.92 (3.68; 5.86)	61	5.10 (4.21; 7.02)	52	0.252
HOMA-IR	1.11 (0.84; 1.42)	60	1.23 (0.92; 1.77)	50	0.196
Total cholesterol (mg/dl)	196 (176; 238)	60	207 (187; 233)	46	0.354
LDL-cholesterol (mg/dl)	121 (99.2; 161.4)	60	137 (115.8; 155.2)	46	0.122
HDL-cholesterol (mg/dl)	53.08± 12.70	60	54.68± 11.81	46	0.524
Triglycerides (mg/dl)	109 (80; 140)	60	93.50 (73; 130.75)	45	0.235
CRP (mg/dl)	0.23 (0.11; 0.39)	55	0.20 (0.07; 0.38)	43	0.278
Adiponectin (µg/ml)	6.52 (3.86; 9.44)	61	9.80 (6.03; 14.30)	47	0.002
Leptin (ng/ml)	9.97 (6.44; 21.79)	61	10.12 (6.64; 19.40)	51	0.698
LAR	1.87 (1.01; 4.27)	61	1.44 (0.71; 3.03)	45	0.142

Data presented as mean±standard deviation or median (1st quartile; 3rd quartile), as applicable. The p-values (*p*) included in the table were obtained with independent samples t-Student tests or Mann-Whitney U tests, as applicable.
AD: Alzheimer's dementia; CRP: C Reactive Protein; HDL: high density lipoprotein; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance; LAR: Leptin to Adiponectin Ratio; LDL: Low density lipoprotein; MCI: Mild cognitive impairment.

No significant differences were observed in glucose and lipid profiles or in C-reactive protein, a general inflammatory marker, when comparing the two groups (Table 4). However, the same did not occur when comparing serum adiponectin levels between AD and MCI

patients, with the former attaining 33% higher levels, contrarily to serum leptin and LAR, which were similar in both groups (Table 4 and Figure 15).

Meanwhile, CSF levels of these adipokines as well as CSF LAR also did not differ between the groups (Table 5 and Figure 15). Comparison of CSF biomarker levels according to diagnosis revealed, as expected, higher t-tau and p-tau, and lower $A\beta_{42}$ concentrations in the AD group ($p<0.001$) (Table 5).

Table 5. Cerebrospinal fluid biomarker and biochemical data of the study population according to baseline diagnosis

	MCI	n	AD	n	*p*
$A\beta_{42}$ (pg/mL)	703.99± 340.18	37	425.58± 162.31	32	<0.001
t-tau (pg/mL)	382.9 (228.2; 520.7)	37	615.75 (449.33; 817.68)	32	<0.001
p-tau (pg/mL)	46.60 (32.7; 64.6)	37	73 (55.075; 85.55)	32	<0.001
t-tau/$A\beta_{42}$	0.74 (0.31; 1.02)	37	1.33 (1.06; 2.50)	32	<0.001
$A\beta_{42}$/p-tau	12.30 (8.67; 27.71)	37	5.75 (4.08; 7.47)	32	<0.001
ATI	0.73 (0.52; 1.29)	37	0.41 (0.25; 0.54)	32	<0.001
Adiponectin (ng/ml)	2.57 (1.85; 4.26)	34	2.93 (1.80; 4.85)	22	0.656
Leptin (pg/ml)	163.07 (122.19; 227.29)	34	147.02 (98.58; 174.38)	22	0.135
LAR	63.63 (34.34; 121.19)	34	41.64 (27.28; 99.53)	22	0.235

Data presented as mean±standard deviation or median (1st quartile; 3rd quartile), as applicable. The p-values (*p*) included in the table were obtained with independent samples t-Student tests or Mann-Whitney U tests, as applicable.

Aβ: Amyloid beta; AD: Alzheimer's dementia; ATI: Amyloid-Tau Index; LAR: Leptin to Adiponectin Ratio; MCI: Mild cognitive impairment; t-tau: total tau; p-tau: phosphorylated tau.

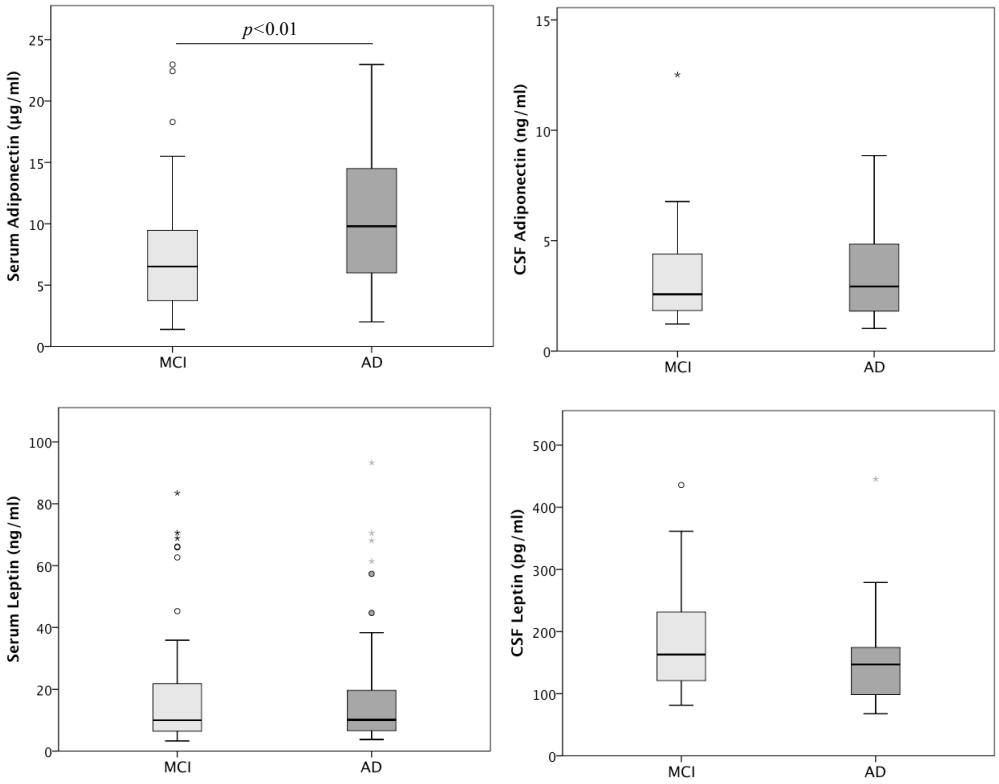


Figure 15. Serum and cerebrospinal fluid (CSF) concentrations of adiponectin and leptin among Mild cognitive impairment (MCI) and Alzheimer's dementia (AD) subjects.

Box and Whisker plots represent median, upper median, lower median and minimum to maximum range.

In order to investigate if serum and CSF levels of each adipokine were related, we conducted a correlation analysis in the combined pool of patients and in each group separately. When considering the entire study population, serum and CSF concentrations of each adipokine showed to be moderately correlated (adiponectin: ρ=0.424, p=0.011; leptin: ρ=0.496, p=0.002), while the analysis of serum and CSF LAR followed the same tendency (ρ=0.408, p=0.015) (data not shown). In the

subgroup analysis, a moderate positive correlation was found for adiponectin in both groups and for leptin in AD patients, although the correlation between CSF and serum leptin concentrations in the MCI group did not reach statistical significance (Figure 16).

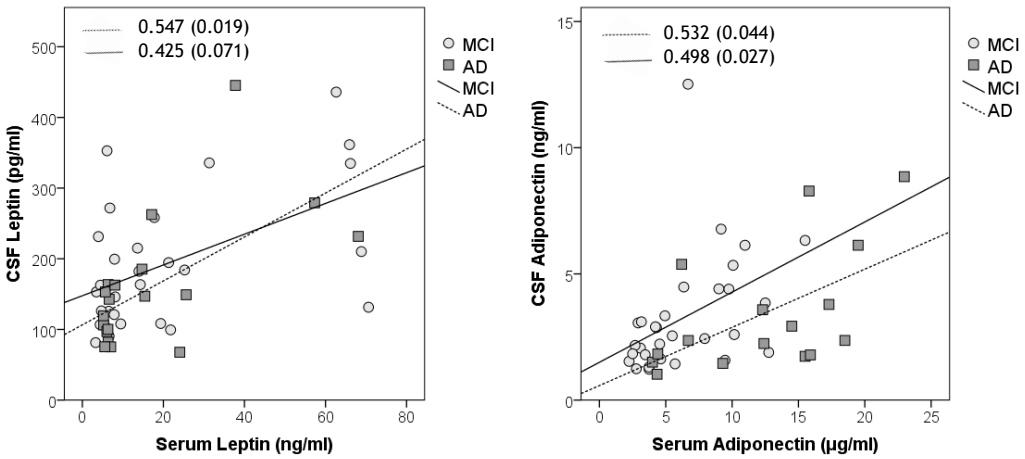


Figure 16. Correlation between serum and cerebrospinal fluid (CSF) levels of leptin and adiponectin.

Data is presented as Spearman correlation coefficient (p-value).

AD: Alzheimer's dementia; MCI: Mild cognitive impairment.

II.4.1.2 Serum adiponectin as staging biomarker of Alzheimer´s disease

A logistic regression model was performed where the patient status (MCI or AD) was taken as the dependent variable and the following independent variables were considered: serum and CSF leptin, serum and CSF adiponectin, insulin and HOMA-IR. Out of the

independent variables, only serum adiponectin was found to significantly contribute to the model. Applying backward elimination, only this variable remained significant, which achieved statistical significance: $\chi^2(1)=9.547$, $p=0.002$, Nagelkerke $R^2=0.114$. A ROC analysis was performed to evaluate the predictive value of serum adiponectin, with the 95% confidence interval for the area under the curve being [0.57, 0.78]. The cut-off 10.85 μg/ml maximized the sum of the specificity and sensitivity, which are 87% and 44%, respectively (Figure 17).

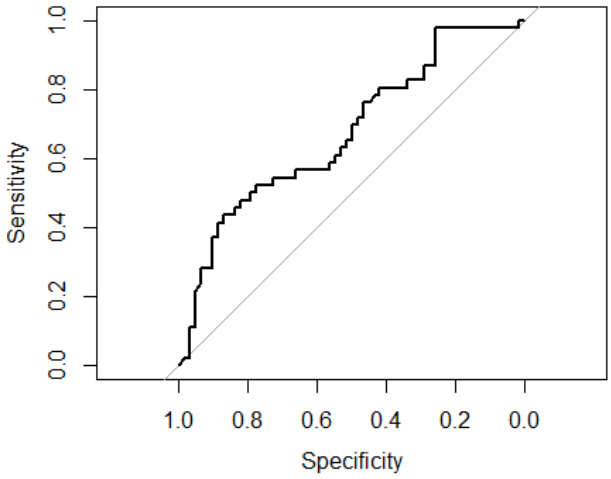


Figure 17. ROC curve for serum adiponectin as predictor of Alzheimer's dementia diagnosis. The reference line represents the ROC (receiver operating characteristic) curve for a statistical test with low discriminatory ability. The area under the ROC curve was 0.673 (95%CI: 0.57-0.78) and cut-off level was 10.85 μg/ml (sensitivity=0.44; specificity=0.87).

II.4.1.3 Alzheimer's disease risk factors and adipokine levels

II.4.1.3.1 Non-modifiable Alzheimer's disease risk factors

In order to determine to which extent the most well-established non-modifiable risk factors for Alzheimer's disease (female gender, age and *APOEε4*) were associated with the two most abundant adipokines, we performed comparative or correlational analysis as appropriated.

Gender

We grouped patients according to gender to compare serum and CSF levels of both adipokines. As expected, serum levels of adiponectin and leptin as well as their ratio were significantly higher in female. Surprisingly, CSF concentrations of these adipokines and CSF LAR did not reflect the differences found in the periphery and were similiar in both gender (Table 6).

Age

In our population, age was positively correlated with serum adiponectin concentration while no association with serum leptin or LAR was observed. Interestingly, CSF leptin and adiponectin concentrations as well as their ratio correlated differently with age. While

CSF adiponectin was higher in older patients, the opposite was observed with CSF leptin and LAR (Table 7).

Table 6. Serum and cerebrospinal fluid adipokine concentration in male and female patients

	Female	n	Male	n	*p*
Serum adiponectin (µg/ml)	8.29 (5.46; 11.10)	75	6.12 (3.45; 9.60)	33	0.034
Serum leptin (ng/ml)	16.23 (7.76; 28.46)	78	6.52 (5.12; 9.13)	34	<0.001
Serum LAR	1.86 (1.128; 4.45)	74	1.15 (0.62; 2.16)	32	0.014
CSF adiponectin (ng/ml)	2.85 (1.82; 3.99)	32	2.37 (1.84; 5.38)	24	0.942
CSF leptin (pg/ml)	163.09 (117.87; 259.38)	32	153.04 (106.75; 182.25)	24	0.118
CSF LAR	53.20 (34.87; 129.66)	32	58.73 (27.92; 95.39)	24	0.252

Data presented as median (1st quartile, 3rd quartile). The p-values (*p*) included in the table were obtained with Mann-Whitney U tests.
CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio.

Table 7. Correlation between adipokine concentration and age

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
Age	0.291 (0.002)	0.070 (0.468)	-0.063 (0.521)	0.368 (0.007)	-0.294 (0.032)	-0.391 (0.004)
	0.326 (0.001)[1]	0.134 (0.186)[1]	-0.067 (0.521)[1]	0.406 (0.007)[1]	-0.366 (0.016)[1]	-0.437 (0.003)[1]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Gender and BMI.
CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio.

APOE genotype

Additionally, we grouped patients according to their *APOE* genotype, comparing ε4 allele carriers (1 or 2 alleles) with non-carriers.

No differences were found in serum or CSF adipokine concentrations between the two groups (Table 8).

Table 8. Serum and cerebrospinal fluid adipokine concentration according to *APOEε4* status

	***APOEε4* non-carrier**	**n**	***APOEε4* carrier**	**n**	***p***
Serum adiponectin (µg/ml)	6.74 (4.22; 10.10)	58	7.99 (5.31; 12.57)	50	0.129
Serum leptin (ng/ml)	9.68 (6.05; 21.87)	62	12.11 (6.91; 22.35)	50	0.489
Serum LAR	1.555 (0.91; 4.25)	58	1.65 (0.93; 2.98)	48	0.645
CSF adiponectin (ng/ml)	2.69 (1.68; 4.46)	27	2.37 (1.89; 3.85)	29	0.880
CSF leptin (pg/ml)	153.04 (119.75; 231.60)	27	162.68 (99.37; 199.43)	29	0.547
CSF LAR	54.25(34.34; 121.19)	27	54.17 (29.74; 90.48)	29	0.678

Data presented as median (1st quartile, 3rd quartile). The p-values (*p*) included in the table were obtained with Mann-Whitney U tests.

APOEε4: apolipoprotein E allele 4; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio.

II.4.1.3.2 Modifiable Alzheimer's disease risk factors

Obesity

In our population, serum and CSF leptin levels were positively correlated with all anthropometric measures evaluated, although the strongest association was observed between serum leptin and BMI, after adjustment for age and gender. On the other hand, neither serum or

CSF adiponectin levels correlated with these parameters. Although serum LAR was positively correlated with BMI , WC and HC, the correlation with WHR did not reach statistical significancy. Furthermore, CSF LAR was correlated with WC and WHR, but not with the other anthropometric measures (Table 9).

Table 9. Correlation between adipokine concentration and anthropometric measurements

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
BMI	-0.088 (0.387)	0.555 (0.000)	0.488 (0.000)	-0.149 (0.330)	0.386 (0.009)	0.289 (0.054)
	-0.056 (0.589)[1]	0.632 (0.000)[1]	0.495 (0.000)[1]	-0.126 (0.422)[1]	0.411 (0.006)[1]	0.300 (0.051)[1]
WC	-0.122 (0.247)	0.432 (0.000)	0.433 (0.000)	-0.127 (0.441)	0.280 (0.085)	0.204 (0.214)
	-0.089 (0.403)[1]	0.551 (0.000)[1]	0.473 (0.000)[1]	-0.181 (0.283)[1]	0.419 (0.010)[1]	0.329 (0.047)[1]
HC	-0.048 (0.649)	0.528 (0.000)	0.497 (0.000)	-0.110 (0.504)	0.339 (0.035)	0.246 (0.131)
	-0.053 (0.617)[1]	0.591 (0.000)[1]	0.514 (0.000)[1]	-0.110 (0.516)[1]	0.328 (0.048)[1]	0.239 (0.154)[1]
WHR	-0.175 (0.096)	0.080 (0.444)	0.143 (0.179)	-0.152 (0.355)	0.122 (0.460)	0.123 (0.454)
	-0.106 (0.322)[1]	0.236 (0.024)[1]	0.207 (0.053)[1]	-0.247 (0.140)[1]	0.405 (0.013)[1]	0.365 (0.026)[1]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Age and Gender.

CSF: cerebrospinal fluid; BMI: body mass index; HC: hip circumference; LAR: leptin to adiponectin ratio; WC: waist circumference; WHR: waist to hip ratio.

Insulin resistance

In healthy conditions, both leptin and adiponectin act as peripheral insulin-sensitizers. Thus, as expected, serum adiponectin concentration was negatively correlated with insulinemia and HOMA-

IR in the combined pool of patients. On the contrary, serum leptin concentration was positively correlated with both these parameters, i.e., higher leptin levels were associated with higher insulin resistance, resembling an obesity-like profile (Table 10). Interestingly, the same pattern of results was found in the CSF but only for adiponectin and LAR, while CSF leptin showed no correlation with insulinemia or HOMA-IR. Furthermore, fasting glycemia was weakly correlated with serum leptin and LAR but no association was found with serum adiponectin or with any of the CSF adipokine concentration (Table 10).

Table 10. Correlation between adipokine concentration and glucose metabolism parameters

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
Fasting glycemia	-0.082 (0.404)	0.221 (0.021)	0.237 (0.016)	-0.175 (0.225)	0.092 (0.526)	0.188 (0.191)
	-0.082 (0.406)[1]	0.218 (0.024)[1]	0.224 (0.024)[1]	-0.196 (0.175)[1]	0.109 (0.457)[1]	0.218 (0.130)[1]
	-0.065 (0.534)[2]	0.213 (0.037)[2]	0.231 (0.028)[2]	-0.180 (0.260)[2]	-0.033 (0.836)[2]	0.115 (0.475)[2]
Fasting insulinemia	-0.304 (0.002)	0.469 (0.000)	0.458 (0.000)	-0.293 (0.053)	0.423 (0.004)	0.449 (0.002)
	-0.309(0.001)[1]	0.504 (0.000)[1]	0.453 (0.000)[1]	-0.288 (0.057)[1]	0.409 (0.005)[1]	0.445 (0.002)[1]
	-0.356 (0.000)[2]	0.404 (0.000)[2]	0.403 (0.000)[2]	-0.441 (0.006)[2]	0.179 (0.311)[2]	0.360 (0.032)[2]
HOMA-IR	-0.290 (0.004)	0.455 (0.000)	0.471 (0.000)	-0.339 (0.028)	0.385 (0.012)	0.462 (0.002)
	-0.301(0.002)[1]	0.471 (0.000)[1]	0.458 (0.000)[1]	-0.342 (0.025)[1]	0.359 (0.018)[1]	0.452 (0.002)[1]
	-0.362 (0.000)[2]	0.382 (0.000)[2]	0.439 (0.000)[2]	-0.495 (0.002)[2]	0.203 (0.257)[2]	0.422 (0.011)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Age, Gender [2] Partial correlation corrected for Age, Gender and BMI.

CSF: cerebrospinal fluid; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance; LAR: leptin to adiponectin ratio.

When analyzing the low $A\beta_{42}$/p-tau group, we found an even stronger negative correlation between insulinemia and HOMA-IR and CSF adiponectin, while the correlation between HOMA-IR and serum adipokines had a similar profile to that found in the combined pool of patients with clinical diagnosis of MCI or AD (Table 11).

Table 11. Correlation between adipokine concentratrion and glucose metabolism parameters in low $A\beta_{42}$/p-tau patients*

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
Fasting glycemia	-0.234 (0.114)	0.359 (0.011)	-0.231 (0.168)	0.155 (0.361)	0.349 (0.017)	0.240 (0.152)
	-0.279 (0.063)[1]	0.379 (0.009)[1]	0.354 (0.018)[1]	-0.251 (0.146)[1]	0.164 (0.346)[1]	0.279 (0.105)[1]
	-0.154 (0.355)[2]	0.225 (0.163)[2]	0.187 (0.268)[2]	-0.094 (0.636)[2]	-0.096 (0.628)[2]	0.039 (0.844)[2]
Fasting insulinemia	-0.375 (0.013)	0.502 (0.000)	-0.354 (0.043)	0.501 (0.003)	0.495 (0.001)	0.509 (0.003)
	-0.349 (0.024)[1]	0.560 (0.000)[1]	0.498 (0.001)[1]	-0.339 (0.062)[1]	0.504 (0.004)[1]	0.532 (0.002)[1]
	-0.322 (0.063)[2]	0.508 (0.002)[2]	0.447 (0.009)[2]	-0.571 (0.004)[2]	0.318 (0.139)[2]	0.485 (0.019)[2]
HOMA-IR	-0.418 (0.006)	0.506 (0.001)	-0.489 (0.004)	0.483 (0.005)	0.536 (0.000)	0.578 (0.001)
	-0.413 (0.007)[1]	0.537 (0.000)[1]	0.527 (0.000)[1]	-0.449 (0.013)[1]	0.445 (0.014)[1]	0.566 (0.001)[1]
	-0.438 (0.010)[2]	0.444 (0.008)[2]	0.486 (0.004)[2]	-0.630 (0.001)[2]	0.263 (0.225)[2]	0.523 (0.010)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Age, Gender [2] Partial correlation corrected for Age, Gender and BMI. *$A\beta_{42}$/p-tau<17.7.
CSF: cerebrospinal fluid; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance; LAR: leptin to adiponectin ratio.

II.4.1.4 Alzheimer´s disease biomarkers and adipokine concentration

Cerebrospinal fluid biomarkers

Out of the three core CSF Alzheimer´s disease biomarkers, only t-tau has shown a significant negative correlation with CSF leptin in AD patients after adjustments for age, gender and BMI. In this group no other adipokine or ratio correlated with these biomarkers (Table 12).

Table 12. Correlation between adipokine concentration and cerebrospinal fluid Alzheimer´s disease biomarkers in AD patients

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
$A\beta_{42}$	0.182 (0.363)	-0.269 (0.151)	-0.168 (0.413)	0.000 (1.000)	0.195 (0.423)	0.079 (0.748)
	-0.101 (0.628)[1]	-0.282 (0.146)[1]	-0.034 (0.873)[1]	-0.283 (0.253)[1]	0.356 (0.141)[1]	0.389 (0.101)[1]
	0.100 (0.680)[2]	-0.389 (0.074)[2]	-0.184 (0.464)[2]	-0.090 (0.787)[2]	0.272 (0.397)[2]	0.114 (0.731)[2]
t-tau	-0.140 (0.488)	-0.129 (0.498)	0.007 (0.974)	0.228 (0.346)	-0.425 (0.071)	-0.433 (0.065)
	-0.049 (0.813)[1]	-0.166 (0.399)[1]	-0.055 (0.800)[1]	0.276 (0.266)[1]	-0.435 (0.061)[1]	-0.557 (0.009)[1]
	-0.214 (0.365)[2]	-0.162 (0.463)[2]	0.024 (0.924)[2]	0.020 (0.952)[2]	-0.597 (0.026)[2]	-0.511 (0.074)[2]
p-tau	-0.267 (0.178)	- 0.164 (0.386)	0.057 (0.780)	-0.139 (0.570)	-0.049 (0.843)	-0.019 (0.940)
	-0.172 (0.401)[1]	-0.300 (0.121)[1]	-0.063 (0.770)[1]	-0.053 (0.837)[1]	-0.142 (0.579)[1]	-0.201 (0.427)[1]
	-0.233 (0.323)[2]	-0.299 (0.176)[2]	-0.008 (0.974)[2]	-0.208 (0.524)[2]	0.064 (0.847)[2]	-0.026 (0.938)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Age and Gender [2] Partial correlation corrected for Age, Gender and BMI.

Aβ: Amyloid beta; AD: Alzheimer's dementia; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio; t-tau: total tau; p-tau: phosphorylated tau.

In MCI patients we found no relation between serum or CSF adipokines and CSF $A\beta_{42}$, t-tau and p-tau (Table 13).

Table 13. Correlation between adipokine concentration and cerebrospinal fluid Alzheimer´s disease biomarkers in MCI patients

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
$A\beta_{42}$	-0.024 (0.893)	0.133 (0.459)	0.031 (0.862)	0.029 (0.873)	-0.035 (0.848)	0.008 (0.964)
	-0.045 (0.804)[1]	0.169 (0.363)[1]	0.050 (0.786)[1]	0.029 (0.880)[1]	-0.021 (0.911)[1]	0.019 (0.921)[1]
	0.018 (0.926)[2]	0.154 (0.443)[2]	-0.057 (0.772)[2]	-0.082 (0.688)[2]	0.013 (0.949)[2]	0.106 (0.601)[2]
t-tau	0.088 (0.621)	0.079 (0.662)	0.027 (0.879)	0.021 (0.911)	-0.306 (0.089)	-0.249 (0.170)
	0.059 (0.746)[1]	0.032 (0.865)[1]	-0.017 (0.926)[1]	0.020 (0.914)[1]	-0.313 (0.081)[1]	-0.251 (0.170)[1]
	0.077 (0.695)[2]	0.201 (0.315)[2]	0.046 (0.818)[2]	-0.009 (0.964)[2]	-0.254 (0.198)[2]	-0.216 (0.278)[2]
p-tau	-0.068 (0.703)	0.112 (0.532)	0.149 (0.400)	0.005 (0.978)	-0.140 (0.442)	-0.112 (0.540)
	-0.070 (0.703)[1]	0.116 (0.533)[1]	0.132 (0.473)[1]	0.003 (0.988)[1]	-0.175 (0.348)[1]	-0.130 (0.488)[1]
	-0.043 (0.826)[2]	-0.014 (0.945)[2]	0.026 (0.894)[2]	0.086 (0.672)[2]	-0.310 (0.110)[2]	-0.284 (0.147)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Age and Gender [2] Partial correlation corrected for Age, Gender and BMI.
Aβ: Amyloid beta; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio; MCI: Mild cognitive impairment; t-tau: total tau; p-tau: phosphorylated tau.

Noteworthy, considering the low $A\beta_{42}$/p-tau subgroup, CSF leptin and t-tau also showed a tendency to correlate, though it did not reach statistical significancy. No other serum or CSF parameter was related with CSF Alzheimer´s disease biomarkers (Table 14).

Table 14. Correlation between adipokine concentration and cerebrospinal fluid Alzheimer´s disease biomarkers in patients with low $A\beta_{42}$/p-tau*

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
$A\beta_{42}$	0.088 (0.552)	0.001 (0.992)	0.131 (0.433)	0.026 (0.875)	-0.003 (0.984)	-0.071 (0.670)
	-0.057 (0.704)[1]	-0.052 (0.725)[1]	-0.016 (0.918)[1]	0.085 (0.621)[1]	0.153 (0.374)[1]	0.012 (0.943)[1]
	0.086 (0.609)[2]	-0.192 (0.236)[2]	-0.186 (0.271)[2]	0.318 (0.099)[2]	-0.134 (0.496)[2]	-0.233 (0.234)[2]
t-tau	0.086 (0.560)	0.096 (0.508)	0.180 (0.279)	-0.343 (0.035)	0.026 (0.861)	-0.329 (0.043)
	0.095 (0.531)[1]	0.084 (0.568)[1]	0.015 (0.923)[1]	0.119 (0.488)[1]	-0.324 (0.054)[1]	-0.306 (0.069)[1]
	0.077 (0.645)[2]	0.158 (0.330)[2]	0.038 (0.824)[2]	-0.027 (0.892)[2]	-0.352 (0.066)[2]	-0.232 (0.234)[2]
p-tau	-0.109 (0.459)	-0.016 (0.912)	-0.103 (0.538)	-0.156 (0.349)	0.081 (0.588)	-0.017 (0.922)
	-0.049 (0.744)[1]	-0.081 (0.585)[1]	0.042 (0.785)[1]	-0.130 (0.450)[1]	-0.172 (0.315)[1]	-0.011 (0.949)[1]
	-0.054 (0.747)[2]	-0.106 (0.516)[2]	0.004 (0.981)[2]	-0.147 (0.455)[2]	-0.205 (0.296)[2]	-0.019 (0.923)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Age and Gender [2] Partial correlation corrected for Age, Gender and BMI. *$A\beta_{42}$/p-tau<17.7. Aβ: Amyloid beta; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio; t-tau: total tau; p-tau: phosphorylated tau.

Additionally, and driven by the evidence that Alzheimer´s disease is disproportionally incident in women and that there is also a gender dimorphism regarding serum adipokine concentration, we performed a correlation analysis between these adipokines and CSF biomarkers in this population. Interestingly, in female patients, a strong positive correlation was found between CSF adiponectin and $A\beta_{42}$, even after adjustments for age and BMI meaning that higher CSF adiponectin levels are associated with higher CSF $A\beta_{42}$ which indicates less aggregated and deposited amyloid (Table 15).

Table 15. Correlation between adipokine concentration and cerebrospinal fluid Alzheimer´s disease biomarkers in female patients

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
$A\beta_{42}$	0.139 (0.397)	0.143 (0.380)	0.051 (0.757)	0.626 (0.000)	-0.077 (0.679)	-0.358 (0.049)
	-0.038 (0.823)[1]	0.194 (0.242)[1]	0.109 (0.520)[1]	0.545 (0.002)[1]	0.068 (0.726)[1]	-0.206 (0.284)[1]
	0.087 (0.643)[2]	-0.065 (0.724)[2]	-0.143 (0.444)[2]	0.590 (0.002)[2]	-0.268 (0.205)[2]	-0.381 (0.066)[2]
t-tau	0.060 (0.716)	-0.174 (0.283)	-0.131 (0.428)	-0.089 (0.634)	-0.334 (0.067)	-0.194 (0.296)
	0.071 (0.678)[1]	-0.174 (0.297)[1]	-0.133 (0.434)[1]	-0.217 (0.257)[1]	-0.293 (0.123)[1]	-0.117 (0.544)[1]
	0.067 (0.720)[2]	-0.179 (0.327)[2]	-0.116 (0.533)[2]	-0.158 (0.461)	-0.226 (0.288)[2]	-0.104 (0.628)[2]
p-tau	-0.012 (0.942)	-0.213 (0.187)	-0.111 (0.501)	-0.229 (0.214)	-0.325 (0.074)	-0.079 (0.674)
	0.033 (0.846)[1]	-0.217 (0.190)[1]	-0.123 (0.467)[1]	-0.314 (0.097)[1]	-0.321 (0.090)[1]	-0.046 (0.811)[1]
	0.034 (0.857)[2]	-0.343 (0.054)[2]	-0.192 (0.300)[2]	-0.226 (0.288)[2]	-0.329 (0.117)[2]	-0.095 (0.658)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Age [2] Partial correlation corrected for Age and BMI.
Aβ: Amyloid beta; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio; t-tau: total tau; p-tau: phosphorylated tau.

Imaging biomarkers

We also assessed the correlation between serum and CSF adipokine concentration and MRI estimates of entorhinal and parahipocampal cortices thickness and hippocampal volume, once atrophy of medial temporal lobe is a well-recognized feature of Alzheimer´s disease.

When considering the entire pool of patients with memory impairment no correlation was observed between adipokine concentration and MTL volumetry (data not shown). However, when we grouped patients with CSF Alzheimer´s disease profile (low $A\beta_{42}$/p-tau),

CSF adiponectin concentration was positively corrrelated with total hippocampal volume (ρ=0.527, p=0.025) and right hippocampal volume (ρ=0.483, p=0.046), even though the correlation with left hippocampal volume was rendered non-significant after BMI adjustment (ρ=0.428, p=0.088) (Figure 18). In addition, a positive correlation between CSF adiponectin and left parahippocampal cortical thickness was also observed.

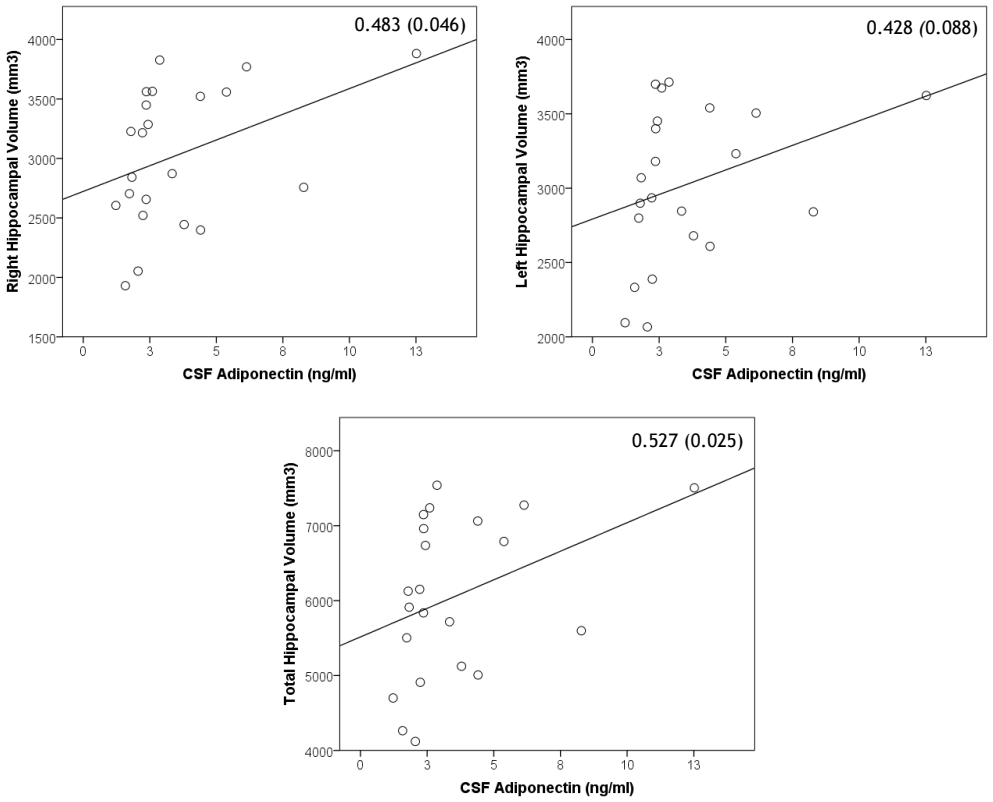


Figure 18. Correlation between cerebrospinal fluid adiponectin concentration and right, left and total hippocampal volumes in patients with low Aβ$_{42}$/p-tau*.

Data presented as Spearman correlation coefficient (p-value). *Aβ$_{42}$/p-tau<17.7.

CSF: cerebrospinal fluid.

Even though serum adiponectin was positively correlated with left hippocampal volume, no association was found with the remained parameters (Table 16). Serum and CSF leptin as well as LAR did not correlate with none of the volumes estimated (Table 16).

Furthermore, and based on the fact that women with Alzheimer´s disease seem to experience faster hippocampal atrophy (Mazure and Swendsen, 2016), we performed a correlation analysis between adipokine concentrations and MRI volumetric measures of MTL structures in this subgroup, though no consistent correlation was observed (Table 17).

Table 16. Correlation between adipokine concentration and medial temporal lobe volumetry in patients with low Aβ42/p-tau*

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
LH EC thickness	-0.152 (0.391) 0.009 (0.960)[1] 0.043 (0.825)[2]	0.069 (0.709) 0.052 (0.790)[1] -0.001 (0.996)[2]	0.061 (0.738) 0.052 (0.784)[1] -0.025 (0.900)[2]	-0.185 (0.411) 0.251 (0.284)[1] 0.206 (0.448)[2]	0.538 (0.010) 0.420 (0.056)[1] 0.310 (0.240)[2]	0.364 (0.096) 0.052 (0.831)[1] -0.049 (0.860)[2]
LH PHC thickness	0.240 (0.172) 0.325 (0.064)[1] 0.331 (0.074)[2]	-0.159 (0.382) -0.233 (0.224)[1] -0.235 (0.248)[2]	-0.299 (0.090) -0.321 (0.084)[1] -0.374 (0.055)[2]	0.162 (0.469) 0.557 (0.006)[1] 0.547 (0.018)[2]	0.147 (0.511) -0.026 (0.913)[1] -0.066 (0.812)[2]	-0.108 (0.632) -0.446 (0.040)[1] -0.446 (0.072)[2]
RH EC thickness	-0.217 (0.217) 0.061 (0.743)[1] 0.165 (0.393)[2]	-0.099 (0.587) -0.091 (0.638)[1] -0.204 (0.298)[2]	-0.029 (0.875) -0.046 (0.810)[1] -0.213 (0.275)[2]	-0.127 (0.572) 0.041 (0.866)[1] -0.020 (0.942)[2]	0.398 (0.068) 0.373 (0.098)[1] 0.352 (0.175)[2]	0.256 (0.249) 0.148 (0.538)[1] 0.144 (0.599)[2]
RH PHC thickness	0.060 (0.735) 0.210 (0.247)[1] 0.257 (0.175)[2]	-0.156 (0.393) -0.305 (0.108)[1] -0.227 (0.266)[2]	-0.360 (0.040) -0.415 (0.022)[1] -0.213 (0.286)[2]	-0.066 (0.772) 0.269 (0.249)[1] 0.318 (0.227)[2]	0.087 (0.700) -0.107 (0.657)[1] -0.096 (0.727)[2]	0.108 (0.631) -0.183 (0.443)[1] -0.196 (0.472)[2]
LH HV	0.178 (0.313) 0.344 (0.049)[1] 0.372 (0.041)[2]	-0.168 (0.357) -0.101 (0.603)[1] -0.074 (0.720)[2]	-0.157 (0.384) -0.163 (0.388)[1] -0.166 (0.408)[2]	0.458 (0.033) 0.324 (0.158)[1] 0.428 (0.088)[2]	-0.150 (0.505) 0.030 (0.902)[1] 0.063 (0.819)[2]	-0.302 (0.171) -0.124 (0.606)[1] -0.234 (0.386)[2]
RH HV	0.053 (0.767) 0.195 (0.284)[1] 0.198 (0.303)[2]	-0.212 (0.244) -0.166 (0.389)[1] -0.129 (0.531)[2]	-0.144 (0.424) -0.148 (0.436)[1] -0.108 (0.593)[2]	0.487 (0.023) 0.368 (0.102)[1] 0.483 (0.046)[2]	-0.193 (0.389) -0.035 (0.887)[1] 0.017 (0.951)[2]	-0.361 (0.100) -0.206 (0.385)[1] -0.321 (0.221)[2]
Total HV	0.128 (0.467) 0.296 (0.096)[1] 0.302 (0.106)[2]	-0.226 (0.213) -0.183 (0.343)[1] -0.145 (0.480)[2]	-0.189 (0.292) -0.201 (0.286)[1] -0.169 (0.400)[2]	0.502 (0.019) 0.413 (0.061)[1] 0.527 (0.025)[2]	-0.154 (0.492) 0.001 (0.998)[1] 0.014 (0.958)[2]	-0.345 (0.116) -0.211 (0.373)[1] -0.338 (0.196)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Estimated Intracranial Volume, Age and Gender; [2]Partial correlation corrected for Estimated Intracranial Volume Age, Gender and BMI. *Aβ42/p-tau<17.7.

Aβ: Amyloid beta; CSF: cerebrospinal fluid; EC: entorhinal cortex; HV: hippocampal volume; LAR: leptin to adiponectin ratio; LH: left hemisphere; p-tau: phosphorylated tau; PHC: parahippocampal cortex; RH: right hemisphere. (n=33 in serum determinations; n=22 in CSF determinations)

Table 17. Correlation between adipokine concentration and medial temporal lobe volumetry in female patients

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
LH EC thickness	-0.005 (0.976)	0.088 (0.594)	0.036 (0.826)	-0.112 (0.656)	0.404 (0.098)	0.327 (0.185)
	0.074 (0.660)[1]	0.068 (0.689)[1]	-0.006 (0.971)[1]	0.039 (0.887)[1]	0.385 (0.141)[1]	0.271 (0.311)[1]
	0.101 (0.563)[2]	0.079 (0.656)[2]	-0.031 (0.863)[2]	-0.056 (0.855)[2]	0.243 (0.424)[2]	0.193 (0.527)[2]
LH PHC thickness	0.197 (0.222)	-0.086 (0.602)	-0.209 (0.202)	0.214 (0.393)	0.104 (0.680)	-0.049 (0.850)
	0.241 (0.146)[1]	-0.139 (0.411)[1]	-0.262 (0.117)[1]	0.379 (0.148)[1]	0.046 (0.866)[1]	-0.154 (0.568)[1]
	0.222 (0.200)[2]	-0.040 (0.822)[2]	-0.201 (0.253)[2]	0.415 (0.159)[2]	0.076 (0.806)[2]	-0.146 (0.635)[2]
RH EC thickness	-0.129 (0.425)	0.031 (0.852)	0.018 (0.912)	-0.119 (0.638)	0.292 (0.239)	0.263 (0.290)
	0.009 (0.957)[1]	0.010 (0.954)[1]	-0.054 (0.751)[1]	0.066 (0.808)[1]	0.231 (0.390)[1]	0.148 (0.585)[1]
	0.075 (0.670)[2]	-0.147 (0.408)[2]	-0.200 (0.256)[2]	0.057 (0.854)[2]	0.160 (0.602)[2]	0.096 (0.754)[2]
RH PHC thickness	0.183 (0.258)	-0.095 (0.566)	-0.206 (0.209)	0.005 (0.987)	0.063 (0.805)	0.137 (0.586)
	0.242 (0.144)[1]	-0.146 (0.390)[1]	-0.266 (0.111)[1]	0.139 (0.608)[1]	0.028 (0.917)[1]	0.057 (0.833)[1]
	0.207 (0.234)[2]	-0.148 (0.405)[2]	-0.273 (0.118)[2]	0.273 (0.366)[2]	0.221 (0.469)[2]	0.075 (0.808)[2]
LH HV	0.101 (0.534)	-0.120 (0.467)	-0.116 (0.482)	0.352 (0.152)	-0.201 (0.422)	-0.366 (0.135)
	0.152 (0.363)[1]	-0.133 (0.434)[1]	-0.145 (0.391)[1]	0.349 (0.186)[1]	-0.247 (0.356)[1]	-0.382 (0.145)[1]
	0.133 (0.446)[2]	-0.318 (0.067)[2]	-0.257 (0.142)[2]	0.364 (0.221)[2]	-0.379 (0.202)[2]	-0.550 (0.051)[2]
RH HV	0.075 (0.646)	-0.121 (0.463)	-0.106 (0.519)	0.416 (0.087)	-0.156 (0.536)	-0.323 (0.191)
	0.127 (0.449)[1]	-0.153 (0.367)[1]	-0.151 (0.373)[1]	0.417 (0.108)[1]	-0.158 (0.559)[1]	-0.309 (0.244)[1]
	0.125 (0.476)[2]	-0.315 (0.070)[2]	-0.247 (0.159)[2]	0.428 (0.145)[2]	-0.290 (0.337)[2]	-0.485 (0.093)[2]
Total HV	0.079 (0.626)	-0.096 (0.561)	-0.086 (0.604)	0.457 (0.058)	-0.166 (0.509)	-0.366 (0.135)
	0.111 (0.509)[1]	-0.122 (0.470)[1]	-0.120 (0.480)[1]	0.474 (0.064)[1]	-0.183 (0.496)[1]	-0.368 (0.160)[1]
	0.106 (0.544)[2]	-0.299 (0.086)[2]	-0.235 (0.181)[2]	0.482 (0.096)[22]	-0.361 (0.225)[2]	-0.560 (0.046)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Estimated Intracranial Volume and Age; [2]Partial correlation corrected for Estimated Intracranial Volume Age and BMI.

Aβ: Amyloid beta; CSF: cerebrospinal fluid; EC: entorhinal cortex; HV: hippocampal volume; LAR: leptin to adiponectin ratio; LH: left hemisphere; p-tau: phosphorylated tau; PHC: parahippocampal cortex; RH: right hemisphere.

II.4.1.5 Neuropsychological tests and adipokine concentration

All patients performed MMSE, MoCA and ADAS-Cog and total scores were investigated in its correlation with serum and CSF levels of both adipokines. Only serum leptin levels demonstrated a tendency to correlate with lower MoCA and higher ADAS-cog scores, i.e., with worse cognitive performance, in the whole cohort after gender, age, education and BMI adjustments, although not reaching statistical significance (Table 18).

Table 18. Correlation between adipokine concentration and neuropsychological tests

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
MMSE	-0.159 (0.117)	-0.129 (0.200)	0.003 (0.977)	0.113 (0.443)	0.126 (0.393)	0.013 (0.932)
	-0.090 (0.382)[1]	-0.046 (0.655)[1]	0.041 (0.697)[1]	0.195 (0.191)[1]	0.169 (0.261)[1]	0.001 (0.993)[1]
	-0.022 (0.842)[2]	-0.149 (0.161)[2]	-0.056 (0.606)[2]	0.203 (0.219)[2]	0.064 (0.705)[2]	-0.057 (0.733)[2]
MoCA	-0.178 (0.211)	-0.071 (0.614)	0.058 (0.689)	0.159 (0.409)	0.013 (0.947)	-0.075 (0.698)
	-0.041 (0.778)[1]	0.060 (0.680)[1]	0.108 (0.469)[1]	0.216 (0.278)[1]	0.091 (0.655)[1]	-0.054 (0.792)[1]
	0.056 (0.712)[2]	-0.287 (0.051)[2]	-0.146 (0.345)[2]	0.186 (0.385)[2]	-0.143 (0.508)[2]	-0.166 (0.441)[2]
ADAS-Cog	0.163 (0.191)	-0.010 (0.936)	-0.086 (0.491)	-0.293 (0.083)	-0.001 (0.997)	0.156 (0.362)
	0.133 (0.296)[1]	-0.056 (0.662)[1]	-0.100 (0.435)[1]	-0.254 (0.143)[1]	-0.046 (0.800)[1]	0.095 (0.594)[1]
	0.085 (0.522)[2]	0.250 (0.056)[2]	0.112 (0.402)[2]	-0.220 (0.251)[2]	0.104 (0.593)[2]	0.138 (0.478)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Gender, Age and Education; [2]Partial correlation corrected for Gender, Age, Education and BMI.

ADAS-Cog: Alzheimer's Disease Assessment Scale-Cognitive subscale; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio; MMSE: Mini Mental State Examination; MoCA: Montreal Cognitive Assessment.

Similarly, the same analysis in the subgroup of patients with Alzheimer's disease CSF profile did not shown any correlation between the results from the neuropsychological tests and adipokine concentrations (Table 19).

Table 19. Correlation between adipokine concentration and neuropsychological tests in patients with low $A\beta_{42}$/p-tau*

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
MMSE	-0.219 (0.153)	-0.150 (0.325)	0.071 (0.649)	0.132 (0.458)	0.104 (0.558)	-0.054 (0.762)
	-0.250 (0.115)[1]	-0.015 (0.927)[1]	0.122 (0.453)[1]	0.057 (0.760)[1]	0.203 (0.274)[1]	0.053 (0.776)[1]
	-0.128 (0.464)[2]	-0.096 (0.578)[2]	0.021 (0.906)[2]	0.210 (0.324)[2]	0.014 (0.948)[2]	-0.041 (0.851)[2]
MoCA	-0.142 (0.551)	0.026 (0.914)	0.154 (0.530)	0.555 (0.026)	0.078 (0.773)	-0.299 (0.261)
	-0.125 (0.633)[1]	0.327 (0.201)[1]	0.357 (0.175)[1]	0.279 (0.357)[1]	0.346 (0.248)[1]	0.050 (0.872)[1]
	0.143 (0.610)[2]	-0.422 (0.117)[2]	-0.261 (0.368)[2]	0.221 (0.513)[2]	0.097 (0.777)[2]	0.065 (0.849)[2]
ADAS-Cog	0.160 (0.408)	-0.019 (0.921)	-0.153 (0.428)	-0.312 (0.147)	0.081 (0.713)	0.243 (0.263)
	0.262 (0.196)[1]	-0.086 (0.669)[1]	-0.159 (0.437)[1]	-0.166 (0.484)[1]	0.013 (0.957)[1]	0.098 (0.681)[1]
	0.207 (0.356)[2]	0.077 (0.726)[2]	-0.011 (0.961)[2]	-0.244 (0.363)[2]	0.227 (0.398)[2]	0.185 (0.492)[2]

Data presented as Spearman correlation coefficient (p-value).[1]Partial correlation corrected for Gender, Age and Education; [2]Partial correlation corrected for Gender, Age, Education and BMI. *$A\beta_{42}$/p-tau<17.7.

Aβ: Amyloid beta; ADAS-Cog: Alzheimer's Disease Assessment Scale-Cognitive subscale; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio; MMSE: Mini Mental State Examination; MoCA: Montreal Cognitive Assessment; p-tau: phosphorylated tau.

However, in female patients both MoCA and ADAS-Cog were moderately correlated with CSF adiponectin, in the sense that higher concentration was associated with better cognitive function. In addition,

CSF leptin and LAR were also inversely correlated with MoCA scores (Table 20).

Table 20. Correlation between adipokine concentration and neuropsychological tests in female patients

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
MMSE	-0.123 (0.312)	0.014 (0.905)	0.123 (0.316)	0.319 (0.086)	0.066 (0.728)	-0.172 (0.364)
	-0.118 (0.343)[1]	0.040 (0.746)[1]	0.145 (0.246)[1]	0.283 (0.152)[1]	0.154 (0.443)[1]	-0.097 (0.631)[1]
	-0.036 (0.784)[2]	-0.072 (0.577)[2]	0.040 (0.760)[2]	0.363 (0.105)[2]	-0.078 (0.737)[2]	-0.245 (0.285)[2]
MoCA	-0.216 (0.228)	0.189 (0.277)	0.253 (0.156)	0.639 (0.008)	-0.065 (0.810)	-0.396 (0.129)
	-0.153 (0.418)[1]	0.185 (0.310)[1]	0.225 (0.233)[1]	0.621 (0.024)[1]	-0.044 (0.888)[1]	-0.337 (0.261)[1]
	-0.020 (0.921)[2]	-0.294 (0.114)[2]	-0.119 (0.547)[2]	0.611 (0.046)[2]	-0.641 (0.033)[2]	-0.627 (0.039)[2]
ADAS-Cog	0.130 (0.383)	-0.163 (0.274)	-0.238 (0.107)	-0.593 (0.003)	-0.023 (0.918)	0.351 (0.101)
	0.129 (0.403)[1]	-0.183 (0.234)[1]	-0.249 (0.103)[1]	-0.517 (0.019)[1]	-0.086 (0.719)[1]	0.235 (0.319)[1]
	0.070 (0.670)[2]	0.213 (0.186)[2]	-0.010 (0.953)[2]	-0.548 (0.028)[2]	0.154 (0.569)[2]	0.352 (0.181)[2]

Data presented as Spearman correlation coefficient (p-value).[1]Partial correlation corrected for Gender, Age and Education; [2]Partial correlation corrected for Gender, Age, Education and BMI.

ADAS-Cog: Alzheimer's Disease Assessment Scale-Cognitive subscale; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio; MMSE: Mini Mental State Examination; MoCA: Montreal Cognitive Assessment.

II.4.2 Higher serum adiponectin is associated with slower Alzheimer's disease progression

II.4.2.1 Baseline characteristics and adipokine profile of MCI subgroups

From the initial 71 MCI patients included in the main study, 67 maintained clinical and neuropsychological follow-up. Twenty-seven MCI patients progressed to AD (MCI-AD) whereas 40 maintained the diagnosis of MCI (MCI-MCI) upon a mean follow-up of 38.2±18.83 months.

Clinical and biochemical data of the participants according to the final diagnosis are summarized in Tables 21 and 22.

There was no significant difference between age, gender, years of education and *APOE* genotype between the groups as well as in anthropometric measurements and in the prevalence of vascular risk factors (Table 21).

Lipid and glycemic biochemical parameters as well as both serum and CSF baseline levels of adiponectin, leptin and LAR were similar when comparing the two groups (Table 22).

Table 21. Demographic, clinical and genetic data of the study population according to diagnosis at follow-up

	MCI-MCI n=40	MCI-AD n=27	*p*
Age (years)	70.68±7.30	72.63±8.23	0.311
Age onset (years)	66.28±7.67	69.6±8.71	0.121
Female gender n(%)	27 (67.5%)	18 (66.7%)	0.943
Education (years)	4 (4, 6.25)	4 (3.5, 6.5)	0.762
***APOEε4* carrier n(%)**	27 (71.1%)	14 (51.9%)	0.114
MCI type (md:Md)	28 (70%):12 (30%)	23 (85.2%):4 (14.8%)	0.268
BMI	26.62±3.92	26.37±3.28	0.795
WC (cm)	97.16±11.00	96.38±9.89	0.781
HC (cm)	102 (97, 108)	101.5 (95.75, 106.25)	0.769
WHR	0.94(0.88, 1)	0.96(0.91, 0.99)	0.705
Hypertension n (%)	22 (55%)	15 (55.6%)	0.964
Type 2 DM n (%)	8 (20%)	5 (55.6%)	0.880
Dyslipidemia n (%)	24 (60%)	20 (74.1%)	0.234
Heart failure n (%)	6 (15%)	2 (7.4%)	0.459
MetS n (%)	17 (36.1%)	13 (33.3%)	0.832

Data presented as mean±standard deviation, median (1st quartile, 3rd quartile) or as number (percentage) of patients, where applicable. The p-values (*p*) included in the table were obtained with independent samples t-Student tests or Mann-Whitney U tests for quantitative variables and with chi-square tests for qualitative variables. [1]MetS defined according to the International Diabetes Federation (IDF).

AD: Alzheimer's dementia; APOE: Apolipoprotein E; BMI: Body Mass Index; DM: Diabetes Mellitus; HC: hip circumference; MCI: Mild cognitive impairment; md: monodomain; Md: multidomain; MetS – Metabolic Syndrome; WC: waist circumference; WHR: waist to hip ratio.

Table 22. Serum and CSF biochemical data of the study population according to diagnosis at follow-up

	MCI-MCI	n	MCI-AD	n	*p*
Serum					
Fasting glycemia (mg/dl)	89.5 (83.25, 98.5)	37	89 (83.5, 97)	27	0.599
Fasting insulinemia (mU/L)	5.15 (3.92, 6.02)	35	4.52 (3.68, 5.68)	22	0.481
HOMA-IR	1.21 (0.93, 1.44)	35	0.99 (0.72, 1.40)	22	0.264
Total cholesterol (mg/dl)	190 (174.5, 231.75)	33	205.5 (185.5, 242.75)	24	0.297
LDL-cholesterol (mg/dl)	112 (89.45, 145.1)	33	139.8 (110.95, 162.75)	24	0.094
HDL-cholesterol (mg/dl)	53.5 (45.25, 60)	33	47 (41, 68)	24	0.349
Triglycerides (mg/dl)	114 (85.75, 151)	33	106 (82.25, 125.5)	24	0.502
Adiponectin (µg/ml)	6.58 (3.52, 9.89)	33	5.97 (4.54, 9.47)	25	0.721
Leptin (ng/ml)	8.79 (5.62, 25.00)	33	12.10 (7.59, 21.41)	25	0.619
LAR	1.39 (0.98, 3.83)	33	2.04 (1.3, 4.41)	24	0.448
CSF					
Adiponectin (ng/ml)	2.75 (1.85, 4.32)	17	2.44 (1.84, 3.11)	15	0.708
Leptin (pg/ml)	162.55 (112.86, 234.64)	17	182.25 (137.03, 209.77)	15	0.986
LAR	55.48 (36.48, 112.65)	17	88.51 (39.70, 124.51)	15	0.817

Data presented as mean±standard deviation or median (1st quartile, 3rd quartile), as applicable. The p-values (*p*) included in the table were obtained with independent samples t-Student tests or Mann-Whitney U tests for quantitative variables.

AD: Alzheimer's dementia; CSF: cerebrospinal fluid; HDL: high density lipoprotein; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance; LAR: leptin to adiponectin ratio; LDL: Low density lipoprotein; MCI: Mild cognitive impairment.

II.4.2.2 MCI-to-AD progression and adipokine concentration

In order to evaluate the accuracy of serum and CSF levels of adiponectin, leptin and LAR in estimating the progression to Alzheimer's type of dementia in MCI patients we performed a ROC analysis (Table 23).

Table 23. Comparison of sensitivity, specificity and AUC for adipokine concentration in predicting MCI-to-AD progression

	Cut-off value	Sensitivity	Specificity	AUC [95% CI]
Serum Adiponectin (µg/ml)	≤8.12	0.720	0.441	0.528 [0.378-0.678]
Serum Leptin (ng/ml)	≥7.41	0.750	0.471	0.539 [0.388-0.690]
Serum LAR	≥1.48	0.720	0.529	0.559 [0.409-0.709]
CSF Adiponectin (ng/ml)	≤2.89	0.733	0.500	0.541 [0.336-0.746]
CSF Leptin (pg/ml)	≥149.51	0.733	0.444	0.496 [0.291-0.702]
CSF LAR	≥68.98	0.600	0.611	0.526 [0.317-0.735]

AD: Alzheimer´s dementia; AUC: area under the curve; CI: confidence interval; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio; MCI: Mild cognitive impairment.

Overall, area under the curve (AUC) values settled around 0.5, meaning that none of the variables represented a good predictor of conversion to dementia in this population.

Additionally, we explored the relation between serum and CSF adipokines and time to progression. Figure 19 shows that serum adiponectin presented a moderate positive correlation with the elapsed time to progression to Alzheimer's type of dementia after adjustment for

age, gender and BMI (ρ=0.484, p=0.049). However, serum leptin and both adipokine CSF concentration as well as their ratio were not correlated with this parameter (Table 24).

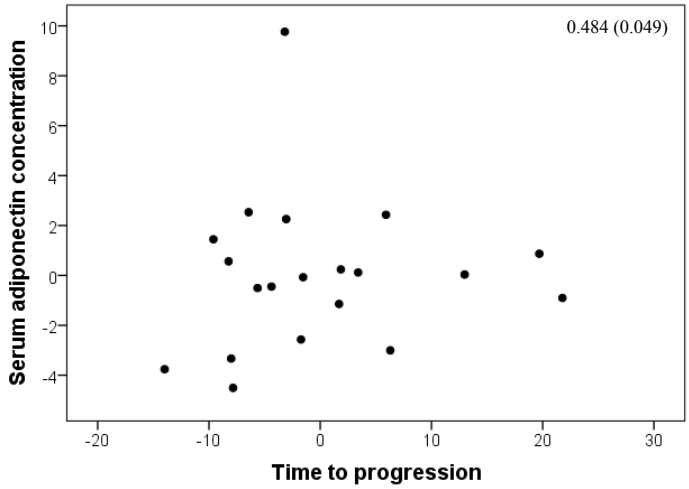


Figure 19. Partial correlation plot displaying the relation between serum adiponectin concentration and time to progression to dementia. Partial correlation derived from the standardized residuals of a multiple regression and presented as Spearman correlation coefficient (p-value) and adjusted for age, gender and BMI.

Table 24. Correlation between adipokine concentration and time to MCI-to-AD progression

	Serum adiponectin	Serum leptin	Serum LAR	CSF adiponectin	CSF leptin	CSF LAR
Time to progression	0.116 (0.615)	0.051 (0.832)	-0.198 (0.391)	-0.129 (0.673)	0.110 (0.720)	0.140 (0.647)
	0.415 (0.077)[1]	0.185 (0.464)[1]	-0.180 (0.461)[1]	-0.068 (0.842)[1]	-0.013 (0.969)[1]	0.076 (0.825)[1]
	0.484 (0.049)[2]	0.058 (0.832)[2]	-0.391 (0.121)[2]	-0.062 (0.864)[2]	-0.038 (0.917)[2]	0.061 (0.867)[2]

Data presented as Spearman correlation coefficient (p-value). [1]Partial correlation corrected for Age and Gender; [2]Partial correlation corrected for Age, Gender and BMI.
AD: Alzheimer's dementia; CSF: cerebrospinal fluid; LAR: leptin to adiponectin ratio; MCI: Mild cognitive impairment.

Chapter II.5

Highlights

Table 25. Highlights of the results arising from clinical investigation

1. Serum adiponectin was 33% higher in AD patients when compared to MCI patients, and may be admitted as a staging biomarker despite low specificity and sensitivity;
2. Adiponectin seems to play a relevant role in female gender since in this group higher CSF adiponectin levels were associated with higher CSF $A\beta_{42}$ and better cognition, though its association with hippocampal volume did not reached significance;
3. In patients with Alzheimer´s disease CSF profile, higher adiponectin levels (particularly in CSF) were associated with greater hippocampal volumes and parahippocampal cortical thickness;
4. In MCI patients, serum adiponectin was associated with slower disease progression, although neither serum or CSF adipokine levels represented good predictors of progression;
5. Lower levels of serum and CSF adiponectin were associated with higher systemic insulin resistance, particularly in patients with Alzheimer´s disease CSF profile.

Chapter II.6

Discussion

The current study is, to the best of our knowledge, the first to simultaneously explore the association of serum and CSF levels of adiponectin, leptin and their ratio with CSF and imaging Alzheimer's disease biomarkers and global neuropsychological scores. It was our intention to focus on the prodromal and early stages of the disease and understand if higher adiponectin serum levels in AD patients could be beneficially associated with disease pathophysiology and cognition. This study also aimed to explore the link between adipokines and the female gender bias in Alzheimer's disease incidence and their influence in the rate of disease progression. According to these aims, the sub-groups explored were composed by patients with the clinical diagnosis of MCI or AD, a subgroup of patients with Alzheimer's disease CSF profile (based on $A\beta_{42}$/p-tau index) and also a female patient sub-group.

Overall, it provides novel biomarker-based evidence of the relationship between adipokines and Alzheimer's disease pathology, highlighting gender differences and proposes a blood-based staging biomarker.

II.6.1 Adipokine levels in MCI and AD patients

Leptin

Serum leptin levels have been associated with cognitive function and risk of Alzheimer's disease even though results from several clinical studies are somehow divergent (Magalhães *et al*, 2015) and dependent on epidemiological factors such as age, gender and race (Oania and McEvoy, 2015). Recent studies have shown that, despite leptin's recognized neuroprotective abilities, in some circumstances central resistance develops with consequent leptin signaling impairment which seems to be the case in obesity and Alzheimer's disease (Bonda *et al*, 2014; Ishii and Iadecola, 2016). Regardless the fact that we did not find any significant differences in serum or CSF leptin levels when comparing MCI and AD patients, which is in accordance to the results presented by Maioli *et al* (2015), Bonda *et al* (2014) had previously described higher CSF leptin in AD patients when compared to MCI. This could be partially explained by the fact that, in opposition to our mild to moderate stage AD cohort, the subjects included in the later study were in the latest pathological stage of disease and a six-fold increase in CSF leptin of Braak VI patients compared to Braak 0-V is described.

Adiponectin

Few clinical studies have inconsistently reported an association

between adiponectin and Alzheimer's disease (for revision see Letra *et al*, 2017) and to date the only meta-analysis on this subject supports the existence of elevated levels of serum adiponectin in AD patients when compared to controls (Ma *et al*, 2016). Accordingly, Waragai *et al* (2016) has recently described higher levels of adiponectin in the serum of MCI and AD patients when compared to controls, but no differences between the two former groups. They have also shown significantly lower CSF adiponectin levels in AD patients compared to MCI and controls, although evident scattered results in the AD group suggest that CSF adiponectin levels may vary according to the severity or pathological stage of the disease. Overall, our results are in line with these authors since serum adiponectin was 33% higher in AD when compared to MCI patients, albeit no differences were observed in the CSF. A possible explanation for this finding is the mild to moderate stage of disease in the AD group which led us to hypothesize that higher peripheral secretion of adiponectin can maintain, for a limited but undetermined period of time, normal CSF levels of this protein in the initial stages of the disease as an effort to compensate central defective adiponectin signaling. Indeed, there is no evidence of intrathecal synthesis of this adipokine and in our study, its concentration in serum and CSF showed a moderate positive correlation favoring the hypothesis of BBB crossing.

Furthermore, we found that a cut-off of 10.85 μg/ml in

adiponectinemia predicted the diagnosis of AD (vs MCI diagnosis) with 87% specificity though with only 44% sensitivity. Although this distinction does not entail considerable practical importance, once MCI vs AD diagnosis lays mostly on clinical aspects, serum adiponectin could be explored as staging biomarker, meaning that it could reflect disease progression. On the other hand, it would also be interesting to evaluate its usefulness in the differential diagnosis with other forms of dementia. Currently, the work of Bossolasco *et al* (2017) was the only to show higher serum adiponectin in a non-Alzheimer's type of dementia (frontotemporal dementia vs controls) with comparable results to AD patients. Although these results counteract the usefulness of high adiponectin as marker of Alzheimer's disease, very few patients have been enrolled in this study (7 FTD; 9 AD; 36 controls) meaning results must be interpreted with caution.

II.6.2 Adipokines and non-modifiable Alzheimer's disease risk factors

This study aimed also to investigate the correlation between adiponectin and leptin levels and the most relevant non-modifiable risk factors for Alzheimer's disease - gender, age and *APOE* genotype.

Gender-based dimorphic expression of adipokines is already well documented although it remains poorly understood (Gui *et al*, 2004). In our population, serum levels of both adiponectin and leptin were, as expected, higher in female patients, although this tendency was not observed in the CSF, contrarily to what was reported by Maioli *et al* (2015) for leptin. Interestingly, Bossolasco *et al* (2017) also did not observe any gender difference in CSF adiponectin levels in patients with amyotrophic lateral sclerosis (ALS), the only study to address gender differences in CSF adiponectin levels to date. Regardless there is no other study addressing this issue, we hypothesize that both adipokines may be transported to the CNS by a non-gender specific BBB saturable system, although in the case of adiponectin, BBB crossing mechanisms are yet to be elucidated.

Even though dementia is not considered a normal part of aging, many age-related cerebral changes may enhance the detrimental effects of Alzheimer's disease, including mitochondrial dysfunction and oxidative stress (Moreira *et al*, 2010). Aging is, indeed, the primary risk factor for the majority of neurodegenerative diseases, including Alzheimer's disease. In our population, aging was associated with higher serum and CSF levels of adiponectin contrarily to what was observed with leptin. Indeed, most of the studies report a significant positive relationship between adiponectinemia and age which may depend not

only on adipose tissue remodeling, but also on others factors such as renal function (Koh *et al*, 2008; Kizer *et al*, 2012*)* or BMI decline (Gustafson, 2012; Besser *et al*, 2014). Recently, adiponectin has been shown to act directly on longevity signaling pathways by activation of AMPK–SIRT1 as well as by positive regulation of PPAR, alleviating oxidative stress, but also by reducing the risk of obesity-related diseases (T2DM, cardiovascular diseases) and cancer (Iwabu *et al*, 2015). In addition, accumulating evidence show that hyperadiponectinemia and insulin sensitivity are key phenotypes of healthy centenarians (Arai *et al*, 2011; Arai and Hirose, 2012) suggesting that adiponectin may alleviate age-related stress in high-metabolic-rate organs, such as the brain.

Finally, we found no significant effect of carrying the *APOEε4* genotype on adipokine levels, suggesting that their serum and CSF concentrations are not tracking any association with the most important genetic risk factor for Alzheimer's disease. However, there is evidence that leptin stimulates *APOE* gene expression in the CNS (Shen *et al*, 2009) and is able to increase *APOE*-dependent Aβ uptake (Fewlass *et al*, 2004).

II.6.3 Adipokines and modifiable Alzheimer's disease risk factors

We further explored the correlation between both adipokines and modifiable risk factors for Alzheimer's disease such as obesity and insulin resistance. Whilst this association is already well defined when using adipokine's circulating levels, this is no longer the case when one consider their CSF concentration.

Overall, leptin has been more consistently associated with BMI and with other central obesity measures when compared to adiponectin. In fact, our results do not support a correlation between anthropometric measures and serum or CSF adiponectin levels, suggesting that this adipokine, unlike leptin, entails a more complex association with obesity and should rather be looked as an adipose tissue dysfunction marker. Such results are to some extent conflicting with other studies which report an association, although weak, between serum adiponectin and BMI, WC and WHR (Okauchi *et al*, 2009; Marques *et al*, 2015).

One of the most relevant effects of adiponectin signal transduction is the increase of insulin-sensitivity in insulin-sensitive tissues and currently, the crosstalk of this adipokine with the insulin-signaling pathway is well defined (Ruan and Dong, 2016) as also is the association between defective brain insulin signaling and Alzheimer's disease pathogenesis, described more than 20 years ago (Hoyer *et al*,

1994). This knowledge made us question whether systemic insulin-resistance could relate with CSF adiponectin levels in cognitive impaired patients. This study shows that not only serum but also CSF adiponectin is negatively correlated with systemic insulin-resistance in patients with biomarker-based Alzheimer's disease diagnosis, suggesting that the central insulin resistance which characterizes this disorder can be boosted by defective central adiponectin signaling and otherwise compensated by recruiting peripheral adiponectin.

II.6.4 Adipokines, Alzheimer´s disease biomarkers and neuropshycological scores

Leptin

The present study reports a moderate negative correlation between CSF leptin and t-tau in AD patients, reinforcing the involvement of this adipokine in tau metabolism. CSF t-tau levels are globally increased after its release from damaged neurons and represents a nonspecific marker of neurodegeneration (Blennow *et al*, 2015). Noteworthy, these results oppose to those presented by Maioli *et al* (2015) and although it is recognized that leptin is capable of reducing tau

phosphorylation (Greco *et al*, 2009), no correlation was observed between leptin levels and p-tau.

An association between higher leptinemia and larger total brain volume in asymptomatic subjects has been reported by Lieb *et al* (2009), though only significant in non-obese participants. This was later reaffirmed by Rajagopalan *et al* (2013) who showed a strong negative association between plasma leptin levels and regional brain atrophy (including temporal lobe) in MCI and AD patients, regardless of their BMI. Nevertheless, we observed no association between serum or CSF leptin levels and MTL volumetric measures.

Finally, higher serum leptin showed a tendency to correlate with worse cognitive performance in the whole cohort, and similarly in the female group we observed a significant inverse correlation between CSF leptin and MoCA scores. Regardless of leptin's neurotrophic and neuroprotective effects (Lee, 2011), aging and Alzheimer's disease-associated modifications in leptin BBB transport can lead to central leptin resistance (Dietrich *et al,* 2008; McGuire and Ishii, 2016), which is the most likely explanation for these results.

Adiponectin

In accordance to other studies (Diehl-Wiesenecker *et al,* 2015; Waragai *et al,* 2016), our results showed no correlation between serum

levels of adiponectin and classical Alzheimer's disease CSF biomarkers. Furthermore, and contrary to our expectations, CSF levels of adiponectin were also unrelated to $A\beta_{42}$, t-tau and p-tau concentrations in MCI and AD patients. Notably, when we separately studied female patients, we observed a strong positive correlation between CSF adiponectin and $A\beta_{42}$ suggesting that higher CSF levels of this adipokine are associated with less soluble $A\beta_{42}$, and eventually less aggregates. These results come into line with the study conducted by Waragai *et al* (2016), the first to demonstrate a correlation between CSF adiponectin and $A\beta_{42}$ as well as with p-tau, although in an heterogeneous cohort (controls, MCI and AD; n=189). However, they have not performed any subgroup analysis based on diagnosis, biomarker profile or gender, and no adjustments for possible confounders such as age, gender, BMI or *APOE* genotype. The co-localization of adiponectin with NFTs in the AD brain is also interestingly described by the same authors suggesting a direct action of this adipokine on pathological hallmarks of the disease.

Regarding imaging biomarkers, we found no robust correlation between CSF adiponectin concentration and MTL volumetry in MCI or AD patients neither in the female sub-group. However, we found a robust correlation between CSF adiponectin and total hippocampal volume as well as with left parahippocampal cortex thickness in the low $A\beta_{42}$/p-tau group, what further supports our hypothesis. To our

knowledge, Waragai *et al* (2016) was the only to report an inverse correlation between CSF adiponectin and hippocampal volume (r=-0.291, $p<0.001$) in a combined pool of patients. Previoulsy, Masaki *et al* (2012) and García-Casares *et al* (2016) had shown an association between lower plasmatic levels of this adipokine and greater hippocampal atrophy in cognitively normal diabetic patients whereas the Mayo Clinic Study of Aging published opposing data (Wennberg *et al*, 2016). In this last study, significant results were restricted to a subgroup of cognitively impaired women with elevated brain amyloid. Interestingly, we also observed a moderate negative correlation between CSF LAR and total hippocampal volume in female patients, and believe that by replicating this study in a larger cohort we would find a correlation with adiponectin CSF levels. Further, in this group we found a consistent correlation between higher CSF concentration of adiponectin and better cognitive performance (measured by MoCA and ADAS-Cog). Overall, the findings herein reported are highly suggestive that adiponectin might be especifically assigned to neuroprotective functions in female gender and its dysfunctional CNS signaling possibly involved in the female-biased incidence of Alzheimer's disease.

II.6.5 Adipokines and MCI-to-AD progression

The progression rate from MCI to dementia is influenced by several factors and the period of time in which this occurs is extremely variable. Kamogawa *et al* (2010) was the first to report that higher peripheral adiponectin levels were associated with reduced risk of MCI in men, altough more recently Wennberg *et al* (2016) reported no association in a similar population. Interestingly, the same authors found otherwise greater odds of MCI in women with higher plasma adiponectin levels, in line with the results reported by the Framingham Heart Study (van Himbergen *et al*, 2012).

Others have described that circulating leptin levels could not be predictive of progression to dementia in MCI patients (Oania and McEvoy, 2015). Our data is concordant with these studies, once we also did not found a correlation between serum adipokines and progression to dementia. However, their CSF concentrations could, in theory, better correlate with the risk of disease progression. To our knowledge, this is the first clinical study to correlate CSF adipokines with the risk of progression from MCI to Alzheimer's type of dementia and to correlate serum and CSF adipokine concentrations with time of progression. Even though our results did not support this hypothesis once CSF levels of adiponectin and leptin were similar in progressors (MCI-AD) and stable (MCI-MCI) patients, higher serum adiponectin levels were associated

with slower progression, suggesting that this adipokine might be protective especially in the early stages of the disease. It is therefore possible to admit that adiponectin contributes to brain resilience and that lifestyle or pharmacological interventions that elevate adiponectin levels may delay dementia phase regardless of the pathological burden.

Finally, CSF leptin-to-adiponectin ratio did not represent an added value in the correlation study with Alzheimer's disease biomarkers compared to individual adipokine concentrations.

Overall, and recognizing the limitations of this study in particular the lack of pre-symptomatic and healthy control groups, it gathers evidence that strongly suggest that adiponectin takes part on Alzheimer's disease pathophysiology.

PART III

ANIMAL

INVESTIGATION

Chapter III.1

Current state of research

III.1.1 Adiponectin and Alzheimer's disease

Few experimental studies have investigated the effects of adiponectin in the CNS, its potential involvement in neuroprotection and the mechanisms by which it may interact with the patho-physiological events of Alzheimer's disease. A summary of the most relevant cellular and animal studies that have explored adiponectin's role in this disorder are listed in Table 26.

Research models have been overall based on cultured hippocampal neuronal cells and wild type or knockout animals treated with adiponectin (globular or full-length) with or without a subsequent insult. Its effects on the glia have been, otherwise, much less explored (Wan *et al*, 2014).

Most studies are consistent in assigning adiponectin a neuroprotective role against diferent toxic insults, including Aβ-induced neurotoxicity (Jeon *et al*, 2009; Qiu *et al*, 2011; Chan *et al*, 2012; Song *et a*l, 2015; Ng *et al*, 2016). Moreover, a couple of studies have reported diminished hippocampal neurogenesis in adiponectin-haploinsufficient and/or adiponectin-deficient mice, which are reversed by icv adiponectin administration (Yu *et a*l, 2014; Zhang *et al*, 2016). This has been, indeed, the preferred route to deliver adiponectin *in vivo*, once its passage to the CNS remains elusive and high concentrations of the protein have to be administrated peripherally to presumably increment

its central levels. Ng *et al* (2016) has recently described that chronic adiponectin deficiency in aged adiponectin-knockout mice lead to Alzheimer's-like pathology and cognitive deficits, reinforcing the relevance of adiponectin in this disease.

Table 26. *In vitro* and animal studies aiming to assess the influence of adiponectin in CNS neuronal and non-neuronal cells involved in cognitive circuits

First author, year	Cell type (origin) or Animal	Adiponectin (admin.)	Treatment	Results
Qiu, 2011	Primary hippocampal neurons (prenatal Sprague Dawley rats)	Recombinant fAd	0.5, 5 and 20 μg/mL for 48h before insult	Adiponectin protects neurons against KA induced excitotoxicity. *Mechanism:* intracellular ROS and apoptosis reduction, by AMPK signalling pathway activation.
Zhang, 2011	Adult hippocampal neural stem cells (Fisher rats)	Recombinant gAd and fAd	0.03–3 μg/ml for 24, 48, 72h and 6 days	Hippocampal NSCs express AdipoR1and R2; adiponectin increases proliferation of adult NSCs but has no effect on apoptosis and differentiation. *Mechanism:* activation of the p38MAPK/GSK3β/β-catenin signalling cascade.
Chan, 2012	Human neuroblastoma cells SH-SY5Y$_{swAPP}$	Recombinant fAd	10 μg/ml for 2h before insult	Adiponectin is protective against Aβ neurotoxicity-induced cytotoxicity under oxidative stress (H_2O_2). *Mechanism:* APPL1-mediated AMPK activation and suppression of NF-kB activation.
Wan, 2014	Human astrocytic cells (U373 MG)	Recombinant gAd	1 and 3 μg/mL for 6, 12, 24 and 48h	Human astrocytes express AdipoR1and R2; adiponectin exerts pro-inflammatory effects on astrocytic cells. *Mechanism:* induction of pro-inflammatory citokine mRNA expression and IL-6 and MCP-1 secretion; ERK1/2, p38 MAPK and NF-kB signalling pathways involved.
Song, 2015	Cortical primary NSCs (ICR mice embryos)	Recombinant fAd	30 μg/ml, for 4 days before insult	At high glucose concentrations, adiponectin inhibits apoptosis and enhances neurogenesis and proliferation in the NSCs, and restores the reduced expression of AdipoR1. *Mechanism:* p53/p21 activation (apoptosis); tailless activation.
Jeon, 2009	4w ♂ ICR mice	Recombinant fAd (icv)	3 μg/g, 24h before insult	Adiponectin pretreatment supressed KA-induced cell death and VEGF, iNOS and NF-kB expression in the hippocampus as well as BBB permeability.

Yu, 2014	8-9w ♂ C57BL/6J mice (WT and ADPN −/−)	Adenovirus expressing adiponectin (icv)	2 µg/animal, 2 weeks before behavioral tests	Adiponectin deficiency diminished the beneficial effects of physical exercise on depression-like behaviors in mice and on hippocampal neurogenesis. Adiponectin treatment reduced depression-like behavior and enhanced hippocampal neurogenesis.
Zhang, 2016	9–12w ♂ C57BL/6J mice (WT, ADPN-/- and ADPN-/+)	Recombinant fAd (icv)	1 µg/animal daily, for 7 days	Adiponectin deficiency decreased dendritic complexity and spine density of dentate gyrus granule neurons and reduced adult hippocampal neurogenesis. Adiponectin treatment increased dendritic spine density and promoted adult hippocampal neurogenesis.
Ng, 2016	9 and 18mo ♂ C57BL/6N mice (WT, ADPN-/-) Human neuroblastoma cells (SH-SY5Y$_{IR}$ and SH-SY5Y$_{swAPP}$)	Trimeric adiponectin	10 µg/ml	Adiponectin deficiency increased anxiety levels, impaired spatial learning and memory, and elicited Alzheimer's-like pathology. *Mechanism:* neuronal insulin resistance by inactivating AMPK-IRS1 signaling. Adiponectin treatment enhanced insulin sensitivity in Aβ overproducing cells. *Mechanism:* AdipoR1-AMPK activation.

Aβ: amyloid beta; AdipoR: Adiponectin Receptor; AMPK: Adenosine Monophosphate-activated Protein Kinase; APPL1: adaptor protein containing the pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1; BBB: Blood-Brain-Barrier; CNS: Central Nervous System; eNOS: endothelial Nitric Oxide Synthase; ERK: Extracellular signal–Regulated Kinases; fAd: full-lenght adiponectin; gAd: globular Adiponectin; GSK: Glycogen Synthase Kinase; icv: intracerebroventricular; IL: Interleukin; IRS1: Insulin receptor substrate 1; KA: Kainic-Acid; MAPK: Mitogen Activated Protein Kinases; MCP-1: Monocyte Chemoattractant Protein-1; NF-kB: nuclear factor of kappa light polypeptide gene enhancer in B-cells; NSCs: Neural Stem Cells; ROS: Reactive Oxygen Species; SH-SY5Y$_{IR}$: Human neuroblastoma cells with insulin resistance; SH-SY5Y$_{swAPP}$: Human neuroblastoma cells expressing Swedish-Amyloid Precursor Protein; VEGF: Vascular Endothelial Growth Factor; w: weeks; WT: wild type. ♂ : male. (Letra *et al*, 2017).

III.1.1.1 Adiponectin and Alzheimer´s disease - common signaling pathways

Adiponectin has been linked to Alzheimer's disease-related pathology by several different mechanisms including insulin-sensitizing, anti-inflammatory, anti-oxidant and anti-apoptotic signaling pathways activation and also by its vascular effects. The main convergence points along this fat-brain axis are summarized below.

Insulin resistance

The increased awareness of DM as a risk factor for Alzheimer's disease together with the metabolic, molecular and biochemical alterations shared by both diseases culminated in the proposal of the term "type 3 diabetes" to designate sporadic Alzheimer's disease. This is a disorder characterized by cerebral glucose dysmetabolism that includes the development of insufficiency and/or resistance to insulin and IGF actions on neuronal survival and cognitive processes (de la Monte and Wands, 2008; De Felice *et al*, 2014). Several studies have highlighted the overlap between insulin resistance and amyloidogenic pathways and support the existence of a bidirectional relation between insulin and Aβ (de la Monte, 2009; Zhao and Townsend, 2009; Blázquez *et al*, 2014; Chami *et al*, 2016). Aβ accumulation promotes downregulation of membrane insulin receptor (IR) and inhibition of IR tyrosine kinase signaling while insulin increases amyloidogenic Aβ production (triggering GSK3α-dependent APP γ-secretase activity) and impedes Aβ transport and clearance (mediated by insulin-degrading enzyme: IDE, lipoprotein receptor protein: LRP and α2-macroglobulin) (Zhao and Townsend, 2009). Additionally, impairment of IR function results in increased activity of GSK3β, since IR activation inhibits this kinase via PI3K/Akt, which leads to enhanced tau phosphorylation and

consequently promotes NFT formation, another neuropathological hallmark of Alzheimer's disease (El Khoury *et al*, 2014).

Similarly, adiponectin and insulin signaling pathways have multiple interactions. Briefly, in the liver, adiponectin inhibits glucose production and upregulates insulin receptor substrate 2 (IRS-2), while in skeletal muscle it increases glucose uptake (promoting GLUT4 translocation to plasma membrane) and ameliorates oxidative status. Adiponectin also indirectly augments insulin sensitivity by inducing fatty acid oxidation, and thus reduces FFA and ectopic lipid deposition. In addition, it potentiates insulin secretion by acting directly on pancreatic β cells, increasing insulin gene expression and regulating local cell apoptosis and proliferation. As an anti-inflammatory adipokine, it may also improve general insulin sensitivity by reducing adipose tissue inflammation (Cheng *et al*, 2014). Overall, adiponectin modulates systemic (and likely also central) insulin resistance and may indirectly alleviate β-amyloid and p-tau burden.

Inflammation

The relevance of neuroinflammation in Alzheimer's disease has been supported by a great amount of evidence that highlights the contribution of inflammatory mediators to disease progression (Morales *et al*, 2014; Heneka *et al*, 2015). Moreover, the identification of

susceptibility genes for the development of Alzheimer's disease which are concomitantly genes coding for immune receptors (eg. TREM2) further consolidated this association (Jonsson *et al*, 2012). Astroglia and microglia are the major sources of pro-inflammatory molecules in the CNS while simultaneously responsible for immunological surveillance and synaptic remodelling (Furman *et al*, 2012; Ji *et al*, 2013). Microglia can be activated by pathological neuronal death or Aβ aggregates promoting its clearance and restoring homeostasis. However, external factors, such as systemic inflammation and obesity, may affect this physiological innate immune response that becomes detrimental if sustained or exaggerated (Heneka *et al*, 2015). Overall, it is recognized that peripheral inflammatory dysregulation can drive neuro-inflammation by promoting sustained glial activation (especially if microglia is already primed as is the case in Alzheimer's disease) and consequently cause functional and structural changes that will culminate in neurodegeneration (Heneka *et al*, 2015).

Dysfunctional adipose tissue is an important source of peripheral pro-inflammatory mediators and thus associated with a systemic chronic low-grade inflammatory status (Gómez-Hernández *et al*, 2016) that is concomitantly cause and consequence of lower adiponectin circulating levels in obese individuals. It is generally accepted that adiponectin is a systemic anti-inflammatory adipokine, capable of macrophage

polarization towards an anti-inflammatory M2 phenotype by inhibiting TNF-α, interferon gamma (INFγ), MCP-1 and IL-6 production as well as increasing anti-inflammatory citokine production (eg IL-10, IL-1Ra) (Turer and Scherer, 2012), and thus able to counterbalance neuroinflammation.

Apoptosis

Severe neuronal loss is a constant feature of Alzheimer's disease (Serrano-Pozo, 2011). However, the underlying mechanisms leading to neuronal cell death are only partially disclosed. Apoptotic signaling pathways are upregulated in Alzheimer's disease (Shafi O, 2016) though other mechanisms such as autophagic cell death, excitotoxicity, necrosis and necroptosis are also involved (Gorman, 2008; Caccamo *et al*, 2017).

An anti-apoptotic role is assigned to adiponectin and carried out by the activation of the enzyme ceramidase and consequent enhancement of its metabolite, sphingosine-1-phosphate (S1p) (Holland *et al*, 2011). S1p is a bioactive sphingolipid derived from sphingosine, which is phosphorylated by 2 sphingosine-kinases (SphK1 and 2), and is involved in survival pathways (Cuvillier *et al*, 1996). It is a potent neuroprotective factor against soluble Aβ-induced apoptosis (Malaplate-Armand *et al*, 2006) and promotor of long-term potentiation, which is essential to memory consolidation (Kanno *et al*, 2010). But the relation

with Alzheimer's disease pathophysiology goes even further. In hippocampus and temporal cortex, decreased levels of S1p have been associated with higher Braak stage, i.e., with higher NFT burden. Interestingly, *APOE* regulates the secretion of S1p, and hippocampal S1p/sphingosine ratio is higher in *APOE*e2 compared to *APOE*e4 carriers, linking this sphingolipid to the most relevant genetic risk factor for late onset Alzheimer's disease (Couttas *et al*, 2014). Thus, S1p constitutes another relevant convergent molecule in adiponectin and Alzheimer's disease metabolisms, supporting adiponectin's involvement in protective strategies against neurodegeneration.

Macro- and microangiopathy

Cerebrovascular dysregulation is thought to be an early event in Alzheimer's disease and contribute to its progression. Several researchers support a vascular etiology for Alzheimer's disease (de la Torre, 2002) and a substantial body of evidence suggests that neurodegeneration can be initiated by chronic cerebral hypoperfusion and dysregulation of cerebral blood flow associated with aging or other classical vascular risk factors such as obesity (Di Marco *et al*, 2015a). In fact, it seems to exist a vicious circle linking Aβ and vascular impairment, in which Aβ reduces eNOS synthesis, upregulates endothelin-1 and induces calcium homeostasis impairment promoting, in association with the cholinergic

deficit, alterations in cerebrovascular reactivity and vasomotion (spontaneous rhythmic modulation of arterial diameter). This will, in turn, affect the clearance of perivascular Aβ and other neurotoxic substances and enhance oxidative stress (Di Marco *et al*, 2015b) contributing to disease progression. AdipoRs are expressed in cerebral vascular endothelial cells and their role as antiatherogenic and anti-hypertensive agents has been well established, though most of the evidence comes from cardiovascular studies. Not surprisingly, low circulating adiponectin levels have been consistently associated with atherosclerotic cerebrovascular disease and increased mortality after ischemic stroke (Chen *et al*, 2005; Prugger *et al*, 2012). Moreover, adiponectin modulates the expression of endothelial adhesion molecules (Ouchi *et al*, 1999; 2000), stimulates eNOS phosphorylation and nitric oxide (NO) production (Chen *et al*, 2003; Nishimura *et al*, 2008) and regulates angiogenesis (Ouchi *et al*, 2004; Shibata *et al*, 2004), possiblly protecting the brain against β–induced vascular impairment.

III.1.2.2 Direct central effects of adiponectin

It has been demonstrated that beyond the indirect effects on the pathological cascade of events described above, adiponectin stimulates neural stem cell proliferation (Song *et al*, 2015; Zhang *et al*, 2011) and

may also be considered a neuroprotective agent by acting directly in neuronal and glial cells. Several models of neuronal toxicity support this hypothesis. Jeon *et al* (2009) and Qiu *et al* (2011) were the first to demonstrate the neuroprotective effects of adiponectin in a kainaic acid-induced excitotoxic model. Subsequent studies using APP transfected-neuroblastoma cells and adiponectin-knockout mice models further corroborated that adiponectin is protective against Aβ neurotoxicity, demonstrating that chronic adiponectin deficiency induces Alzheimer's-like pathology (including microglial activation and astrogliosis) as well as cognitive impairment (Chan *et al*, 2012; Ng *et al*, 2016). The molecular mechanisms involved include mostly AdipoR1/adaptor protein containing the pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif 1 - APPL1/AMPK (Jeon *et al*, 2009; Qiu *et al*, 2011; Chan *et al*, 2012) and also AdipoR1/APPL1/IRS1/2 downstream signalling events (Ng *et al*, 2016). APPL1 links the AdipoRs to its downstream targets, modulating glucose and lipid metabolism as well as vascular functions. It is also a key binding partner of IRS1/2 enhancing insulin signaling transduction. Interestingly, in neurons, APPL1 has been found to modulate the PI3K/Akt pathway stimulating synaptogenesis (Majumdar *et al*, 2011), NMDA-receptor-dependent prosurvival signaling (Wang *et al*, 2012) and the activation of the phosphatidylinositol triphosphate (PIP3) pathway in response to long-

term potentiation induction and thus important for synaptic plasticity (Fernández-Monreal *et al*, 2016). A distinct distribution and accumulation of APPL1 in Alzheimer's disease brains reinforce a possible involvement of this adaptor protein in the synaptic modifications associated with this disease (Ogawa *et al*, 2013) and may constitute the main link between adiponectin and Alzheimer's disease.

Two recent studies have on the other hand demonstrated that osmotin, a natural homolog of adiponectin, counteracts amyloidogenic Aβ production and aggregation, tau hyperphosphorylation, synaptic dysfunction and hippocampal neurodegeneration through the regulation of PI3K/Akt/GSK3β (Ali *et al*, 2015) and/or AMPK/SIRT1 (Shah *et al*, 2016) signaling pathways.

Remarkably, neuropathological data, though still very limited, favors the existence of a direct interaction between adiponectin and the histological hallmarks of Alzheimer's disease (Vingtdeux *et al*, 2011; Waragai *et al*, 2016). It suggests that this adipokine can have a buffering effect on the aggregates' neurotoxicity, which may be mediated by AMPK, once adiponectin co-localizes with NFTs in Alzheimer's disease brains (Waragai *et al*, 2016) and AMPK accumulates around amyloid plaques of neurons bearing NFTs (Vingtdeux *et al*, 2011). Moreover, adiponectin has shown remarkable effects on dendritic growth, arborization, and spinogenesis in mature granule neurons of

hippocampal dentate gyrus and thus able to act as a neurotrophic protein (Zhang *et al*, 2016).

Beyond the recognized peripheral regulation of inflammation by adiponectin, its effects on microglia and astrocytes are almost unexplored. Chabry *et al* (2015) showed that icv delivered adiponectin has potent anti-inflammatory effects on microglia of corticosterone-treated mice, by reducing the level and expression of pro-inflammatory genes (IL-1β, IL-6, TNFα, inhibitor of kappa-light-chain-enhancer of activated B cells: IκB-α) and increasing mRNAs of anti-inflammatory mediators (Arginase 1 - Arg1, IL-10). To our knowledge there is only one study that has determined the effect of globular adiponectin on astrocytic cells with results indicating a pro-inflammatory effect involving IL-6, MCP-1, IL-1β and IL-8 (Wan *et al*, 2014). Further investigation is imperative to understand the impact of adiponectin on CNS inflammation, taking into account that inflammation may be beneficial in the earliest phases of Alzheimer's disease (Chakrabarty *et al*, 2010, 2012).

Chapter III.2

Hypothesis and Aims

Results from our clinical research demonstrated that serum adiponectin levels were raised in AD patients when compared to MCI patients, though CSF levels were similar in both groups. Accordingly, we hypothesized that in mild to moderate AD there is a raise in serum adiponectin levels in order to compensate a central signaling deficiency, at least temporarily, once it has been described lower CSF levels of adiponectin in later stages of the disease (Waragai *et al*, 2016). Nevertheless, studies that modulate peripheral adiponectin availability aiming to disclose its central effects are lacking, and presently we cannot affirm that an elevation of serum adiponectin levels will have a beneficial influence on Alzheimer's disease pathology.

To test our hypothesis, a high-fat diet model was chosen in order to mimic the most common mechanism contributing to human obesity. Attention was focused on the hippocampus since it represents a critical structure for learning and memory, especially affected in Alzheimer's disease.

III.2.1 Main objectives

- To determine if peripheral delivery of adiponectin improves hippocampal insulin sensitivity;
- To determine if peripheral delivery of adiponectin prevents high-fat diet-induced hippocampal neuronal loss, inflammation, synaptic protein expression and spatial memory.

III.2.2 Secondary objectives

- To assess safety of long-term subcutaneous globular adiponectin administration;
- To determine if peripheral delivery of adiponectin prevents high-fat diet-induced glucose intolerance, lipid metabolism and adipose tissue dysfunction.

Chapter III.3

Research design and Methods

III.3.1 Reagents

Unless otherwise stated, all reagents were purchased from Merck Darmstad (Germany), Sigma-Aldrich (EUA) or Panreac Química SA (Spain).

Primary antibodies used in the western blot and immuno-histochemistry analysis were directed to β-actin (A5441, Sigma-Aldrich, USA), AMPK, p-AMPK, IkBα, PPARγ, AKT, p-AKT, IRS-1, p-IRS-1, p-HSL, phospho-GSK3β ser9 (#2532, #2535, #9242, #2443, #9272, #4058, #3194, #3070, #4139 and #9336 Cell Signaling, USA), GSK3β, phospho-Tau ser396, IR (sc-81462, sc-101815, sc-57342, Santa Cruz, USA), Tau (MN1010, Thermofisher, USA), F4/80, HSL, AdipoR1, Ob-R and phospho-IR (Ab74383, Ab45422, ab70362, ab5593 and ab60946, Abcam, UK), AdipoR2 (#PA1-1071, Thermo Scientific, USA), T-cad (#ABT121, Milipore, USA), SNAP-25, Synaptophysin (#S5187, #S5768, Sigma-Aldrich, USA), Syntaxin-1, Synapsin-1 (#110011, #106001, Synaptic Systems, USA).

III.3.2 Animal Diet

A high-fat diet was chosen with the objective of increasing animal total weight and adipose tissue mass. A period of 4 months was chosen based on different lengths of time used in previous studies (Winocur and

Greenwood, 2005; Morrison *et al*, 2010; Pistell *et al*, 2010). Table 27 describes and compares the nutritional composition of both standard and high-fat diets.

Table 27. Nutritional composition of standard and high-fat diets

	Standard diet Standard diet AO3 (Safe, France)	**High-fat diet** Purified Diet 231 HF(Safe, France)
Proteins (%)	21.4	26.9
Lipids (%)	5.1	39.7
Carbohydrates (%)	45	10.1
Calories (Kcal/kg)	2830	5053

III.3.3 Adiponectin production

Based on the existing evidence that adiponectin's globular domain (gAd) is more potent than the full-length protein (fAd) (Fruebis *et al*, 2001; Almer *et al*, 2011) and also capable of passing the BBB to exert its effects on the CNS (Chen *et al*, 2009), we have chosen to produce and administrate gAd rather than a fAd isoform. The dosis was calculated based on the normal concentrations of the protein in circulation and in preliminary studies in which different dosis were administrated and metabolic impact as well as toxicity were evaluated.

Cloning of adiponectin gene

Adiponectin cDNA of 744 base pair was obtained from SinoBiological (China) inserted in a plasmid, pMD18-T (MG50636-M). The DNA sequence coding for the globular domain of the protein was localized, ranging from the residue 330 of the 744 base pair cDNA (GCT) to the end (TGA), amplified and cloned into an expression vector containing a potent fusion tag, rPPure of Hitag® Biotechnology, to solubilize and increase the yields of the recombinant protein obtained (www.hitag.pt). Primers were designed to match adiponectin cDNA and introduce NcoI, at 5' position, and XhoI, at 3' position, restriction enzyme recognition sites, in order to clone this gene segment into Hitag®'s rPPure platform (Figure 20).

The 444 base pair modified adiponectin globular domain was amplified by Polymerase Chain Reaction (PCR) and cloned into a plasmid pGEM Teasy (Promega, CA, USA), which was sequenced in an external laboratory (MWG Operon, Germany). The insert was then cloned into a Hitag®'s rPPure plasmid, which contains a 69 amino acid residues ORF (opening reading frame) coding to the Fh8 protein at the N-terminal side of the adiponectin insert, and its identity was reconfirmed by sequencing. The plasmid was used to transform Escherichia coli BL21 strain.

A. Sequence of adiponectin gene

GCTTATGTGTATCGCTCAGCGTTCAGTGTGGGGCTGGAGACCCGCGTCACTGTTCCCAATGTACCCATTCG
CTTTACTAAGATCTTCTACAACCAACAGAATCATTATGACGGCAGCACTGGCAAGTTCTACTGCAACATTC
CGGGACTCTACTACTTCTCTTACCACATCACGGTGTACATGAAAGATGTGAAGGTGAGCCTCTTCAAGAA
GGACAAGGCCGTTCTCTTCACCTACGACCAGTATCAGGAAAAGAATGTGGACCAGGCCTCTGGCTCTGT
GCTCCTCCATCTGGAGGTGGGAGACCAAGTCTGGCTCCAGGTGTATGGGGATGGGGACCACAATGGACT
CTATGCAGATAACGTCAACGACTCTACATTTACTGGCTTTCTTCTCTACCATGATACCAACTGA

B. Primers designed to amplify the globular domain of adiponectin

ADIPOQ Mut Fwd: 5'-gagcctg**CCATGG**cgGCTTATGTG-3'
First 7 residues of 5' primer region match pMD18-T cDNA right before the globular domain, bold region shows *NcoI* restriction site, underline "cg" residues maintain the right coding frame, and last 3' 9 residues match the globular domain of Adiponectin.

ADIPOQ Mut Rev: 5'-cgatcctct**CTCGAG**TCAGTTGGT-3'
First 9 residues of 5' primer region match the pMD18-T plasmid after the Adiponectin cDNA STOP codon, bold region shows *XhoI* restriction site, underline "TCA" residues are the STOP codon, and last 3' 6 residues match the globular domain of Adiponectin.

Figure 20. Sequence of adiponectin gene (A) used to clone and amplify the globular fraction with the primers described (B).

Protein expression and purification

Pre-cultures of the clone were grown overnight in LB medium at 37ºC, supplied with kanamicine as the selection antibiotic. After this, 40mL precultured media were added to 2-litre flasks containing 400mL fresh culture media and grown (37ºC, 3 h) to a final OD600nm of 0.4–0.6. Then, isopropyl-b-D-1-thiogalacto-pyranoside (IPTG, 1mM) was added and cultures grown (37ºC, 200 rpm, 4 h). After that, bacteria cells were recovered by centrifugation (4000 rpm, 20 min, 4ºC) and used to extract the protein. For that, cell pellets were re-suspended in phosphate buffered solution (native buffer) and submitted to freeze and thaw cycles, from -80ºC to 37ºC, and sonication. Soluble fraction was isolated by

centrifugation (10000 rpm, 20 min) and filtered (0.45 µm). Protein purification was performed using a DuoFlow Fast Performance Liquid Chromatography apparatus (Bio-Rad). His-trap columns (GE Healthcare, USA) were used to isolate the target protein due to its 6xHis tags. Briefly, columns were equilibrated with 30mL Native Buffer containing 20mM Imidazole (IMZ); sample loading with a flux of 1 ml/min; first wash with 40mL Native Buffer containing 20mM IMZ; second wash with 40mL Native Buffer containing 40mM IMZ and 10% glycerol; protein elution with Native Buffer containing 300mM IMZ, collecting 10 fractions of 2mL each.

Protein was purified by fast protein liquid chromatography (FPLC) based on UV absorption at 240 nm and fractions were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 21). The protein was quantified by the colorimetric Bradford assay (Bio-Rad, USA).

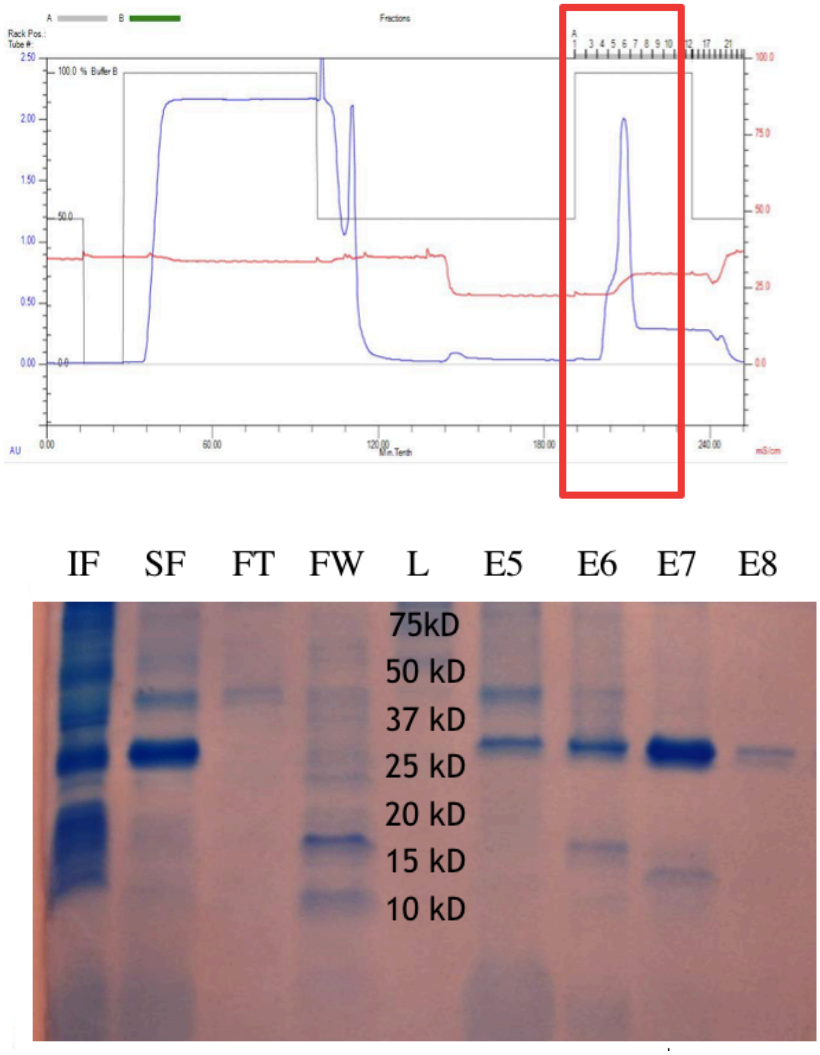


Figure 21. Adiponectin purification by fast protein liquid chromatography (FPLC) based on its UV absorption (top) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile of the elution fraction (bottom). In FPLC, time (minutes) is represented in the X axis, and the absorbance (240 nm) in the Y axis. The blue line shows the protein absorbance at 240 nm during column loading, wash and elution, by this same order across time. The peak of protein elution is well defined at the end of the graphic where the fractions are collected. Red line shows the conductivity and grey line the buffer exchange.

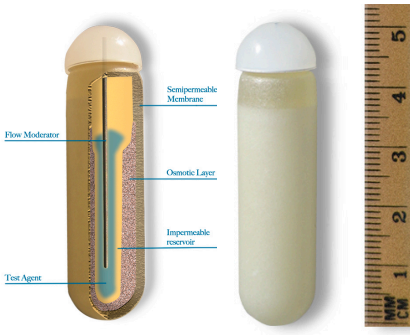
E5: Elution 5; E6: Elution 6; E7: Elution 7; E8: Elution 8; IF: Insoluble fraction; FT: Flow-Through; FW: First wash; L :Ladder; SF: Soluble fraction.

III.3.4 Osmotic pump characteristics and implantation

In order to continuously delivery adiponectin to the animals, we used miniature osmotic pumps with approximately 4 weeks duration (Alzet® 2ML4, USA). General characteristics of the pumps are summarized in Table 28.

Minipumps were filled with 2.7 mg of gAd dissolved in phosphate buffered saline (PBS, 98 µg/day continuous delivery) or with PBS only, according to the manufacturer instructions. After, they were implanted subcutaneously in the back of anesthetized animals (ketamine chloride, 75 mg/kg, im, Parke-Davis, Ann Arbor, USA and choloropromazine chloride, 2.65 mg/kg, im, Laboratórios Vitória, Portugal). A small incision was made in the skin between the scapulae and a small pocket was formed by using a hemostat, spreading the subcutaneous connective tissues apart. The incision was then closed by continuous closure with a 3/0 short-term absorbable suture of braided and coated polyglycol acid (Safil Quick®, B Braun, Aesculap AG, Tuttlingen, Germany).

Table 28. Osmotic Pump technical description (Alzet® 2ML4, USA)	
Length	5.1 cm
Diameter	1.4 cm
Weight (empty)	5.1 g
Total Displaced Volume	6.5 ml
Pumping Rate	2.5 µl/hr (± 0.05 µl/hr)*
Duration	28 days*
Reservoir Volume	2000 µl (2 ml)
Materials	
Flow moderator cap	Polyethylene
Pump outer membrane	Cellulose Ester Blend
Pump drug reservoir	Thermoplastic
	Hydrocarbon Elastomer



* Nominal Performance (at 37°C). (Retrieved from www.alzet.com).

III.3.5 Animal models

We studied 12 month old male Wistar rats, from our breeding colony (Faculty of Medicine, University of Coimbra). Animals were kept under standard ventilation, temperature (22–24ºC), humidity (50–60%) and light (12 h light/12 h darkness) with free access to water and food. At 8 months old, rats were divided in two groups, the first one was maintained with standard diet (CTL), while the second one was maintained with high-fat diet during 4 months. This second group was subsequently divided into three subgroups: a group of rats was treated during the last 28 days with globular adiponectin through a subcutaneous minipump with continued release (HFDA). Another group

was also subjected to minipump implantation, with continued PBS (vehicle) administration (HFDV). A third subgroup was maintained with no treatment (HFD). A total of 12 to 14 animals were used per group to permit the allocation of part of the rats to behavioral tasks.

The experimental protocol (Figure 22) was approved by the local Institutional Animal Care and Use Committee and all procedures followed the principles mentioned in the Declaration of Helsinki and were performed by licensed users (Portuguese Veterinary Authority and FELASA).

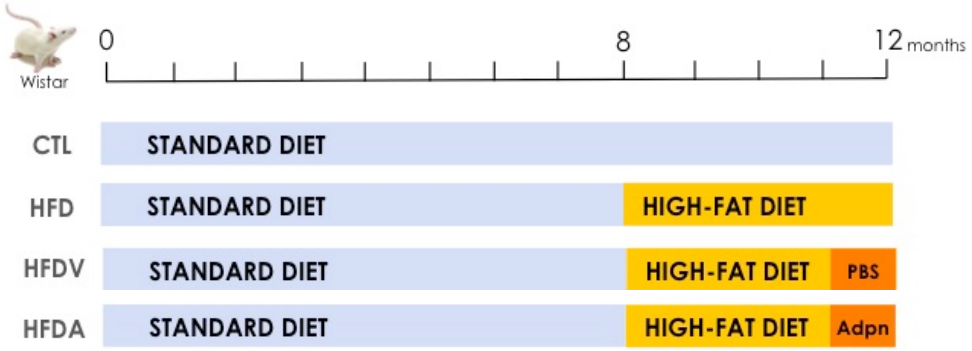


Figure 22. Graphical representation of the experimental protocol.

CTL: controls; HFD: high-fat diet group; HFDV: high-fat diet with vehicle (PBS: phosphate buffered saline) group; HFDA: high-fat diet with adiponectin (Adpn) group.

III.3.6 Sample collection and storage

After the experimental period, animals were anaesthetized with ketamine chloride (75 mg/kg, im, Parke-Davis, Ann Arbor, USA) and chlorpromazine chloride (2.65 mg/kg, im, Laboratórios Vitória, Portugal) and blood was collected by cardiac puncture. Serum and plasma were collected using BD Vacutainer and BD Vacutainer K3E tubes with 5.4 mg EDTA (UK), respectively. Blood was centrifuged at 2500xg and 4ºC during 15 minutes, and plasma and serum were aliquoted and stored at -80ºC. Afterwards animals were decapitated and brain and adipose tissue removed.

Epididymal and subcutaneous adipose tissues were then stored in 10% formalin or frozen at -80ºC for further immuno-hystochemical and western blotting analysis. In the same rats, both hippocampi were dissected and stored at -80ºC until further processing for western blotting, while the rats used in behavioral tests had their whole brain post-fixed for 24 hours in 4% paraformaldehyde, then dehydrated in 20% sucrose in 0.1 M phosphate-buffered saline solution (PBS, in mM: 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.47 KH_2PO_4; pH 7.4) for 24 hours and stored at −80°C for posterior sectioning and immunohistochemistry analysis.

III.3.7 Blood determinations

Glucose and insulin

In overnight (18 hours) fasted rats, glucose tolerance test was performed by measuring glycemia in blood collected in the tail vein 2 hours after intraperitoneal glucose administration (1.8 g/Kg) using a glucometer (Bayer, Germany). In another day, after an overnight fasting and immediately before sacrifice, glycemia and HbA1c were measured in blood collected in the tail vein using a glucometer (Bayer, Germany) and A1CNow system (Bayer, Germany), respectively.

Serum insulin concentration was determined using the Rat Insulin ELISA Kit (Mercodia, Sweden) and HOMA was calculated as following: HOMA-IR = [(If)*(Gf)]/ 22.5, where (If) is the fasting insulin level (mU/ml) and (Gf) is the fasting glucose level (mmol/L).

Lipids

Serum cholesterol (total and HDL) and triglyceride levels were determined using commercial kits (Olympus-Diagnóstica, Produtos de Diagnóstico SA, Portugal). Plasmatic FFA levels were assessed spectrophotometrically using the Half-micro Test (Roche Diagnostic, Germany).

Adipokines

Serum concentrations of adiponectin and leptin were determined by ELISA using the Rat Adiponectin Immunoassay Kit and the Rat Leptin Immunoassay Kit (Invitrogen), respectively.

III.3.8 Adipose tissue analysis

Insulin signaling, lipolysis and inflammation assessment

For quantification of proteins involved in insulin signaling, lipolysis and inflammation by western blot, adipose tissue sections of 300 mg were homogenized in a buffer containing 25 mM Tris, 150 mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 10 mM PMSF and 40 ml/g tissue of proteases inhibitor cocktail (Sigma, USA), pH=7.4 and centrifuged (14000 rpm, 20 min, 4ºC). Supernatants were collected, centrifuged again and protein concentration was determined using the BCA method (Pierce, USA). Samples were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with TBST solution (25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween, pH=7.6) supplemented with 5% BSA. Membranes were incubated overnight at 4ºC with the respective primary antibodies and during 2 hours at room

temperature with the secondary antibodies (anti-mouse, GE Healthcare, UK and anti-rabbit, Bio-Rad, USA). Membranes were incubated with ECF or ECL and revealed using the Typhoon system (GE Healthcare Life Sciences, UK) or the Versadoc system (Bio-Rad, USA). Membrane analysis was performed using the software Image Quant (Molecular Dynamics, USA).

Adipocyte area and inflammatory markers

In order to determine the adipocyte area, hematoxylin-eosin staining was performed in deparaffinized adipose tissue sections (4 mm). The number of adipocytes was determined in each field and the mean adipocyte area was determined in at least 8 fields/rat. Images were captured in a Motic microscope (China) using a Zeiss camera (Germany).

Staining of F4/80 (a macrophage marker) was performed after paraffin removal, hydration and blocking. Sections were incubated with primary antibodies overnight (4ºC) and with secondary antibody-peroxidase during 2 hours (room temperature) (IHC peroxidase Kit, Chemicon, USA). Diaminobenzidine (DAB) was used as the enzyme substrate. Finally, sections were stained with haematoxylin, dehydrated and mounted with mounting medium.

III.3.9 Hippocampal determinations

Neuronal morphology and survival

Hippocampal slides were immersed in xylene (Sigma-Aldrich, USA) for 5 minutes followed by immersions in 95% ethanol (Sigma-Aldrich, USA) for 3 minutes and 70% ethanol for 3 minutes before being placed in a container with distilled water for 3 minutes. The slides were then immersed in cresyl violet staining solution for 8 minutes at 60°C and washed again in a container with distilled water for another 3 minutes. The previous steps were repeated in reverse, first by dipping the slides in 70% ethanol for 3 minutes, then 95% ethanol for 2 minutes, followed by one immersion in 100% ethanol for 1 minute. The final two immersions were in xylene solution for 5 minutes each. Slides were then coverslipped with permanent mounting medium and left air dried overnight. Sections were examined with a Axio Lab.A1 microscope (Zeiss, Germany). Images of the hippocampal subregions from each animal group, were captured using the same settings and an equivalent area of each subregion was considered.

Neuroinflammation

Hippocampal cryosections (30 µm thickness) were cut in a

cryostat (Leica CM3050S, Nussloch, Germany) and used for free-floating immunohistochemistry. Slices were washed twice with 0.1 M PBS, blocked/permeabilized (5% bovine serum albumin, 0.1% Triton X-100, 2 hours, room temperature) and incubated with the primary antibody at 4 °C during 48 hours (mouse anti-GFAP, 1:500, Merck Millipore, Darmstadt, Germany). After washing in 0.1 M PBS, sections were incubated with the secondary antibody (donkey anti-mouse, 1:500, Invitrogen, Waltham, MA, USA) plus 4',6-diamidino-2- phenylindole (DAPI, 1:5000) at room temperature during 2 hours. From this point forward, the slices were protected from light, washed three times with 0.1 M PBS and then mounted on gelatinized slides using DAKO glycergel mounting medium. Sections were examined with a LSM 710 Meta Confocal laser scanning microscope (Zeiss, Germany). Images of the hippocampal subregions from each animal group were captured using the same settings and an equivalent area of each subregion was considered.

Glucose metabolism and synaptic proteins

In the preparation of total extracts, hippocampi were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, supplemented with complete miniprotease inhibitor cocktail tablets and 1 mMDTT) and the resulting homogenate was sonicated (4 pulses, 2

seconds each) and then centrifuged (14000 rpm, 20 min, 4ºC). Supernatants were collected, centrifuged again and protein concentration was determined using the BCA method (Pierce, USA). Samples were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with TBST solution (25mM Tris-HCl, 150mM NaCl, 0.1% Tween, pH=7.6) supplemented with 5% BSA. Membranes were incubated overnight at 4ºC with the respective primary antibodies and during 2 hours at room temperature with the secondary antibodies (anti-mouse, GE Healthcare, UK and anti-rabbit, Bio-Rad, USA). Membranes were incubated with ECL and revealed using the Versadoc system (Bio-Rad, USA). Membrane analysis was performed using the software Image Quant® (Molecular Dynamics, USA).

III.3.10 Hippocampal-dependent learning and memory assessment

Morris Water Maze test was performed as described by Morris *et al* (1982). It consists of a circular swimming pool (1.7 m in diameter x 0.8 m in height) containing 25 °C tap water of 60 cm depth. Inside the water, a target Plexiglas platform of the size of 10 x 10 cm was hidden 1.5 cm beneath the water surface. There were 4 marked starting points

on the inner wall of the pool and also distant visual cues on the walls (Figure 23). The training session consisted of 4 consecutive trials in which the animals (n=4-6/group) were placed in the maze starting from the four cardinal positions (North, South, East and West) and then were allowed to freely swim for 60 seconds or until finding the submerged platform. If they failed to find the platform within this period, they were gently guided to it. The animals were allowed to remain on the platform for 10 seconds after escape and were removed from the tank for 30 seconds before being placed at the next starting point.

Test session was carried out on the 5th day and consisted of a single probe trial where the platform was removed from the maze and each rat was allowed to swim for 60 seconds. The time spent in the correct quadrant of the removed platform and the number of crossings of the original place of the platform counted in the analysis (Soares *et al*, 2013). All trials were video-recorded to accurately score the latency of escape from the starting point to the platform and swimming pathway, using an image analyser (ANY-maze, Stoelting, USA).

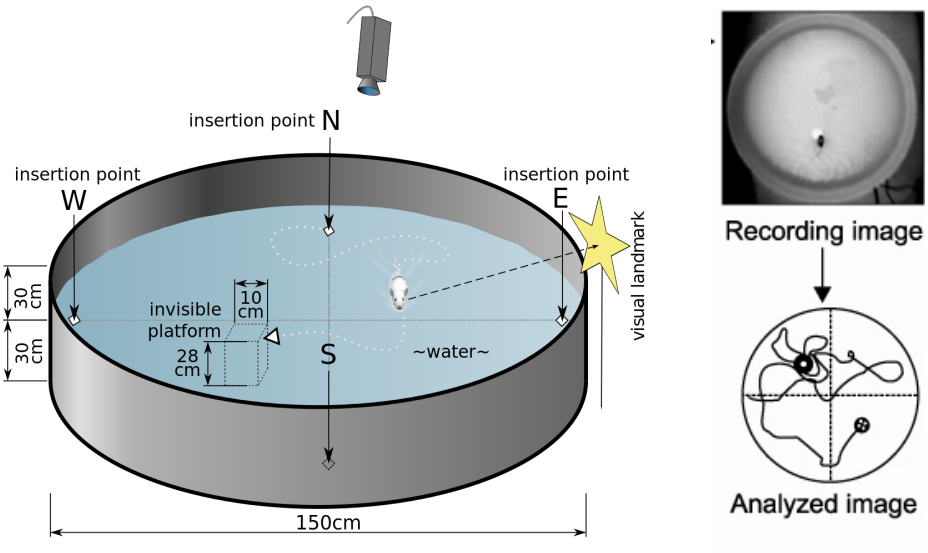


Figure 23. Schematic representation of the equipment used to performe the Morris Water Maze test and example of image recording and analysis (adapted from Koo *et al*, 2007 and https://en.wikipedia.org/wiki/Morris_water_navigation_task).

III.3.11 Statistical analysis

Each quantitative variable was assessed for normality resorting to Shapiro-Wilk test. For normally distributed variables, statistical differences between the groups were assessed by one-way ANOVA and Tukey *post-hoc* test was applied. Results are described by its mean and standard deviation. Since not all the data from behavioral test followed a normal distribution, non-parametric Wilcoxon test was used for paired samples and Kruskal-Wallis for comparing two or more independent samples followed by Dunn's *post hoc* testing. The level of significance adopted was 0.05.

Chapter III.4

Results

III.4.1 Chronic adiponectin administration through a subcutaneous minipump reverts high-fat diet induced glucose intolerance and adipose tissue dysmetabolism in Wistar rats

III.4.1.1 Effects of high-fat diet and adiponectin administration on body weight, adipocyte area and adipokine profile

As expected, high-fat diet-fed rats experienced an increase in total body weight although this was not entirely observed in the vehicle group (Table 29). Adiponectin administered in the last month induced a decrease in body weight, which became similar to the weight of control rats (Table 29 and Figure 24).

Table 29. Body weight and glucose, insulin and adipokine determinations in peripheral blood

	CTL	HFD	HFDV	HFDA
Body weight (g)	532±16	647±24**	565±30	509±21#
Fasting glycemia (mg/dl)	72±2	72±3	81±5	62±3*
Fasting insulinemia (pmol/L)	220±38	278±49	257±46	327±85
HbA1c (%)	5.0±0.2	4.9±0.1	4.8±0.3	4.6±0.2
Adiponectin (µg/ml)	44±6	50.2±4.5	40.5±3.9	45.1±8.7

CTL: controls; HbA1c: glycosylated hemoglobin; HDL: High-Density Lipoprotein; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin.

*Different from CTL; #Different from HFD. One symbol $p<0.05$; two symbols $p<0.01$.

Data is presented as mean±SEM; n=8/group.

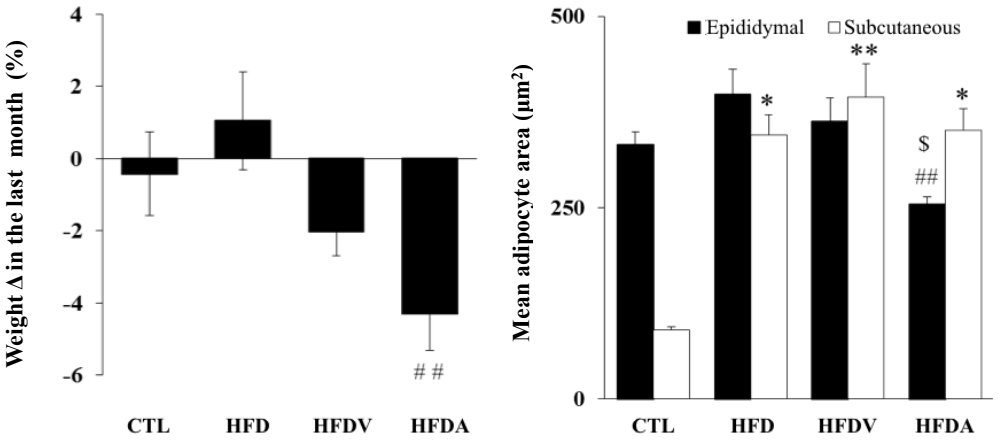


Figure 24. Body weight variation (Δ) in the last month (left panel) and mean epididymal and subcutaneous adipocyte area (right panel).

CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin.

*Different from CTL. #Different from HFD. $Different from HFDV. One symbol $p<0.05$; two symbols $p<0.01$.

Data is presented as mean±SEM; n=8/group.

All high-fat diet-fed groups (HFD, HFDV and HFDA) showed increased subcutaneous adipocyte area. Instead, in HFD and HFDV rats no alterations were observed in the area of epididymal adipocytes when compared with controls, dissipating the difference between the areas of the two deposits observed in control rats. In the HFDA group, a significant reduction of epididymal adipocyte area was observed when compared with HFD and HFDV rats (Figure 24).

High-fat diet further caused an increased accumulation of Periodic Acid-Schiff (PAS)-positive and fibrotic materials in both adipose tissue

depots, which was reduced by adiponectin treatment especially in subcutaneous adipose tissue (Figure 25).

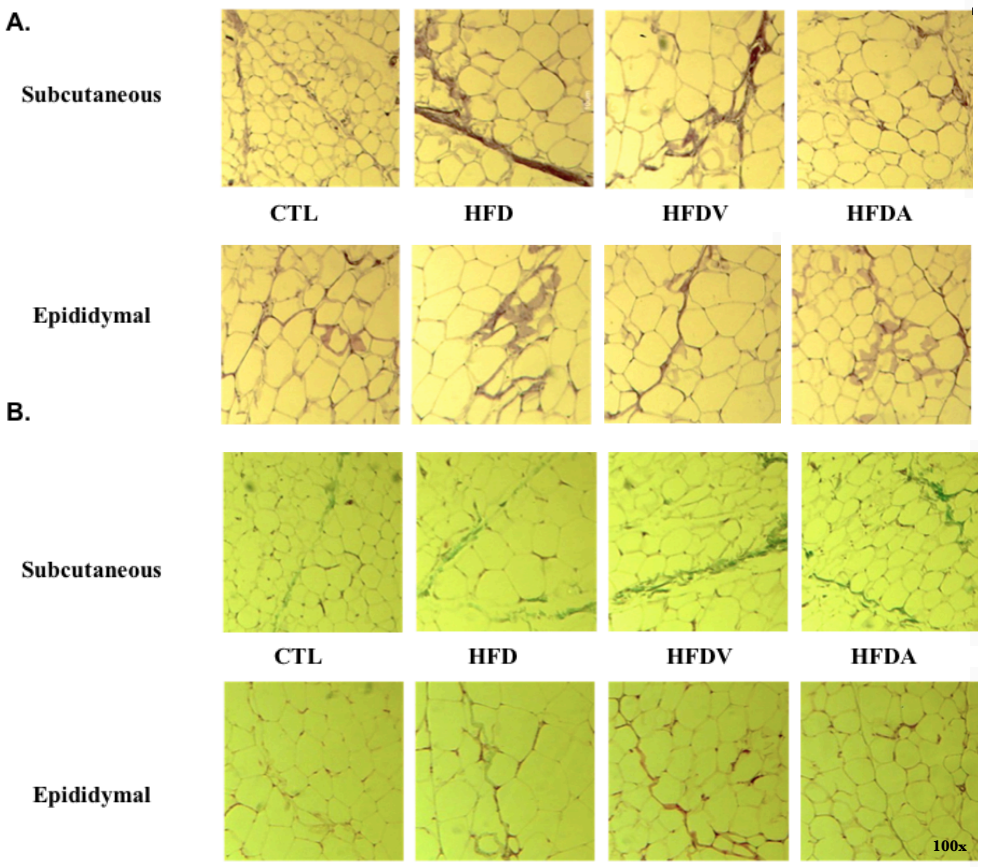


Figure 25. Representative photomicrographs for PAS staining (A) and Masson's trichrome staining (B) of subcutaneous and epididymal adipose tissues.
CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin; PAS: Periodic Acid-Schiff.

In accordance with increased body weight and adipocyte hypertrophy, serum leptin levels were significantly higher in HFD and HFDV rats (Figure 26), though alterations in adiponectinemia were not

observed (Table 28). In the HDFA group, serum adiponectin levels were not increased probably as a consequence of low-dose continuous subcutaneous administration (Table 29). However, alongside with total body weight and epididymal adipocyte area, adiponectin administration significantly reduced serum leptin levels, improving leptin to adiponectin ratio which showed a tendency to normalize (Figure 26).

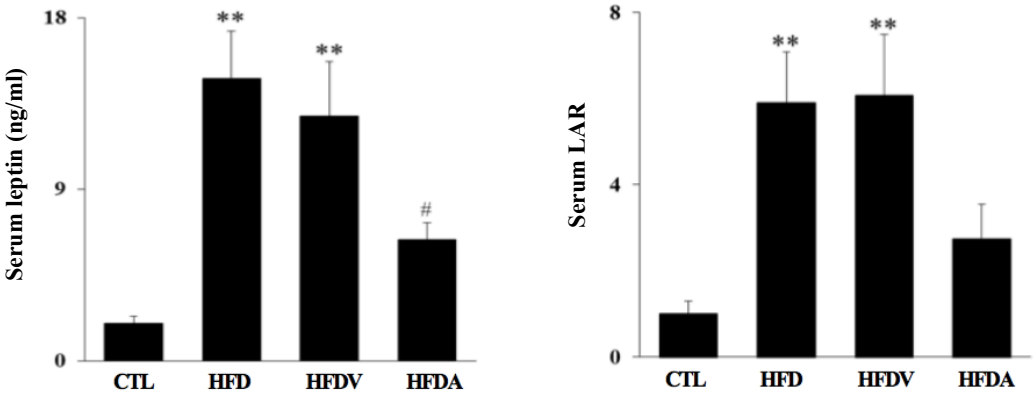


Figure 26. Serum leptin levels (left panel) and leptin to adiponectin ratio (LAR) (right panel).

CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin.

*Different from CTL. #Different from HFD. One symbol $p<0.05$; two symbols $p<0.01$.

Data is presented as mean±SEM; n=8/group.

III.4.1.2 Effects of high-fat diet and adiponectin administration on glucose profile and adipose tissue insulin signaling

High-fat diet did not change fasting glycemia or HbA1c levels when compared to the control group (Table 29). Moreover, no significant alterations were observed in fasting insulinemia and in the insulin resistance index HOMA (Table 29 and Figure 27).

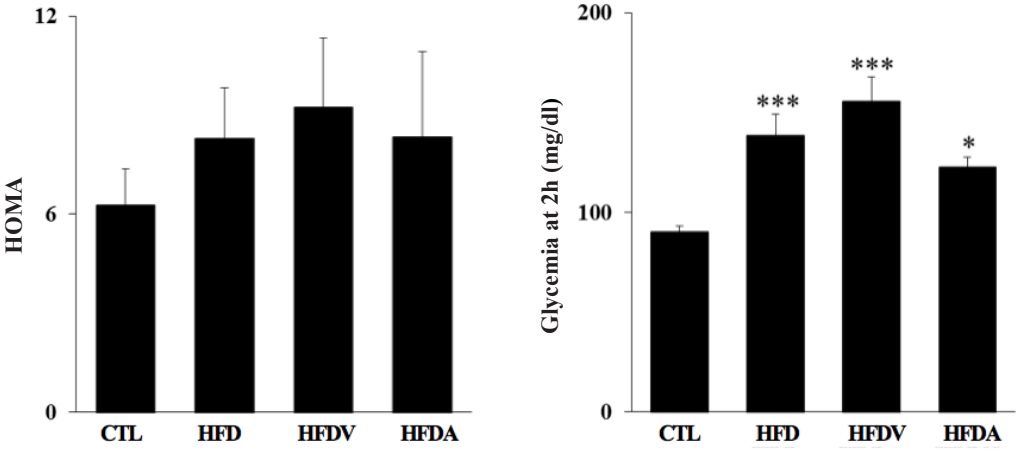


Figure 27. Insulin resistance index HOMA (left panel) and glycemia at 2 hours after an intraperitoneal glucose administration (IPGTT) (right panel).
CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin; HOMA: Homeostatic model assessment.
*Different from CTL. One symbol $p<0.05$; three symbols $p<0.001$.
Data is presented as mean±SEM; n=8/group.

However, adiponectin treatment resulted in decreased fasting glycemia in comparison to controls (Table 29). Despite no differences were observed in fasting glycemia, high-fat fed-rats showed increased glycemia 2 hours after an intraperitoneal glucose administration

(IPGTT), i.e., lower glucose tolerance. This was also observed in the vehicle-treated group and was partially reverted by adiponectin, since the difference to the control group was decreased (Figure 27).

Furthermore, in HFD and HFDV rats' epididymal adipose tissue we observed decreased levels of both total and phosphorylated (Tyr895) IRS-1, while only in the first group were total IRS-1 subcutaneous adipose tissue levels significantly lower than those found in controls. On the other hand, adiponectin treatment restored total IRS-1 levels in epididymal adipose tissue and counteracted the high-fat diet-induced decrease of IRS-1 activation (Figure 28). Despite lower IRS-1 levels and activation in high-fat fed rats, no significant alterations were observed for total and active (phospho Ser473) Akt levels, one of its downstream effectors (Figure 29).

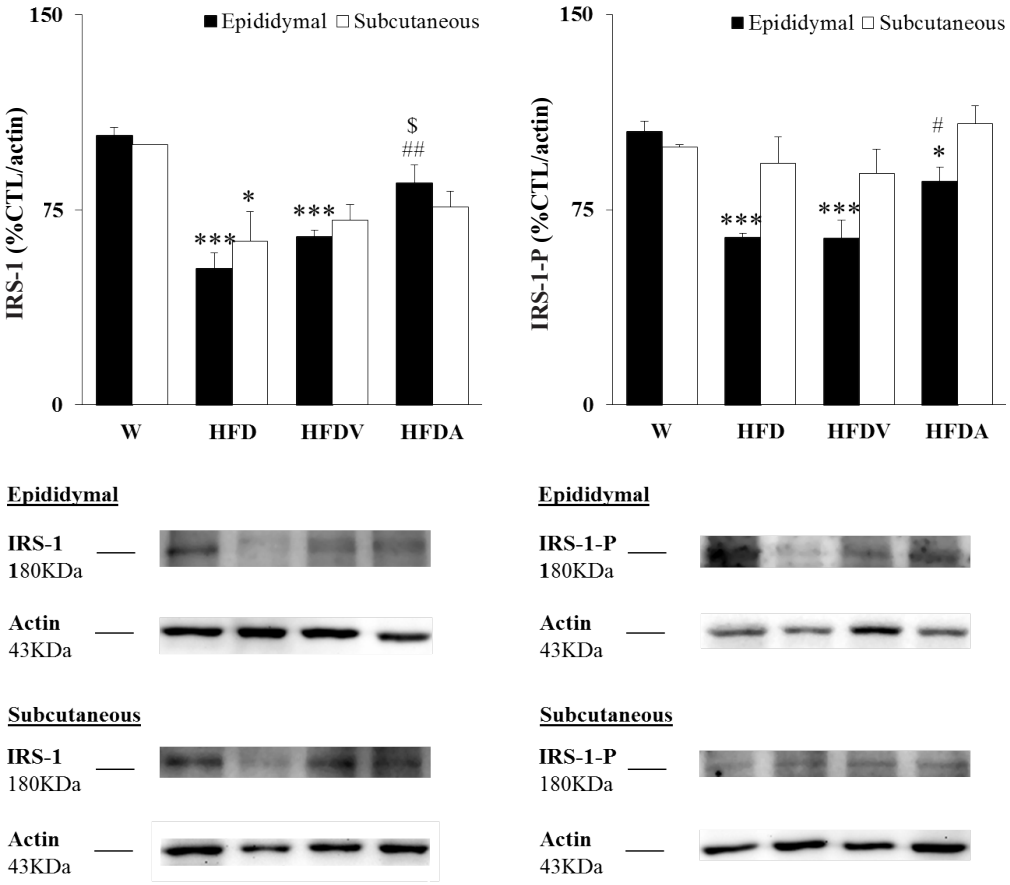


Figure 28. Total (left panel) and phosphorylated (right panel) levels of insulin receptor substrate 1 (IRS-1 and IRS-1-P, respectively) in epididymal and subcutaneous adipose tissues. Representative western blots are shown below.

CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin.

*Different from CTL. #Different from HFD. $Different from HFDV. One symbol $p<0.05$; two symbols $p<0.01$; three symbols $p<0.001$.

Data is presented as mean±SEM; n=8/group.

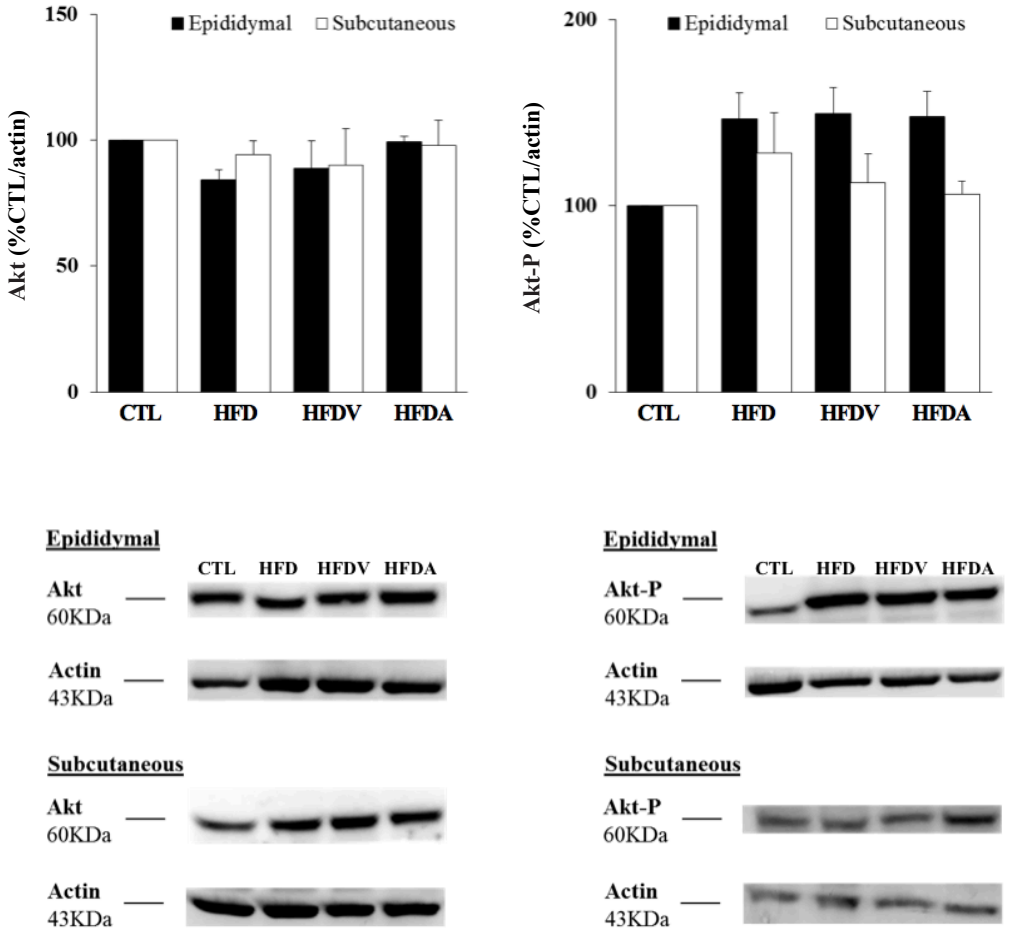


Figure 29. Total (left panel) and phosphorylated Akt (right panel) in epididymal and subcutaneous adipose tissues. Representative western blots are shown below.

CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin.

Data is presented as mean±SEM; n=8/group.

III.4.1.3 Effects of high-fat diet and adiponectin administration on lipid profile and lipolysis regulation in adipose tissue

Although no significant differences were observed in cholesterol and triglycerides levels between the experimental groups, HFD rats presented increased plasma FFA when compared with controls (Table 30). This was not observed in adiponectin-treated rats, though it was not observed in vehicle-treated rats either (Table 30). Increased plasma FFA levels result largely from insufficient oxidation and dysregulation of lipolysis in adipose tissue, which in turn are strongly controlled by AMPK and HSL, respectively.

Table 30. Lipid profile determinations in peripheral blood

	CTL	HFD	HFDV	HFDA
Triglycerides (mg/dl)	85±7	100±5	91±9	83±8
Total cholesterol (mg/dl)	108±5	124±10	110±7	96±9
Non-HDL cholesterol (mg/dl)	48±2	53±5	48±3	42±4
Free fatty acids (mM)	0.44±0.005	0.71±0.09*	0.62±0.06	0.53±0.07

CTL: controls; HbA1c: glycosylated hemoglobin; HDL: High-Density Lipoprotein; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin.

*Different from CTL. One symbol $p<0.05$.

Data is presented as mean±SEM; n=8/group.

No significant differences were observed regarding total and phosphorylated (Thr172) AMPK levels (Figure 30) as well as in total HSL levels of both adipose tissues between the groups (Figure 31).

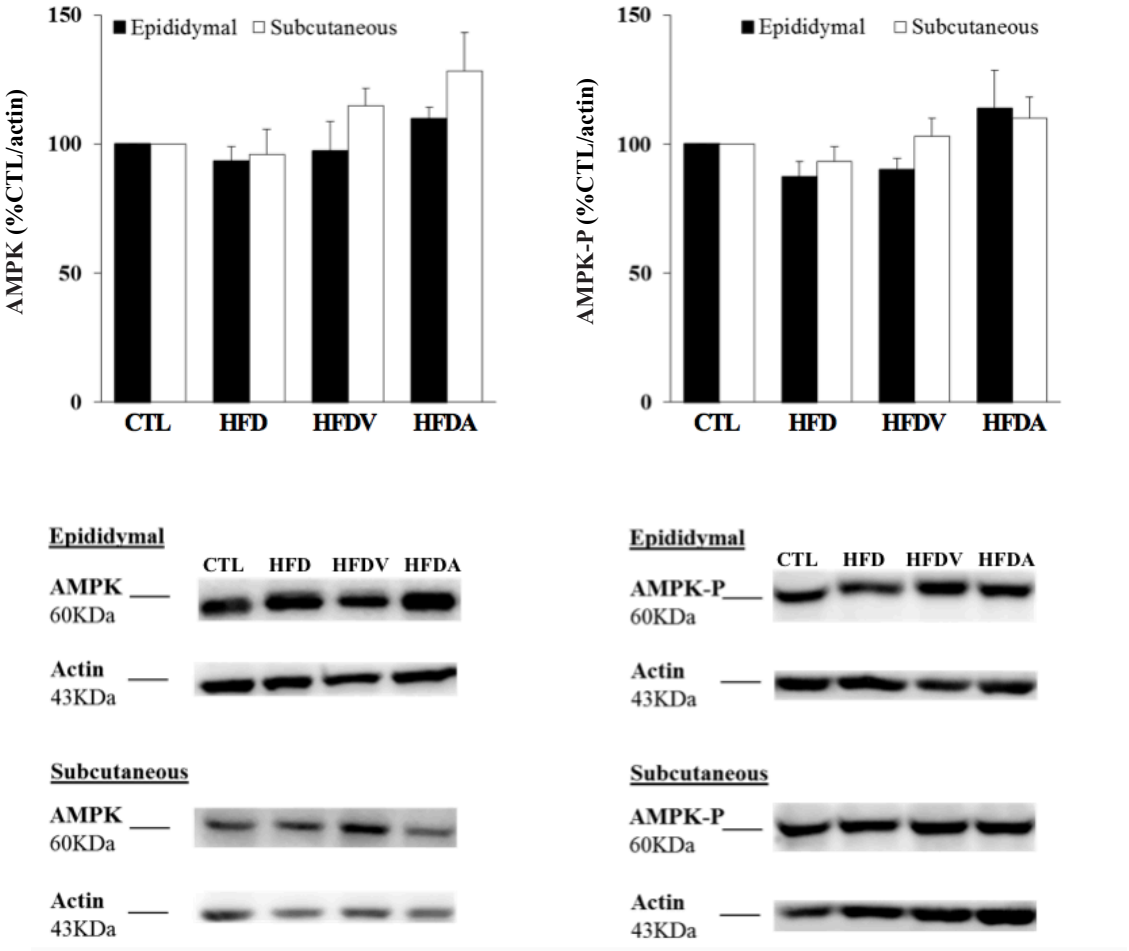


Figure 30. Total (left panel) and phosphorylated (right panel) AMPK levels in the epididymal and subcutaneous adipose tissues. Representative western blots are shown.

AMPK: 5' adenosine monophosphate- activated protein kinase; CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin.

Data is presented as mean±SEM; n=8/group.

However, HFD rats showed decreased HSL activation (Ser563) in epididymal adipose tissue when compared with controls. Similar results were observed in the vehicle-treated group, but not in the adiponectin-treated group, which partially inhibited such effects (Figure 31).

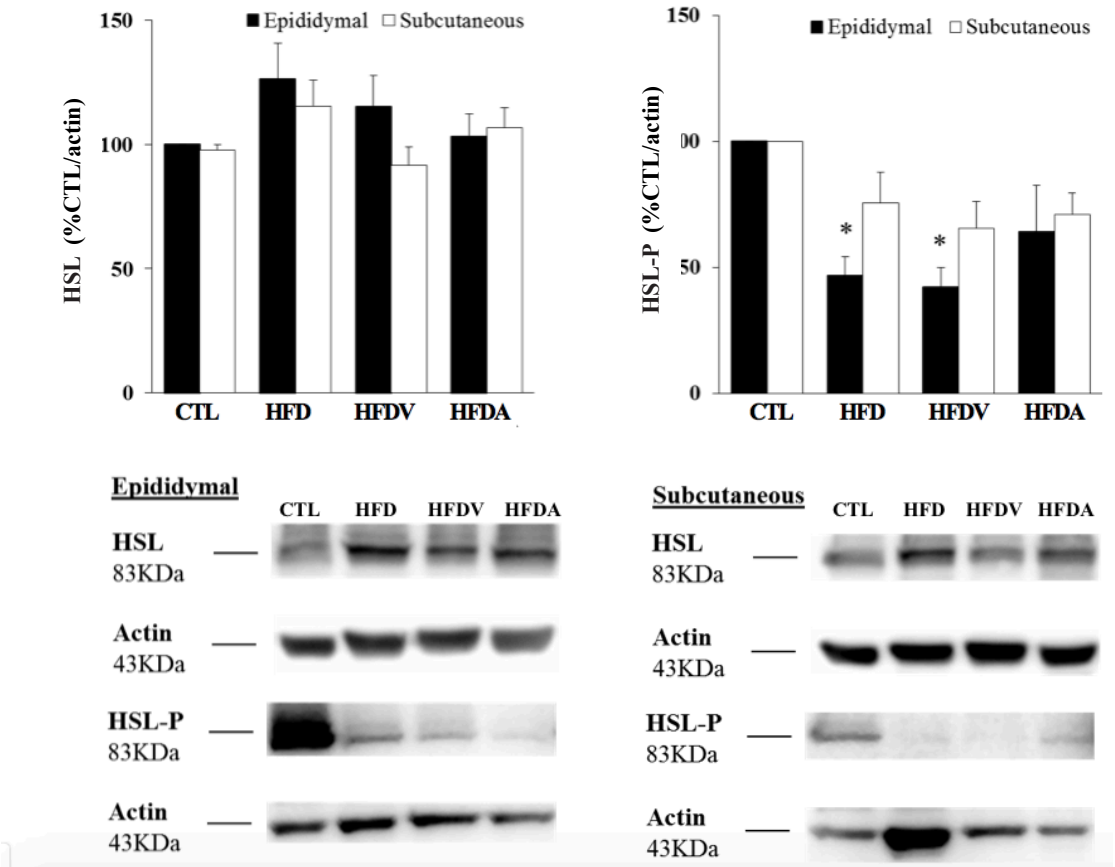


Figure 31. Total (left panel) and phosphorylated (right panel) HSL levels in the epididymal and subcutaneous adipose tissues. Representative western blots are shown.

CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin; HSL: hormone-sensitive lipase.

*Different from CTL. One symbol $p<0.05$.

Data is presented as mean±SEM; n=8/group.

High-fat diet also induced a decrease in PPARγ levels, a key transcriptional factor controlling adipokine gene expression, in both adipose tissues while adiponectin administration prevented such decrease, especially in the epididymal depot where increased levels of this transcriptional factor were observed in comparison with HFD group (Figure 32).

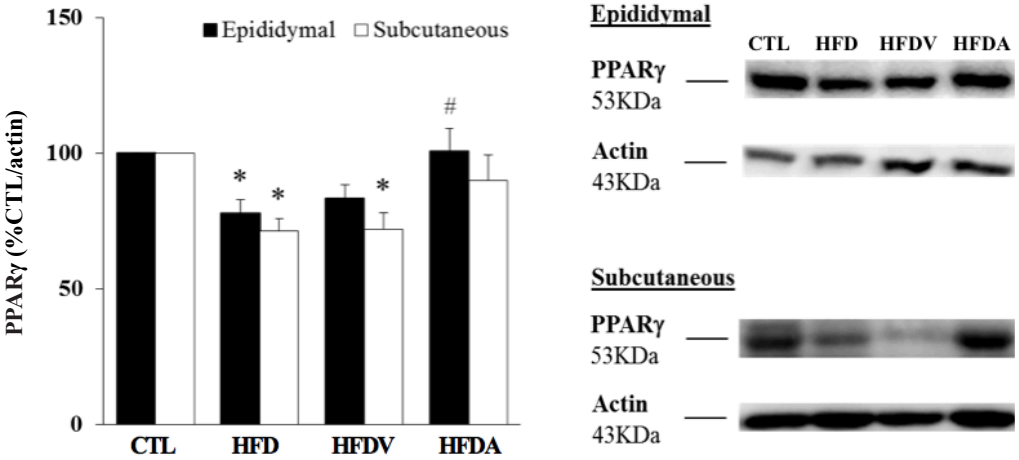


Figure 32. PPARγ levels in the epididymal and subcutaneous adipose tissues. Representative western blots are shown in the right panel.

CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin; PPARγ: peroxisome proliferator-activated receptor gamma.

*Different from CTL. #Different from HFD. One symbol $p<0.05$.

Data is presented as mean±SEM; n=8/group.

III.4.1.4 Effects of adiponectin on adipose tissue inflammation

Activation of inflammatory mechanisms in adipose tissue can be demonstrated through the determination of nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-kB) inhibitor alpha (IkBα) levels, as well as by demonstrating adipose tissue macrophage infiltration using a F4/80 membrane marker.

In both epididymal and subcutaneous adipose tissues, high-fat diet induced a decrease in IkBα levels, suggesting that cellular inflammatory mechanisms were triggered (Figure 33).

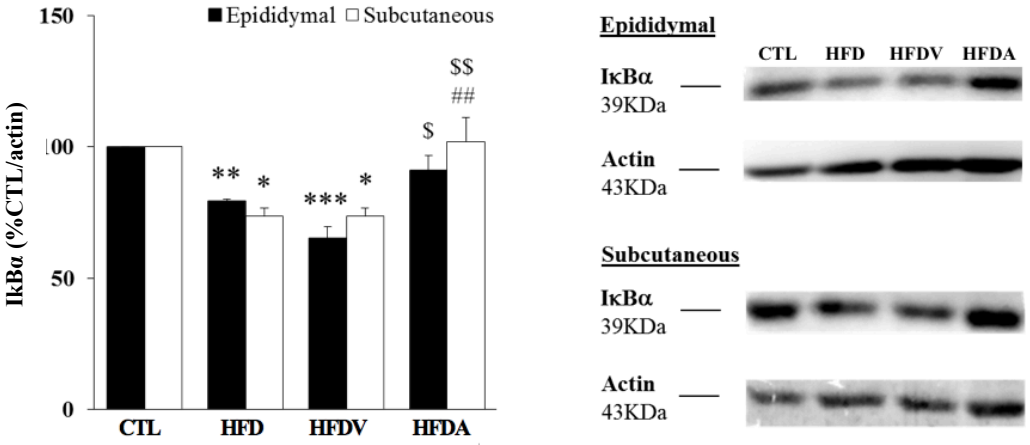


Figure 33. IkBα levels in epididymal and subcutaneous adipose tissues. Representative western blots are shown in the right panel.

CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin; IkBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha.

*Different from CTL. #Different from HFD. $Different from HFDV. One symbol $p<0.05$; two symbols $p<0.01$; three symbols $p<0.001$. Data is presented as mean±SEM; n=8/group.

However, F4/80 levels showed no differences between the groups, although HFD and HFDV rats exhibited F4/80-positive staining in multiple areas from both adipose tissues, as opposed to what was observed in controls. Interestingly, IkBα levels were fully reverted and F4/80-positive regions were reduced by adiponectin treatment (Figures 33 and 34, respectively).

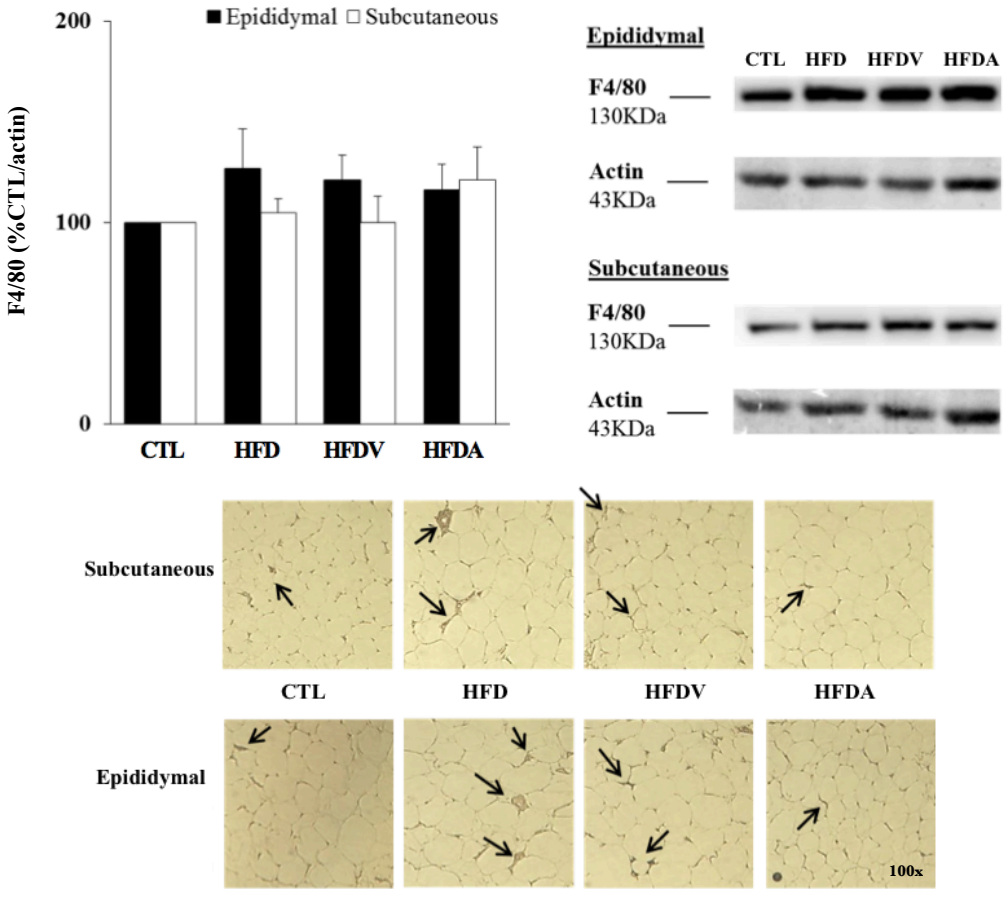


Figure 34. F4/80 levels (top panel) and representative photo-micrographs for F4/80+ areas (bottom panel; black arrows) in epididymal and subcutaneous adipose tissues.

CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin. Data is presented as mean±SEM; n=8/group.

III.4.1.5 Effects of high-fat diet and adiponectin administration on hippocampal insulin signaling pathway

In the hippocampus, high-fat diet did not induce alterations in total or phosphorylated (Tyr1163) insulin receptor levels when compared with controls. However, adiponectin treatment increased total insulin receptor levels when compared to HFD and HFDV and its active form when compared to controls (Figure 35).

To determine if the PI3K/Akt pathway was activated downstream in the HFDA group and given that it represents an important pathway in the regulation of neuronal survival, we measured Akt in the rat hippocampal tissue. Total Akt levels were significantly elevated in the adiponectin-treated rats when compared to HFD, HFDV and control groups, but no differences were observed in phosphorylated Akt (Ser473) levels (Figure 35).

Moreover, insulin-mediated translocation of GLUT4 (mediated by Akt) is thought to be an essential mechanism through which hippocampal neurons can rapidly use glucose during hippocampal-dependent tasks. Thus, we assessed the expression of this transporter in the hippocampus, but no differences were found between the groups (Figure 35).

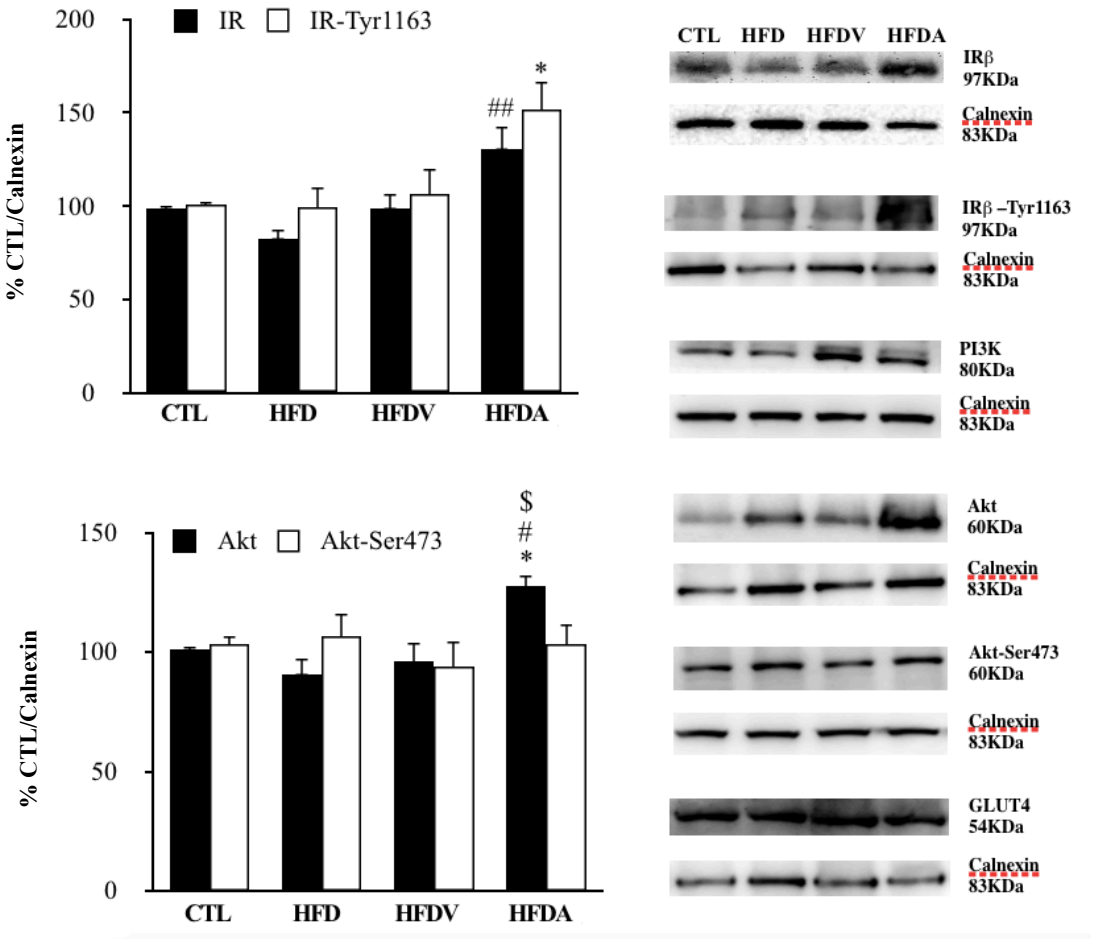


Figure 35. Hippocampal total and phosphorylated insulin receptor (IR) and Akt levels (left panel). Representative western blots are shown, including GLUT4 (right panel). CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin; GLUT4: glucose transporter type 4.
*Different from CTL. #Different from HFD. $Different from HFDV. One symbol $p<0.05$. Data is presented as mean±SEM; n=8/group.

Considering that GSK3β is the major responsible for tau phosphorylation and is inactivated by phosphorylation at Ser9 by Akt, we also looked into hippocampal levels of total and phosphorylated GSK3β and tau (in Ser9 and Ser396, respectively), which were similar in all groups (Figure 36).

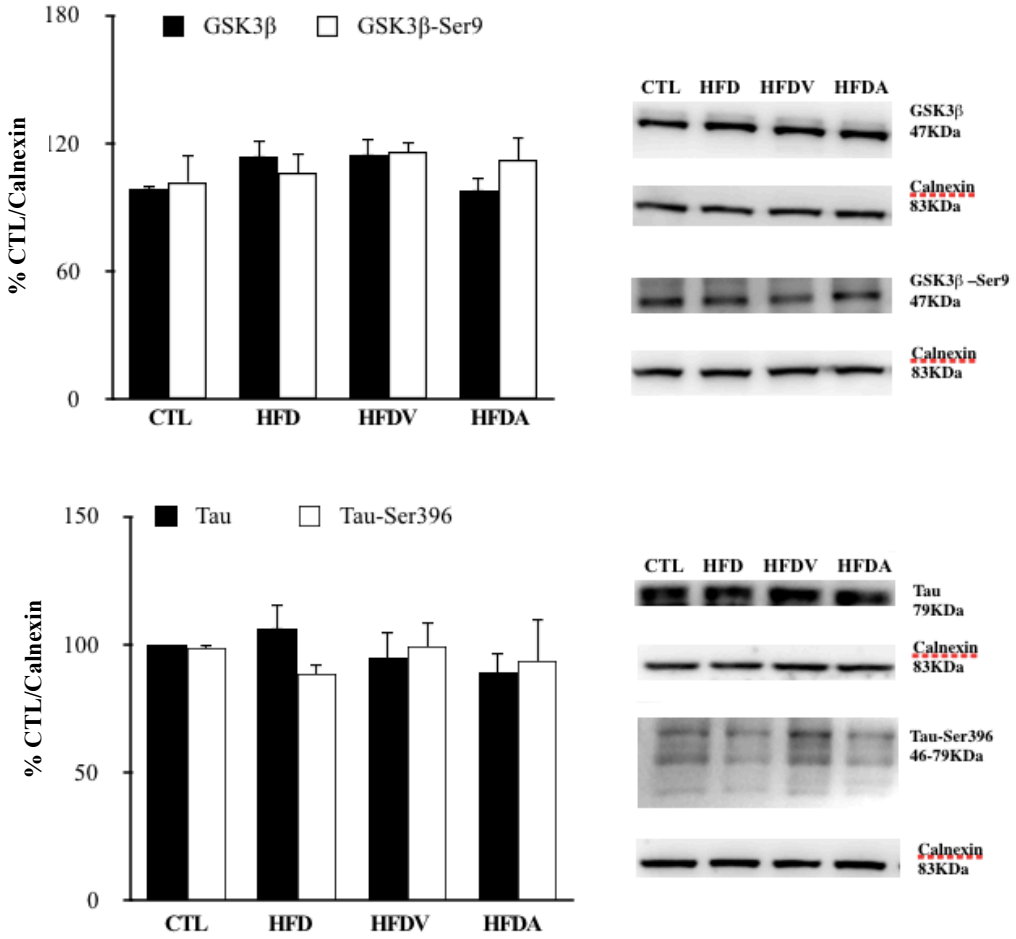


Figure 36. Hippocampal total and phosphorylated GSK3β (top panel) and tau (bottom panel) levels. Representative western blots are shown).

CTL: controls; GSK3β: glycogen synthase kinase 3 beta; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin.

Data is presented as mean±SEM; n=8/group.

III.4.1.6 Effects of adiponectin in hippocampal AdipoR expression and AMPK activation

AdipoR1, AdipoR2 and T-cad are widely distributed in the hippocampus and their expression was determined in all experimental groups. Although no significant differences were observed, T-cad expression showed a tendency to be increased in HFD and HFDV rats and to normalize after adiponectin administration (Figure 37).

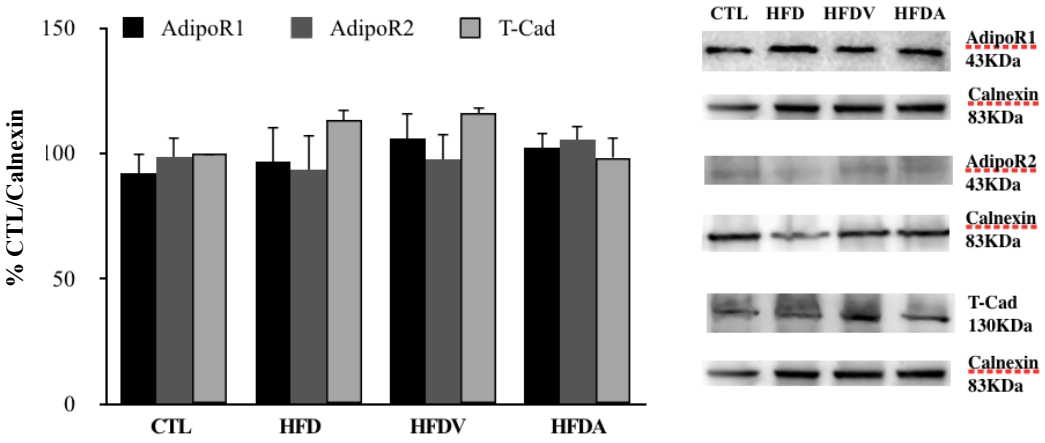


Figure 37. Adiponectin receptors expression in the hippocampus. Representative western blots are shown.

AdipoR: adiponectin receptor; CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin; T-cad: T-cadherin.

Data is presented as mean±SEM; n=8/group.

To determine the capacity of peripherally administrated adiponectin to enhance the activation of hippocampal AMPK, one of its

main downstream effectors, we quantified its total and phosphorylated levels. As shown in Figure 38, AMPK levels were not altered by high-fat diet consumption or adiponectin treatment.

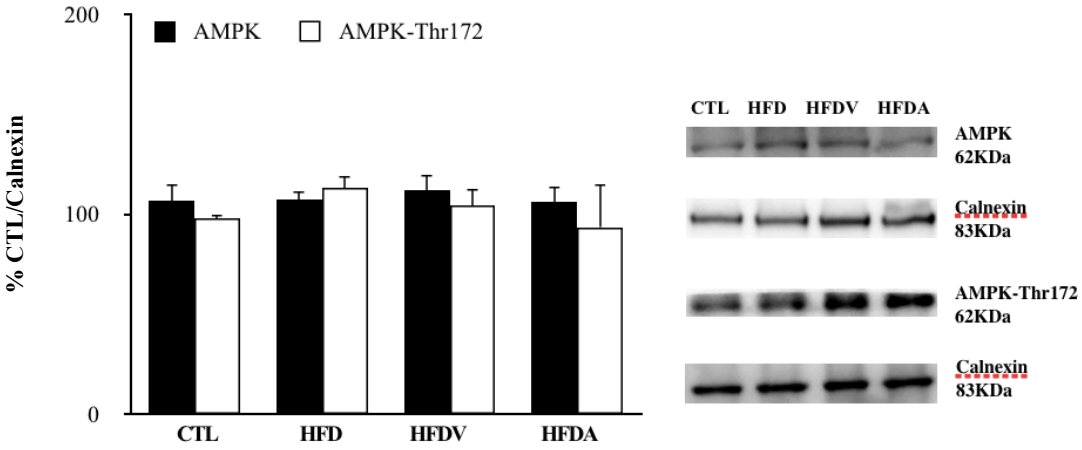


Figure 38. Hippocampal total and phosphorylated AMPK levels. Representative western blots are shown.
AMPK: 5' adenosine monophosphate-activated protein kinase; CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin.
Data is presented as mean±SEM; n=8/group.

III.4.1.7 Effects of adiponectin in presynaptic protein expression, neuroinflammation and neuronal death

Since presynaptic protein expression is traditionally altered in Alzheimer's disease, we assessed hippocampal levels of two SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptor) proteins (syntaxin and synaptosomal-associated protein 25 - SNAP25)

and two vesicle proteins (synaptophysin and synapsin). No significant differences in any of these parameters were observed between the groups (Figure 39).

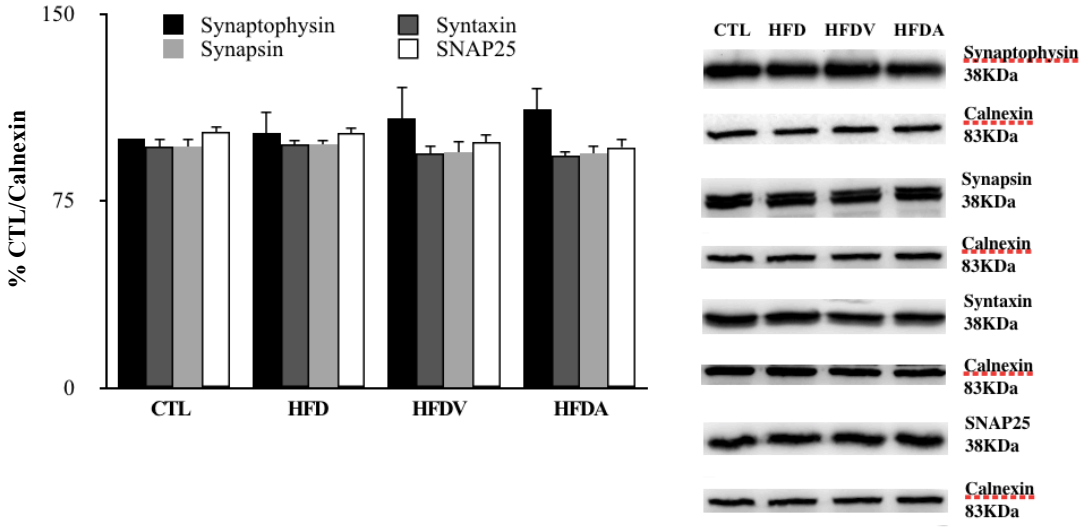


Figure 39. Hippocampal presynaptic proteins expression. Representative western blots are shown.

CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin; SNAP25: synaptosomal-associated protein 25.

Data is presented as mean±SEM; n=8/group.

We also investigated whether the peripheral administration of adiponectin could influence neuronal morphology and survival. Hystological analysis of hippocampal sections revealed preserved cresyl violet staining of Nissl bodies in cornus amonis 1 (CA1), cornus amonis 3 (CA3) and dentate gyrus (DG) regions among the different groups (Figure 40).

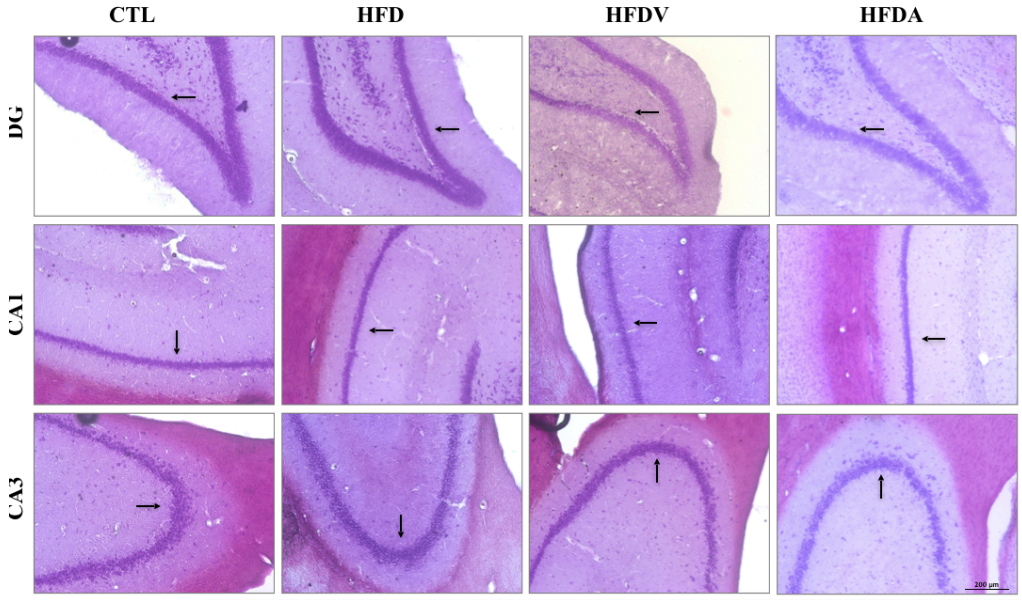


Figure 40. Cresyl violet staining of hippocampal coronal sections (30 µm thickness). Representative images depicting DG, CA1 and CA3 subregions are shown including DG's granule cell layer and CA1 and CA3's pyramidal cell layers (arrows).
Scale bar: 200 µm.
CA: (cornus amonis); CTL: controls; DG: dentate gyrus; HFD: high-fat diet. CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin. n=4/group.

Therefore, the consumption of high-fat diet did not induce significant hippocampal neuronal loss and thus it was not possible to evaluate the capacity of adiponectin in preventing metabolic-induced neuronal damage.

Once adiponectin has recognized systemic anti-inflammatory effects, we also investigated its potential to mitigate astrogliosis, a pathological hallmark of Alzheimer's disease. However, immuno-

reactivity of glial fibrillary acidic protein (GFAP) in CA1, CA3 and DG hippocampal regions was indistinguishable between groups (Figure 41).

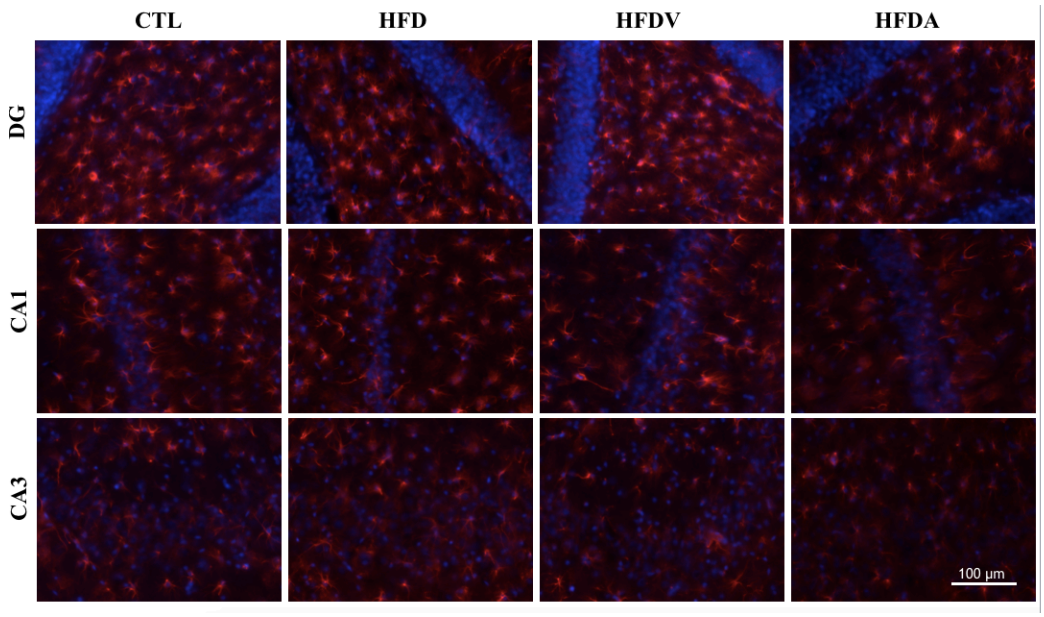


Figure 41. Hippocampal GFAP immunoreactivity (in red) from hippocampal coronal slices (30 µm thickness). Nuclei were stained with DAPI (in blue). Representative images depicting DG, CA1 and CA3 subregions are shown.
Scale bar: 100 um.
CTL: controls; HFD: high-fat diet; HFDV: high-fat diet and vehicle; HFDA: high-fat diet and adiponectin; DG: dentate gyrus; CA: (cornus amonis); GFAP: glial fibrillary acidic protein; DAPI: 4',6-diamidino-2phenylindole. n=4/group.

III.4.1.8 Effects of adiponectin in hippocampal-dependent cognitive tasks

To determine the effect of exogenous adiponectin in hippocampal-dependent spatial learning and memory we used Morris Water Maze task. In consecutive 4 days of the hidden platform tests, the escape latency in all groups showed a gradual decrease (Figure 42).

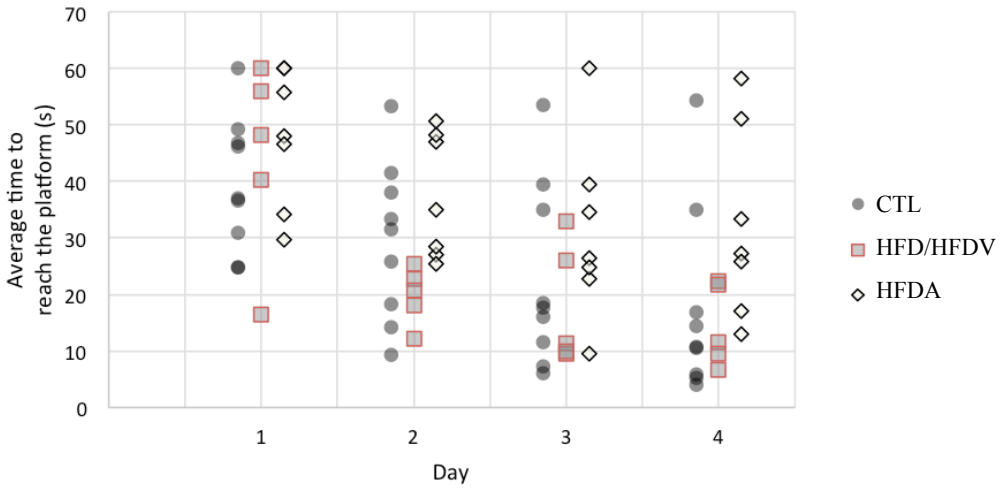


Figure 42. Morris Water Maze spatial learning and memory task performance during learning sessions. Results are expressed as mean of latency to find the platform (in seconds - s) for each animal in each of the training days (4).
CTL: controls (n=9); HFD/HFDV: high-fat diet/high-fat diet and vehicle (n=5); HFDA: high-fat diet and adiponectin (n=7).

Both controls and adiponectin-treated rats significantly reduced the time spent to reach the platform when comparing the first and last days (CTL: p=0.021; HFD/HFDV: p=0.080; HFDA: p=0.018). Though

statistical significance was not attained for the HFD/HFDV group, the results from CTL and HDFA groups are rather scattered and do not translate what is more clearly represented in Figure 42.

Memory function was tested on the 5th day with the platform removed. Measured variables included: (a) time spent in the correct quadrant, i.e., where the platform was placed, (b) time spent in the opposite one, (c) latency to reach the original position of the platform for the first time, and finally (d) the number of crossings in the correct quadrant. All groups presented similar performances in the variables tested (Figure 43), meaning that high-fat diet did not impair learning or spacial memory and thus we were not able to evaluate preventive effects of adiponectin in this context. During the experiment, some animals presented clinical signs of respiratory distress (particularly affecting HDF and HFDV groups) and were excluded. Once HFD-fed rats from both groups did not display significant differences in their behavioural performance, we have gathered their results in one group (HFD/HFDV).

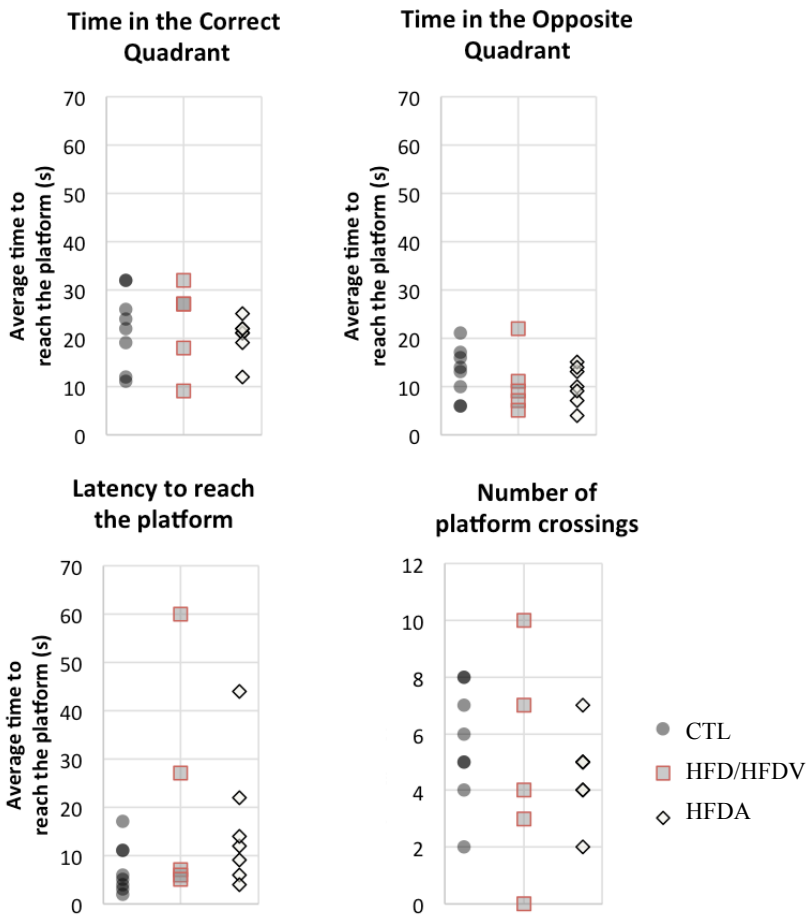


Figure 43. Morris Water Maze spatial learning task performance during the test session, on the 5th day. Time in the correct and in the opposite quadrants (upper panels) as well as latency to reach the original place of the platform and number of crossings (lower panels) are represented for each group.

CTL: controls (n=8); HFD: high-fat diet/high-fat diet and vehicule (n=5); HFDA: high-fat diet and adiponectin (n=7).

Chapter III.5

Highlights

Table 31. Highlights of the results arising from animal investigation

1. Long-term subcutaneous administration of globular adiponectin reduced body weight and epididymal/visceral adipocyte size, and has also improved serum LAR;
2. Long-term subcutaneous administration of globular adiponectin restored high-fat diet-induced epididymal adipose tissue dysfunction (insulin signaling, lipolysis, and inflammation), improving systemic metabolism;
3. Long-term subcutaneous administration of globular adiponectin improved hippocampal insulin signaling in aged Wistar rats, suggesting that it may contribute to brain resilience to age-related neurodegenerative diseases;
4. High-fat diet did not induce neuronal loss, neuroinflammation, synaptic protein or memory impairment, compromising the study of potential neuroprotective actions of adiponectin.

Chapter III.6

Discussion

III.6.1 The effects of high-fat diet in systemic and adipose tissue metabolism

Obesity is a complex disease that has a multifactorial etiology. Of particular significance is the excessive consumption of dietary fat, which along with physical inactivity, is the major causative factor of obesity. For this reason we decided to use a diet-induced obesity rat model instead of monogenetic models. A great amount of evidence show that several weeks of a semi-purified diet with a fat content of at least 40% can lead to obesity, hyperglicemia, hypertriglyceridemia, and hyperleptinemia if administered to susceptible rodent strains such as Wistar rats, mimicking the pathophysiology of human obesity and metabolic syndrome (Buettner *et al,* 2007). As expected, after 4 months of high-fat diet (70% energy from fat), animals increased their body weight and subcutaneous adipocyte area. This was not observed in the epididymal adipose tissue, but 12 month old Wistar rats have already increased adipocyte area in this fat depot when compared to 6 month old rats, and thus limited buffering capacity (data not shown).

Systemic effects of high fat diet in rodents are, on the other hand, dependent on animal strain, fat type (e.g. lard, corn oil, safflower oil, fish oil) and diet length, so its effects on blood glucose, insulin and lipid levels are somehow discrepant across studies (Buettner *et al*, 2007). Although in our high-fat fed rats no significant differences were observed in fasting

glucose and lipid metabolic markers (glycemia, insulinemia, HbA1c, cholesterol and triglycerides), there was an increase in glycemia 2 hours after a glucose intraperitoneal overload and in plasmatic free fatty acids. This was accompanied by impaired activation of the insulin pathway in the epididymal adipose tissue, the correspondent in humans to visceral adipose tissue and the depot most implicated in obesity-associated dysmetabolism. In this tissue, IRS-1 levels (total and activated) were decreased by the diet, despite the fact that downstream Akt levels were unaltered, suggesting that this decrease was probably not severe enough to affect GLUT4 expression or translocation to the cell surface, although we did not assess this particular parameter.

Meanwhile, the alterations described above for epididymal adipose tissue were not observed in the subcutaneous WAT. This comes in line with evidence showing that subcutaneous WAT is a depot less predisposed to insulin resistance and inflammation related with high-fat diet consumption and consequently able to better respond or adapt to conditional changes in metabolic demands (Sierra Rojas *et al*, 2016). Accordingly, only in the epididymal adipose tissue was lipolysis significantly impaired as a consequence of high-fat diet consumption taking into consideration the decreased levels of activated HSL in this tissue, although both depots had decreased PPARγ levels. This nuclear receptor is responsible for promoting insulin sensitivity due to its control

over lipid esterification, explaining the increase in plasmatic FFA levels observed in the HFD group, but also due to the inhibition of inflammatory pathways such as NF-κB. Moreover, PPARγ is a key transcriptional factor in the control of adipokine gene expression, including adiponectin (Ram, 2003; Guilherme *et al*, 2008). Notwithstanding, serum adiponectin levels were not reduced by the diet, as expected. In humans and rodents, obesity is classically associated with a decrease in adiponectinemia and in the expression of adiponectin in the adipose tissue (Ouchi *et al*, 2011). Interestingly, some authors have reported that lower adiponectin mRNA levels do not always reflect a parallel decrease in serum protein concentration, emphasizing the complexity of post-translational control mechanisms of circulating adiponectin levels. In our study, beside not having assessed adiponectin mRNA levels we also did not adjust its concentration to (increased) adipose tissue mass or body weight, what in some authors' opinion is a more appropriated approach (Barnea *et al*, 2006). Moreover, we did not discriminate adiponectin isoforms and thus it was not possible to determine if the diet caused any shift on the HMW/Low molecular weight (LMW) ratio, what could better reflect metabolic distress. Total and HMW levels have been described as proportional by most studies, but differential effects of each isoform as well as their tissue-specific actions are largely unexplored. Finally, high-fat diet induced

hyperleptinemia, as expected by the increase in fat amount. This is typically found in obese individuals, contributing to the development of leptin resistance and further aggravation of metabolic syndrome (Stern *et al*, 2016). On account of the elevation in leptin levels in the HFD groups, serum LAR was increased. This ratio is considered an important and reliable marker of adipose tissue dysfunction (Satoh *et al*, 2004).

In summary, these data show that high-fat diet consumption by adult Wistar rats results in visceral adipose tissue dysfunction and decreased glucose tolerance, which are associated with impaired insulin sensitivity.

III.6.2 The effects of peripheral administration of adiponectin in systemic and adipose tissue metabolism

To study the peripheral effects of adiponectin we have performed for the first time a prolonged and controlled subcutaneous administration of the recombinant globular domain of this adipokine, which was produced using an innovative strategy (Hitag's rPPure platform). Adiponectin circulates in high levels in plasma, making its chronic administration difficult from the economic point of view. Until now, several research groups have developed different strategies to achieve sustained *in vivo* hyperadiponectinemia. Gene therapy

approaches included transgenic mice or adenovirus-mediated adiponectin overexpression in adipose tissue (Satoh *et a*., 2005; Bauche *et al*, 2007) and injection of adiponectin-expressing plasmids in the skeletal muscle (Kandasamy *et al*, 2012). All of these originated higher plasma adiponectin levels, resulting in higher energy expenditure due to increased AMPK and Akt activation in liver and skeletal muscle, and improved glycemic profile. Using an experimental setup with daily adiponectin intraperitoneal administration during 7 days to obese mice, Masaki *et al* (2003) showed that adiponectin increased resting metabolic activity, with increased expression of UCPs in adipose tissue, what appears to counteract the findings of Qiao *et al* (2014). Data from these studies suggest that globular and HMW forms of adiponectin may have different effects on BAT. In the study conducted by Xu *et al* (2003), a subcutaneous minipump with continued full-length adiponectin infusion (30 μg/day) was implanted during 14 days in mice fed a high-fat ethanol-containing diet. Continued adiponectin infusion resulted in reduced liver injury with increased lipid oxidation and decreased inflammation. In our study, for the first time we report the effects of a long-term (28 days) continued globular adiponectin infusion (98 μg/day) on systemic and adipose tissue metabolic markers of high-fat fed non-obese and non-diabetic rats. We performed a more prolonged administration with a smaller dosage, when matched for body weight.

Our results demonstrate that chronic peripheral adiponectin administration resulted in the reduction of body weight, suggesting that the raise in serum adiponectin may contribute to the weight loss observed in patients with incipient dementia (Johnson *et al*, 2006). It also resulted in decreased epididymal adipocyte area with consequent decrease in serum leptin and LAR as well as in increased IkBα (NF-kB inhibitor) and PPARγ levels. Adiponectin administration also resulted in the increase of IRS-1 activation in epididymal adipose tissue and consequently in its sensitivity to insulin, which was associated with decreased fasting glycemia and glucose tolerance improvement. Importantly, HFD and HFDV groups showed serum glucose levels over 140 mg/dl 2 hours after intraperitoneal glucose administration, the threshold for considering the existence of glucose intolerance. This was not observed after adiponectin treatment. More, we show that this type of therapeutic approach may be useful to improve adipose tissue function. We have not evidenced an increase in serum adiponectin levels or AMPK activation, most probably due to the method of administration, i.e., low dosis of adiponectin was continuosly delivered instead of high dosis in specific time points. More, chronic adiponectin infusion was well tolerated and no toxicity was denoted taking into account hepatic and renal injury markers (data not shown). Overall, this study establishes the potential of globular adiponectin in improving

adipose tissue function, and thus metabolic homeostasis in obesogenic conditions, as well as its safety.

III.6.3 Hippocampal effects of high-fat diet and peripheral administration of adiponectin

After characterization of systemic and adipose tissue effects of peripherally delivered adiponectin, attention was focused on the rat hippocampus, once it is especially susceptible to Alzheimer's pathology and is one of the brain structures with higher expression of adiponectin receptors (Thundyil *et al*, 2012).

Many studies have linked high-caloric and high-fat diets with impaired hippocampal function, especially when associated with aging. Worse performance on spatial learning and memory tasks, reduced hippocampal dendritic spine density, reduced long-term potentiation and BDNF levels have been described (Freeman *et al,* 2014). Although we have not observed deleterious diet-induced effects such as synaptic disruption, astrogliosis, disruption of the insulin/PI3K/Akt/GSK3β/tau pathway or spatial memory impairment in the hippocampus, we report for the first time beneficial hippocampal effects of prolonged peripheral administration of adiponectin. It ameliorated insulin sensitivity, as we also reported in the adipose tissue of these animals, even though it did

not alter t-tau or p-tau levels. It would probably be necessary to add a more direct neurotoxic insult to better determine if adiponectin was able to prevent tau hyperphosphorylation. Notwithstanding, adiponectin treatment was able to enhance the activation of insulin receptor when compared to aged controls, suggesting that this adipokine may have beneficial effects on aging-related metabolic disturbances and thus contribute to hippocampal resilience against neurodegeneration. Interestingly, and although in our model the expression of hippocampal adiponectin receptors was unaltered after the diet or adiponectin administration, it was recently described that mRNA of both AdipoR1 and 2 is elevated in wild type rats after restraint stress, and that this phenomenon is impaired in Alzheimer's transgenic animals. Indeed, there is some evidence supporting the existence of downregulated expression of AdipoRs in this disorder, which may compromise its effects against neuronal insults (Várhelyi *et al*, 2017).

Gathering all data and current knowledge, it is reasonable to speculate that adiponectin can be considered a potential therapeutic target in Alzheimer's disease. Besides lifestyle modifications (such as diet and physical exercise) and bariatric surgery, which are able to increase adiponectin secretion, there is growing interest in pharmacological strategies that target adiponectin, AdipoR or its downstream signalling

pathways. Several drugs have shown their ability to increase plasma adiponectin levels (Montecucco *et al*, 2009) and presumably its cerebral levels although none are currently approved for the treatment of Alzheimer's disease (Dufouil *et al*, 2005; Fakhfouri *et al*, 2012; O'Caoimh *et al*, 2014; Cheng *et al*, 2016; Mcguinness *et al*, 2016; Zhou *et al*, 2016). Although representing the most obvious replacement strategy, direct peripheral administration of adiponectin (intravenous or intraperitoneal) has important limitations. These include: (1) its high concentration in plasma (μg/mL) with the need of high dosis administration and elevated costs; (2) the existence of different isoforms with unclear action/site of action; and (3) possible feedback loops in which adiponectin down-regulates its own production and AdipoR expression (though only demonstrated for AdipoR2). Similar to leptin and insulin, adiponectin peripheral resistance has also been described and may represent a major obstacle to its peripheral administration (Sun *et al*, 2009). Central resistance to leptin and insulin are likewise described in obesity and Alzheimer's disease, but whether the same occurs with adiponectin remains unexplored. If adiponectin could be delivered to the CNS, some of the limitations described above would be overridden, including the amount of protein required. Various non-invasive methods, such as drug lipophilic transformation, prodrugs and intranasal drug delivery are already widely used in neurodegenerative diseases research. Intranasal

globular adiponectin treatment has been used in mice models of α-synucleinopathies with beneficial effects in disease progression (Sekiyama *et al*, 2014). However, the utilization of AdipoR agonists has been suggested as the most promising therapeutic approach (Okada-Iwabu *et al*, 2013; Kadowaki *et al*, 2014), though specific molecular key mediators in the adiponectin signalling pathway, such as APPL1, may also be suitable candidates in the search for therapeutic targets. As more accessible alternatives, natural products have been studied as anti-neurodegeneration agents. The most paradigmatic example is osmotin, present in fruits and vegetables, that may act as an AdipoR agonist.

PART IV

CONCLUSIONS AND FUTURE DIRECTIONS

Taken together, the results reported in this thesis provide evidence that adiponectin may influence the risk of developing Alzheimer's disease as well as its progression rate. It further expands the knowledge on how increased serum adiponectin levels observed in these patients can reflect a metabolic integrated effort against neurodegeneration, and, in addition, that serum adiponectin may be admitted as an accessible staging biomarker, most likely integrated in peripheral biomarker panels that could be, in the future, used in clinical settings.

The results arising from our research also highlight the gender specific role that adipokines, particularly adiponectin, may assume in the crosstalk between the brain and adipose tissue. In our perspective, these findings indicate that research on Alzheimer's disease should always include gender specific outcomes in clinical studies, as well as include female animals, seldom used in experimental research. Furthermore, gender differences in adipose tissue's physiology and its complex connections with the CNS should be included in the list of potential contributors to the female-biased AD incidence. This will be one of the focus of our future research. Ultimately, the contribution of the raise in serum adiponectin to the weight loss observed in MCI patients that progress to dementia must also be accounted and further explored.

Finally, we have demonstrated that adiponectin is able to improve adipose tissue function and induce not only systemic but also central metabolic benefits, which comprised insulin sensitivity improvement in the hippocampus. We also demonstrated that prolonged globular adiponectin administration is well tolerated and does not produce any side effect although, as previously discussed, it will be very difficult to used it in clinical settings and agonists are being currently tested.

Moreover, adiponectin may exert its neuroprotective actions by activating anti-inflammatory and anti-apoptotic signaling pathways. None of these pathways were specifically investigated in this exploratory study but will be addressed in future research. Adiponectin also participates in calcium fluxes and mitochondrial metabolism, highly implicated in Alzheimer's disease pathogenesis, though no report exists on this specific action on the hippocampus, like is the case of other signaling pathways such as p38MAPK, Rab5 and ERK. Studies focusing these questions will offer valuable insights into the mechanisms underlying cerebral pleiotropic actions of this adipokine. Further investigation is also required to clarify key gaps in our understanding of adiponectin's physiology, particularly how its passage to the CNS is regulated, the exact function of each isoform and their receptor-dependent and independent downstream pathways. This would certainly facilitate data interpretation and enhance congruency of the results

delivered by clinical research. Likewise, it is important to explore if other CNS areas such as frontal and temporal cortices are also susceptible to adiponectin modulatory actions and elucidate if adiponectin can influence other neurodegenerative diseases. On the other hand, it is important to improve the knowledge on the effects of adiponectin in glial cells, once they are relevant players on Alzheimer's disease pathophysiology and this adipokine is involved in systemic inflammation. To clarify these questions other models and approaches should be explored, namely Alzheimer's transgenic mice and icv continuous adiponectin administration.

Furthermore, one must not overlook that adipose tissue is a source of peripheral amyloid precursor protein (Joachim *et al*, 1989), which expression is up-regulated under obesogenic conditions and positively correlated with insulin resistance, hyperinsulinemia, inflammation (Lee *et al*, 2008) and Aβ plasmatic levels (Lee *et al*, 2009). In addition, it was recently recognized that peripheral-derived Aβ can enter the brain, contribute to Alzheimer's disease pathology and induce functional neuronal deficits (Bu *et al*, 2017). Whether obesity-induced alterations on adipose tissue's secretome influence the regulation of adipocyte APP processing machinery, Aβ peripheral production and clearance or the expression of proteins involved in Aβ efflux/influx through the BBB are some of many fundamental questions, the answer

to which would help to better understand the relationship between obesity and Alzheimer's disease.

Ultimately, our study contributes to support adiponectin's involvement in the association between mid-life obesity and late-onset Alzheimer's disease and further encourage research in this field. It is important to aknowledge that currently only about 20% of patients with Alzheimer's dementia are being treated with the approved symptomatic therapies, and it is estimated that the proportion of patients having access to new drugs, probably more expensive and intravenous, will be even lower (Frisoni *et al*, 2017). It is thus important to outline that, even whith the approval of new drugs, effective and aggressive strategies for the prevention of Alzheimer's disease are imperative and a thorough understanding of its modifiable risk factors, including obesity, may have an important impact on reducing the burden of this devastating disease.

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