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Glucose Metabolism, Alzheimer's Disease and Trasnthyretin, is there a link?

Sofia Cordeiro Tavares

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University of Porto

Faculty of Medicine

Experimental Biology Department

Masters in Neurobiology

IBMC- Institute of Molecular and Cellular Biology

i3S- Institute for Research and Innovation in Health

Glucose metabolism, Alzheimer's Disease and Transthyretin, is there a link?

Student: Sofia Tavares

Supervisor: Isabel Cardoso, PhD

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"Wherever you go, go with all your heart." [Confucius]

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"I hear and I Forget. I see and I remember. I do and I understand."

[Confucius]

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[Henry Ward Beecher]

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"Ser feliz é reconhecer que vale a pena viver

Apesar de todos os desafios, incompreensões e períodos de crise.

(…)

É atravessar desertos fora de si, mas ser capaz de encontrar

Um oásis no recôndito da sua alma…

(…)

É ter coragem para ouvir um "Não"!!!

É ter segurança para receber uma crítica,

Mesmo que injusta…

Pedras no caminho?

Guardo todas, um dia vou construir um castelo…"

[Fernando Pessoa]

Abstract

Alterations in glucose metabolism are associated not only with diseases such as Diabetes Mellitus (DM) type 1 and 2 (T1DM and T2DM, respectively) but also with neurodegenerative disorders such as Alzheimer's Disease (AD) and other dementias.

Transthyretin (TTR), a 55 kDa homotetrameric protein known for its role in transport of T_4 and retinol, has also been reported to be altered in metabolic disorders. For instance, in T2DM and in Gestational Diabetes Mellitus (GDM) TTR is elevated, whereas in T1DM TTR is decreased. TTR was also found to promote insulin release to protect against β-cell death and to bind to glucose-regulated proteins, to regulate glucagon, recovery of glucose levels, suggesting that TTR may play important roles in glucose homeostasis by regulating the expression of glucagon. Moreover, TTR, which is also decreased in CSF and plasma of AD patients, is the major Aβ peptide sequestering protein in CSF avoiding Aβ aggregation and toxicity and thus known to be protective in AD.

Therefore, this project aimed to investigate the role of TTR in glucose metabolism and the implications it might have in AD.

Here, we showed, using both *in vivo* and *in vitro* models, that TTR is involved in glucose metabolism at the periphery and at the BBB, since TTR influences the internalization of glucose, through 1) the modulation of upstream proteins in the liver, such as glucose transporters and possibly 2) the modulation of downstream pathways in the BBB.

We began by evaluating the effect of TTR in glucose metabolism, by measuring the glucose levels in plasma from mice with different TTR genetic backgrounds (with two copies of the TTR gene: TTR+/+; with only one copy of the TTR gene: TTR+/-; and without TTR: TTR-/-). Our results suggested that insufficient amount of TTR impairs uptake of glucose from blood, since mice with insufficient TTR (TTR+/- mice) had significantly higher plasma glucose levels, although, and confirming the results obtained by another group, plasma glucose levels were not altered in TTR-/- animals, as compared to TTR+/+.

Given the importance of the liver both in glucose homesotasis and in TTR production and catabolism, we also evaluated glucose in the culture media of hepatocytes, using both an hepatoma cell line ($HepG_2$ cells) and, aiming at bringing the model closer to the *in vivo* situation, primary hepatocytes derived from TTR+/+, TTR+/- and TTR-/- mice. As for the $HepG_2$ cells, no alterations in glucose levels in their culture media were observed, when cells were incubated with human recombinant TTR (hTTR). In relation to the primary hepatocytes, interestingly, the results confirmed the observations in the plasmas, as TTR+/− hepatocytes presented the highest glucose levels. Importantly, addition of hTTR in the media of TTR+/− hepatocytes partially rescued the phenotype and significantly decreased the glucose levels. We also observed that glucose in the supernatants of TTR−/− hepatocytes was higher than in the TTR+/+, differently from the measurements in plasmas, but still not as high as in the TTR +/− supernatants.

Given the extreme importance of glucose metabolism in the brain and the related alterations in several disorders, we also performed a similar study at the BBB, using the hCMEC/D3 cell line, demonstrating that the presence of TTR decreased the glucose levels in supernatant media of these cells too.

To further understand if the observed decreased glucose metabolism was a consequence or a cause of the TTR insufficiency in mice, we evaluated the effect of different concentrations of glucose in TTR levels. We observed no differences in TTR expression, which suggests that glucose alterations do not precede TTR insufficiency, but instead it is the other way around: alterations in TTR are prior to the changes in glucose levels.

Next, we decided to investigate the mechanism behind the observed glucose levels, by analyzing the effect of TTR on glucose transporters, namely GLUT1, by immunocytochemistry. Here, we observed that in the liver cellular models, but not in our BBB model, TTR increased the expression of GLUT1, suggesting that TTR modulates the upstream proteins in glucose metabolism at the liver.

In order to address the impact of TTR in glucose signaling cascade, we then evaluated the effect of TTR in the expression of PKM1/2 (at the transcript level) by qRT-PCR and, although not statistically significant, we observed increased expression of this gene in the presence of TTR in hCMEC/D3 cells, but not in $HepG_2$ cells, suggesting a downstream modulation of TTR in glucose signaling pathways in our BBB model.

Overall, our results suggest that TTR might have a protective role in glucose metabolism, although by modulating through different pathways at the BBB and at the liver.

Keywords: Alzheimer's Disease, Amyloid β peptide, Glucose metabolism, Blood-brain barrier, Periphery, Transthyretin.

Resumo

Alterações no metabolismo da glicose estão associadas não só com doenças como a Diabetes Mellitus tipo 1 e 2 (DMT1 e DMT2, respetivamente), mas também com doenças neurodegenerativas, como a Doença de Alzheimer (DA) e outras demências.

A transtirretina (TTR), uma proteína homotetramérica de 55 kDa, conhecida pelo seu papel no transporte de T_4 e retinol, encontra-se alterada em diversas desordens metabólicas. Por exemplo, na Diabetes Mellitus, a TTR é encontrada em níveis mais elevados na DMT2 e na Diabetes Mellitus Gestacional (DMG), enquanto que na DMT1 se encontra em níveis mais baixos. Também foi demonstrado que a TTR pode promover a liberação de insulina, de forma a proteger as células β da apoptose, bem como ligar-se a proteínas reguladoras da glicose, regular o glucagon e recuperar os níveis de glicose, o que sugere que a TTR desempenha importantes funções na homeostase da glicose através da regulação do glucagon.

Além disso, a TTR, que se encontra diminuída no fluido cérebroespinal e plasma de pacientes com DA, é a principal proteína sequestradora do péptido Aβ no fluido cérebroespinal, evitando a toxicidade e agregação do Aβ e, portanto, contribuindo para a sua função neuroprotetora na DA.

Portanto, este projeto teve como objetivo investigar o papel da TTR no metabolismo da glicose e as implicações que isso possa ter na DA.

No nosso trabalho, demonstramos, usando modelos *in vivo* e *in vitro*, que a TTR está envolvida no metabolismo da glicose e modula tanto a barreira hematoencefálica (BHE) como a periferia, uma vez que 1) influencia a internalização da glicose, através da 2) modulação das proteínas a montante no fígado, tais como os transportadores da glucose e, possivelmente, na 3) modulação das vias a jusante no BHE.

Em primeiro lugar, começou-se por avaliar o efeito da TTR no metabolismo da glicose através da medição dos níveis de glucose em plasma de ratos com diferentes backgrounds genéticos de TTR (com duas cópias do gene da TTR: TTR+/+; com apenas uma cópia do gene da TTR: TTR+/-; e sem TTR: TTR-/-). Estes resultados sugeriram que a insuficiência de TTR prejudica a captação de glicose do sangue, uma vez que os ratos com insuficiência de TTR (ratos TTR+/-) apresentaram níveis plasmáticos de glicose significativamente maiores, embora, e confirmando os resultados obtidos por outro grupo, os níveis de glicose no plasma não se alteraram nos animais TTR-/-, comparativamente aos TTR+/+.

Dada a importância do fígado não só na homeostase da glicose, como também na produção e catabolismo da TTR, também avaliamos a glicose nos meios de cultura de hepatócitos, utilizando uma linha celular de hepatomas (células $HepG_2$) e hepatócitos primários derivados de ratos TTR+/+, TTR+/- e TTR-/-, visando aproximar o modelo da situação *in vivo*. Relativamente às células $HepG_2$, não se verificaram alterações nos níveis de glicose no seu meio de cultura, quando incubadas com TTR humana recombinante (hTTR). Quanto aos hepatócitos primários, curiosamente, confirmou as observações obtidas nos

plasmas, sendo que os hepatócitos TTR+/- apresentaram on níveis de glicose mais elevados. De salientar que, a adição de hTTR no meio dos hepatócitos TTR-/- levou a uma recuperação parcial do fenótipo e a um decréscimo significativo dos níveis de glicose. Também observamos que os níveis de glicose nos sobrenadantes dos hepatócitos TTR-/- eram mais elevados que dos TTR+/+, diferente das medições observadas nos plasmas, mas ainda assim não tão elevado como nos TTR+/-.

Dada a extrema importância do metabolismo de glicose no cérebro e alterações que neste ocorrem relacionadas com diversos distúrbios, também realizamos um estudo semelhantes na BHE, utilizando a linha celular hCMEC/D3, demonstrando que, na presença de TTR, os níveis de glicose diminuem no subernadante destas células.

Para entender melhor se a diminuição do metabolismo da glicose seria uma consequência ou uma causa da insuficiência de TTR nos ratos, avaliamos o efeito de diferentes concentrações de glicose nos níveis da TTR. Aqui, não foram observadas diferenças na expressão de TTR, o que sugere que as alterações na glicose não precedem a insuficiência de TTR, mas em vez disso o oposto, ou seja, alterações na TTR são anteriores às mudanças nos níveis de glicose.

Em seguida, investigou-se o mecanismo responsável pelos níveis de glicose, através da análise do efeito da TTR nos transportadores da glicose, principalmente o GLUT1, por imunocitoquímica. Aqui, observou-se que nos modelos celulares de fígado, mas não no modelo da BHE, a TTR aumenta a expressão do GLUT1, o que sugere que a TTR modula as proteínas ascendentes do metabolismo da glucose, no fígado.

Assim, de forma a avaliar o impacto da TTR na cascata de sinalização da glicose, avaliou-se o efeito da TTR na expressão do gene PKM1/2 (ao nível da transcrição), através da execução de um qRT-PCR e, embora não estatisticamente significativo, observamos um aumento na expressão deste gene na presença de TTR nas hCMEC/D3, mas não nas células $HepG_2$, sugerindo uma modulação descendente, por parte da TTR, nas vias de sinalização da glicose no nosso modelo de BHE.

De um modo geral, os nossos resultados sugerem que a TTR pode desempenhar um papel protetor no metabolismo da glucose, embora modulando diferentes vias ao nível da BHE e do fígado.

Palavras-chave: Doença de Alzheimer, Péptido β-amiloide, Metabolismo da Glicose, Barreira hematoencefálica, Periferia, Transtirretina.

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Abbreviations

AD	Alzheimer's Disease
Aβ	Amyloid-beta peptide
ADAM	A Disintegrin and metallproteinase
AICD	APP intracellular domain
ApoE	Apoliprotein E
ApoE1	Apolipoprotein E1
APP	Amyloid-beta precursor protein
ATP	Adenosine triphosphate
BACE-1	Beta-secretase 1
BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CNS	Central nervous system
CSF	Cerebrospinal fluid
αCTF	C38 carboxyl-terminal
DAPI	4',6'-diamidino-2-phenylindole
dH_2O	Destillated water
DM	Diabetes Mellitus
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EBM2	Endothelial-cell basal medium 2
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FAD	Autosomal Dominant Familial Alzheimer's Disease

FAP	Familial Amyloid Polyneuropathy
FBS	Fetal bovine serum
FFA	Free fatty acids
FOXO1	Forkhead box protein O1
GCK	Glucokinase gene
GDM	Gestational Diabetes Mellitus
GLUT	Glucose transporters
GLP	Glucagon-like peptide
GIP	Gastric inhibitory polypeptide
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GK	Glucokinase
hCMEC/D3	Immortalized human cerebral microvascular endothelial cell line
$HepG_2$	Immortalized human liver cancer cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGP	Hepatic glucose production
HLA	Human leukocyte antigen
HNF	Hepatic nuclear factor
HNF1A	Hepatic nuclear factor 1-alpha
ICV	Intracerebroventricular injection
IDE	Insulin degradative enzyme
IgG	Immunoglobulin G
IGF-1	Insulin-like growth factor I
IR	Insulin receptor
IRS	Insulin responsive substracts
kDa	Kilo dalton

KO	Knockout
LRP1	Low-density lipoprotein receptor-related protein 1
LRP2	Low-density lipoprotein receptor-related protein 2
MHC	Major histocompatibility complex
MODY	Maturity onset diabetes of the young
NCBI	National center of biotechnology information
NEP	Neprilysin
NPY	Neuropeptide Y
NTF	Neurofibrillary tangles
O/N	Overnight
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline Tween-20
PHF	Paired helical filaments
PI3K	Phosphoinositide 3-kinase
PKM1/2	Pyruvate kinase M1/2 isoforms
PM	Plasma Membrane
PMSF	Phenylmethylsulfonyl fluoride
PNS	Peripheral Nervous System
PSEN 1	Presenilin 1
PSEN 2	Presenilin 2
PVDF	Polyvinylidene fluoride
qRT-PCR	Real-time polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RBP	Retinol binding protein
RT	Room temperature
sAPPα	Soluble amyloid-beta precursor protein alpha

SLGT	Sodium-linked glucose transporters
SP	Senile plaques
SSA	Senile system amyloidosis
TH	Thyroid hormone
Tg	Transgenic
TTR	Transthyretin
T_4	Thyroxine
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
UDP	Uridine diphosphate glucose
WB	Western Blot
WE	William's E medium

Introduction

1. Energy metabolism

Our body requires energy to function properly, especially the brain, a very complex structure, that requires high amounts of energy that provides it sensitive to changes in energy fuel supply and mitochondrial function. This complex structure utilizes 25%, approximately, of the total body glucose, and the majority of which is used to transduce energy through glycolysis and mitochondrial oxidative phosphorylation to support synaptic transmission (Yin *et al*., 2016).

This energy provided by glucose consumption, through food intake, is possible by the liver function, a crucial organ that is responsible for the maintenance of normal glucose homeostasis, which means that it produces glucose during fasting – glycogenolysis and gluconeogenesis (Aronoff *et al*., 2004) - and stores it postprandially.

Circulating glucose is derived from three sources: intestinal absorption during the fed state, where there's a gastric emptying; glycogenolysis and gluconeogenesis, which are hepatic processes (Aronoff *et al*., 2004).

1.1. Liver glucose metabolism

The liver is a complex organ comprising multiple different functions. It acts as a factory, being responsible for body energy metabolism, namely through the metabolism of proteins, carbohydrates, lipids, iron, copper, and acts also as a detoxifier (Sendensky, 2011). Regarding glucose metabolism, hepatocytes are the main cell type in the liver, where blood glucose enters through glucose transporter 2 (GLUT2) and is then released through other transporters or even other mechanisms (Rui, 2014). In the fasting state, the synthesis of glucose in the liver, de novo, from precursors such as lactate, gluconeogenic amino acids and glycerol (gluconeogenesis) is important to provide the organism with glucose. In the feeding state, when glucose is already available, gluconeogenesis must be inhibit (Barthel & Schmoll, 2003). These mechanisms together result in a net of hepatic glucose production (HGP).

1.1.1. Glycogen metabolism

The glycogen metabolism is important both in the fed and fasted states, as previously mentioned, but mainly after a meal, in which glucose is converted to glycogen, in order to remove glucose from the portal vein (Agius, 2008), as well as providing a storage form of glucose that can be used in the fasted state (Han *et al*., 2016). The main precursor for glycogen synthesis is glucose-6-phosphate (G6P), that results from the phosphorylation, by glucokinase (GK), of glucose that was up taken from circulation via GLUT2 (Rui, 2014). Then, the G6P is

transformed into glucose-1-phosphate (G1P) by phosphoglucomutase, that is subsequently converted into UDP-glucose by UDP-glucose phosphorylase (UGP) and, finally, glycogen synthase converts it into glycogen, that is stored post-prandial (Adeva-Andany *et al*., 2016). The amount of glycogen formed is determined by the amount of glucose uptake, as well as by the amount of glycogen already present (Radziuk & Pye, 2001) and the accumulation of this glycogen in the liver during the fed state provides storage of glucose used in the fasting state (Han *et al*., 2016).

1.1.2. Gluconeogenesis

Gluconeogenesis is the de novo glucose synthesis under long periods of fasting or starvation (Han *et al*., 2016), using as main sources lactate, amino acids and glycerol (Barthel & Schmoll, 2003), which are generated in the liver or delivered to the liver through the circulation from extrahepatic tissues (Rui, 2014) (**Figure 1**).

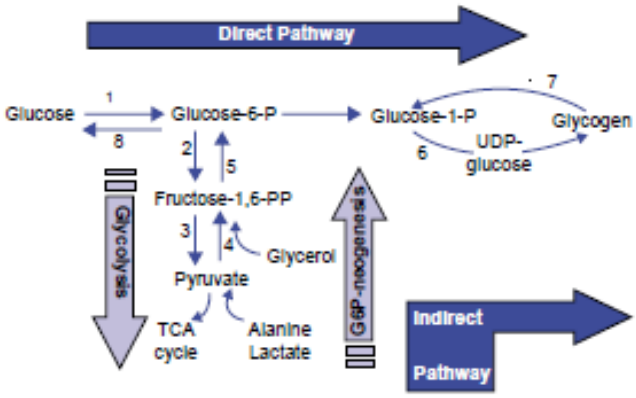

Figure 1 - Mechanisms of glucose regulation (Roden & Bernroider 2003).

1.2. Normal physiology of insulin secretion and action

1.2.1. Insulin structure and function

Insulin was first described as a result of a collaboration between the surgeon Frederick G. Banting and his students assistant, winning the Nobel Prize for Physiology or Medicine (Bilous & Donnelly, 2010).

Insulin is molecule formed by two polypeptide chains linked by disulphide bridges and it is found, in circulation, as a monomer with a molecular weight of 6 kDa. This hormone, discovered more than 75 years ago (Shepherd & Kahn, 1999b) is the most important, as well as the only β-cell hormone known to lower blood glucose concentrations (Aronoff *et al*., 2004). Firstly, it is synthetized as a larger precursor, preproinsulin, which is cleaved by protease

activity, prohormone convertases (Rutter *et al*., 2015), to proinsulin, which is then stored in vesicles in the Golgi of β-cell. Still, in an early stage of development, in the secretory granules, this proinsulin is converted into insulin and C-peptide (**Figure 2**), that are then released by exocytosis, when the granules are transported to the cell surface, being the actin cytoskeleton and cyclic GTPases mediators of the biphasic insulin release (Bilous & Donnelly, 2010).

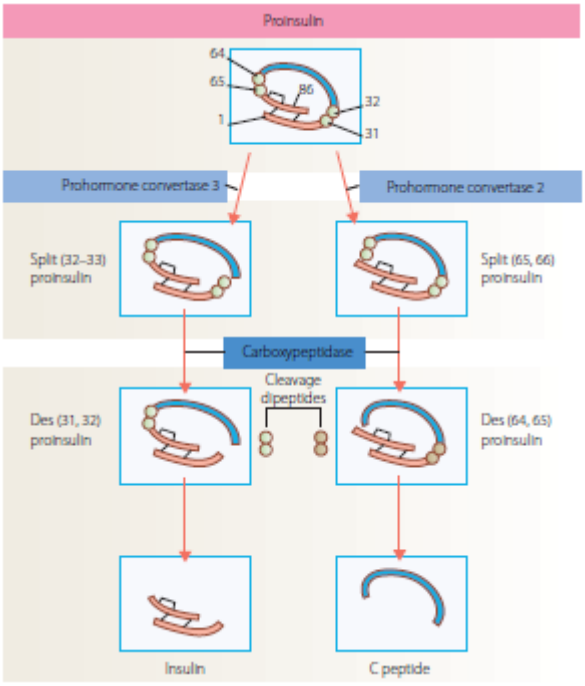

Figure 2 - Biosynthesis and processing of insulin (Bilous & Donnelly 2010).

Regarding insulin functions, insulin, among a group of other hormones (glucagon, GLP-1, cortisol, growth hormone), is known to control the glycogenolysis and gluconeogenesis, in order to maintain circulating glucose within normal concentrations (Petersen *et al.,* 2017). Besides that, it is also responsible for glucose and fat uptake through triggering the translocation of the intracellular glucose transporter GLUT4 to the plasma membrane (Ward, 2015); stimulating the storage of glycogen and nucleic acids (fat) in the liver, muscle and adipocyte tissue (Saltiel & Kahn, 2001), through the stimulation of glycogenesis, lipogenesis (increased activation and phosphorylation of acetyl-coA carboxylase (Wilcox, 2005); and reducing glucose production and release by the liver through glycogenolysis (Ward, 2015), whenever there's an excess of glucose in the blood circulation.

1.2.2. Insulin secretion and regulation

For the proper function of insulin response, β-cell have to function properly, because their sensitivity to glucose is determined by the peripheral tissues' sensitivity to insulin action

(Bell & Polonsky, 2001). Synthesis and secretion of insulin is regulated by both nutrient and non-nutrient secretagogues, regarding environmental stimuli and the interplay of other hormones (**Figure 3**). The nutrient secretagogues that trigger insulin are mainly glucose; the non-nutrient secretagogues act through neural stimuli, namely cholinergic and adrenergic pathways or peptide hormones, for example, GLP-1, or even cationic amino acids (Wilcox, 2005).

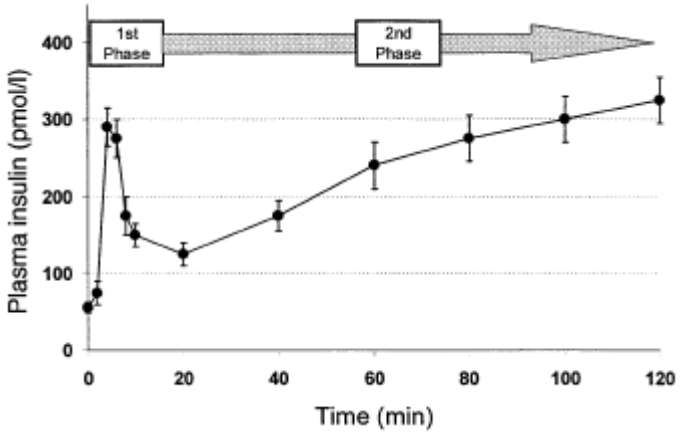

Figure 3 - Phases of insulin secretory responses over time (Pratley & Weyer 2001).

This glucose-insulin dose-response (**Figure 4**), in response to a dose stimulation of about 8 mmol/L of glucose, is primarily determined by the activity of glucokinase, which is expressed in higher levels in the pancreatic β-cell and the liver (Bell & Polonsky, 2001).

On one hand, in the β-cells, once glucose enters via GLUT2, the glucokinase acts as a sensor of glucose, controlling glucose entry into the glycolytic pathway and its metabolism, which further triggers the release of insulin into the circulation (Bilous & Donnelly, 2010). The latter mechanism may be activated through hormones.

On the other hand, in the liver the glucokinase, stores glucose as glycogen, particularly in the postprandial state (Bell & Polonsky, 2001), in other words after eating a meal.

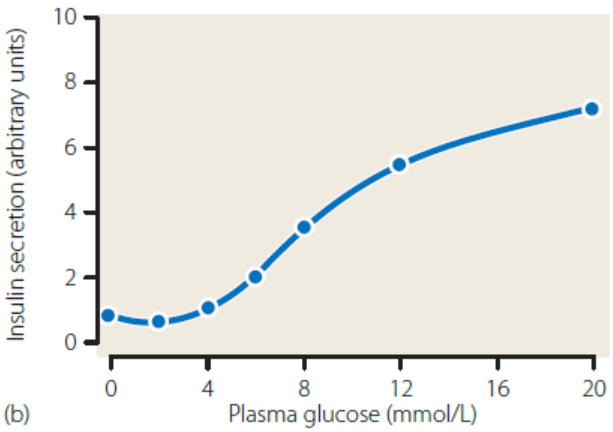

Figure 4 - Glucose-insulin dose-response (Bilous & Donnelly 2010).

Besides the response of insulin to ingestion of glucose, there is also the incretin effect, which are two hormones: glucagon-like peptide-1 (GLP-1), secreted by L cells, and gastric inhibitory polypeptide (GIP), secreted by K cells of the upper jejunum wall (Bilous & Donnelly, 2010) responsible for insulin release in the GI tract (Wilcox, 2005). The GLP-1 is also responsible for the inhibition of glucagon release, delays in the gastric emptying and reduction of appetite.

Moreover, other genes involved in insulin secretion are HNF-1α, HNF-1β and HNF-4α, that are transcription factors that control gene expression during the embryonic development and in adults tissues, and that are also involved in glucose transport, glycolysis and mitochondrial metabolism (Bell & Polonsky, 2001).

1.2.3. Insulin receptors and binding

For insulin act properly, it needs to bind to insulin receptors. The insulin receptor (IR) has two α and two β glycoprotein subunits and insulin binds to the extracellular α subunit, generating ATP, which binds to the intracellular β subunit and phosphorylates it, conferring tyrosine kinase activity and, subsequently, insulin responsive substrates (IRS). These IRS, which then bind to other signaling molecules mediating further cellular actions of insulin. There are four known IRS proteins: IRS-1, IRS-2, IRS-3 and IRS-4. The first two, which are the most well characterized, are mainly in the skeletal muscle and liver, respectively, and IRS-1 mediates mitogenic effects of insulin and is the ligand for glucose sensing to insulin, whereas the IRS-2 mediates insulin peripheral actions and growth of β-cells (Wilcox, 2005).

1.2.4. Glucose transporters (GLUTs)

In order to organs to be supplied with glucose, systems of transport are required for maximal glucose transport, and these are the cellular transporters of glucose: the GLUTs and sodium-linked glucose transporters (SLGT), which are restricted to the intestine and kidney, transporting glucose against a glucose-concentrations gradient by using, as an energy source, sodium cotransport (Shepherd & Kahn, 1999a). The types of GLUTs which are best characterized are: GLUT1, GLUT2, GLUT3, GLUT4, GLUT5, GLUT8, GLUT9, GLUT10 and they all have distinct substrate specificities, kinetic properties and tissues distributions that determine their functions (Bilous & Donnelly, 2010; Shepherd & Kahn, 1999b). Among them, GLUT1 to 4, which belong to the class I, are the most important in the transport of glucose (Navale & Paranjape, 2016) (**Table 1**).

Table 1. Summary of the properties of the glucose transporters (Bilous & Donnelly, 2010; Shepherd & Kahn, 1999b; Zhao & Keating, 2007).

Protein	Site of expression	Function
GLUT1	Ubiquitously expressed in tissues and cultured cells	Mediates basal and non-insulin mediated glucose uptake
GLUT2	Islet β-cells, liver, intestine, kidneys	Glucose sensor, together with glucokinase, being a high-capacity low-affinity transport
GLUT3	Brain and nerve cells	Non-insulin mediated glucose uptake to the brain
GLUT4	Muscle, adipose tissue, heart	Insulin-stimulated glucose uptake

The main insulin-responsive glucose transporter is GLUT4, that is also known to have interactions with GLUT1, in adipose tissue and muscle. During insulin signaling, the vesicles at the basal state, where GLUT4 is, are translocated to the plasma membrane, where they fuse with GLUT1 allowing glucose to enter the cell (Bilous & Donnelly, 2010; Zhao & Keating, 2007). This process is reversible, being the vesicles with GLUT4 recycled.

Regarding the central nervous system (CNS), the most studied transporter is GLUT1, due to the fact that it is one of the major transporters in supplying brain cells with glucose. Despite of the many studies until now, little is known about the structure and mechanism of GLUT1.

In the peripheral nervous system (PNS), the most studied transporter is GLUT4, since it is directly involved in the trafficking of proteins, cellular components, related with insulin in order to maintain body glucose homeostasis. A dysregulation at these levels can lead to obesity and type 2 diabetes, although the underlying mechanism is still poorly understood (Thorens, 2014).

The liver was recognized to play a predominant and important role in the disposal of meal glucose, regarding its position between the portal circulation draining the gut and the systemic circulation acting as a filter (Radziuk & Pye, 2001). In the liver, the main cell type are the hepatocytes, as previously mentioned, and in these cells GLUT2 is the predominant hepatic glucose transporter in humans, through which blood glucose enters, but also facilitates glucose release to the bloodstream, whereas in the pancreas GLUT2 contributes to glucose-mediated insulin secretion (Adeva-Andany *et al*., 2016)

Importantly, the regulation of these transporters before and after the beginning of lactation indicates that their expression and function is mainly regulated by hormonal changes (Zhao & Keating, 2007).

Overall, in normal subjects, these proteins, together with other mechanisms, maintain the glucose homeostasis, allowing a balance between the glucose released from the liver and

intestine into the blood circulation and the glucose uptake into the peripheral tissues (muscle and adipose tissue), which, therefore, maintain insulin at lowers levels in the fasting state and at higher levels at mealtimes (Bilous & Donnelly, 2010).

1.3. Liver and brain crosstalk

The liver's metabolism is regulated by the CNS via sympathetic nervous system, which promotes HGP and mobilization of metabolic fuels for extrahepatic tissues, and parasympathetic nervous system, which suppresses sympathetic nervous system action and inhibits HGP, but also promotes fuel storage in the liver (Rui, 2014).

As previously described, insulin has direct effects on the liver by inhibiting glycogenolysis, but it also has indirect effects mainly through the activation of hypothalamic insulin signaling, which includes the modulation of feeding behavior, neuropeptide Y expression, hypoglycemia counter-regulation and autonomic outflow, in order to regulate glucose production (Puglianiello & Cianfarani, 2006). Importantly this suppression of HGP is possible through the hyperpolarization of neurons due K_{ATP} channel activation (Inoue, 2016), involving the insulin receptor, IRS and PI3K (Puglianiello & Cianfarani, 2006). This input relays to the motor nucleus of the vagus nerve in the brainstem, leading to the activation of efferent vagal fibers, which innervate the liver (Pocai *et al*., 2005).

Moreover, leptin, another circulating hormone, also plays a role in glucose homeostasis, through the stimulation of melanocortin-independent pathways involving insulin-like pathways, such as PI3K signaling cascade (Puglianiello & Cianfarani, 2006).

Overall, this crosstalk is important for the body's metabolic and nutritional status because CNS has a major role in the control of insulin sensitivity and glucose homeostasis. An onset hypothalamic resistance to multiple signs can contribute to the susceptibility of insulin resistance, which means that diabetes can be a disorder with underlying CNS defects (Puglianiello & Cianfarani, 2006).

2. Diabetes Mellitus (DM)

Diabetes was first described by Aretaeus in the second century, but the adjective Mellitus was introduced by John Rollo (d. 1809). The knowledge on Diabetes Mellitus (DM) had many contributors, besides the ones mentioned, and Claude Bernard and Paul Langerhans were also involved (Bilous & Donnelly, 2010). DM consists of a metabolic disorder that comprises multiple etiologies (**Figure 5**) and affects about 30.3 million people worldwide at all ages, but with great incidence in the older people (for Disease Control, Prevention, and others 2017).

According to WHO and American Diabetes Association, DM presents a variety of symptoms, due to the fact that it presents several pathogenic processes comprising a range from autoimmune destruction of the pancreatic β-cells to abnormal insulin action (American Diabetes Association, 2004). Since its prevalence is increasing drastically, the WHO estimates that by 2030 there will be 370 million adults with diabetes, almost the double from today's number (Ozougwu, 2013), which is concerning given the long-term consequences that patients can suffer, namely blindness, kidney dysfunction, cardiovascular disease, which are the principal cause of morbidity and mortality (Alkayyali & Lyssenko, 2014). In Europe, about 6-8% of the population suffers from diabetes and 90% of them has Type 2 Diabetes Mellitus (T2DM) and 10% Type 1 Diabetes Mellitus (T1DM), being T2DM the most common and fastest increasing type of diabetes both in Europe and worldwide (Groop & Pociot, 2014).

The current classification of DM is based on its etiology and there are four categories: T1DM, T2DM - which are the most common - gestational diabetes and other specific types of diabetes, caused by diseases of the exocrine pancreas, genetic syndromes, and many other (Bilous & Donnelly, 2010).

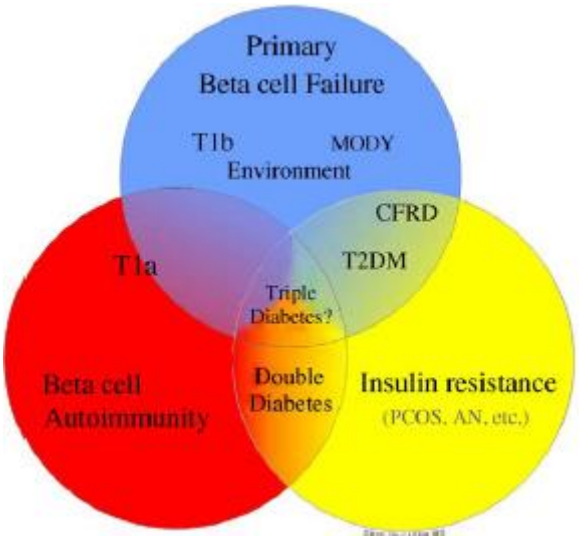

Figure 5 - Characterization of diabetes (Ize-Ludlow & Sperling, 2005).

2.1. Genetics

For many years that the knowledge about the role of genetic factors as a risk of DM is known, although there is a lack of knowledge about the underlying genes, which is referred as missing heritability (Manolio *et al*., 2009). In 1935, Hinsworth established two types of diabetes (Ali, 2013): T1DM, which is characterized by absolute insulin deficiency resulting from autoimmune destruction of pancreatic β-cells, and is more common among children and young adults (Groop & Pociot, 2014) and T2DM, which is heterogeneous metabolic disorder resulting

from decreased sensitivity of tissues to insulin (Pullakhandam *et al*. 2012), that commonly occur in the adulthood, more likely at 65 years old or older (Chatterjee *et al*., 2017).

There is a long history of studies regarding the genetics of diabetes, but with the development of genome-wide maps, genome sequencing and understanding of human population genetics, it was possible to gain knowledge on the underlying mechanism, in a population-based manner (Ali, 2013; Florez *et al*., 2003; Groop & Pociot, 2014).

On one hand, T1DM is considered as a complex genetic trait, which means is not only associated with multiple genetic loci but also with environmental factors. Until now, two chromosomal regions are known, namely the human leukocyte antigen (HLA) region, within the major histocompatibility complex (MHC) in the chromosome 6p21.3, which provides a strong clustered in families of about 40-50% and the insulin gene region in the chromosome 11p15, being T1DM very specific to pancreatic β-cells, since insulin or insulin precursors may act as autoantigens (Groop & Pociot, 2014). Moreover, in T1DM, the risk for monozygotic twins is higher (100% concordance) than for dizygotic twins (Florez *et al*., 2003), due to the dependent degree of genetic identity with the proband, among family members (Groop & Pociot, 2014).

On the other hand, T2DM results from an interaction between environmental factors (obesity, stress, sedentary lifestyle, age) and a hereditary component of about 20-80%. However, this family risk relies on the great impact of the environmental factors, since studies indicate that there is a higher risk of developing diabetes later in life. Nevertheless, this risk is not only due to genetic factors, but also due to epigenetic processes such as intrauterine and pregnancy (Ali, 2013), which can give rise to Gestational Diabetes Mellitus (GDM), characterized by glucose intolerance during pregnancy (Chen *et al*., 2017). Despite that, the genetic risks of T2DM are not completely defined, but it known that the monogenic diabetes of the young (MODY) can be caused by mutations in the hepatocyte nuclear factor-1A (HNF1A) and in the glucokinase gene (GCK) (Ali, 2013).

Overall, there's evidence that genetics are implicated in T1DM and T2DM, although the genetic contribution differs for each, being the genetic risk for T1DM greater than for T2DM, as well as the genes involved in both (Rich, 2006).

2.2. Pathophysiology

2.2.1. Pancreatic β-cells decline and insulin dysfunction

As previously described, the pancreatic β-cells are crucial for the normal response to insulin resistance by increasing their output of insulin according to the tissues needs. However, the decline of the function of these cells can include cytokine-induced inflammation (oxidative

stress, endoplasmic reticulum stress, inflammation), obesity, overconsumption of saturated fat and free fatty acids (FFA), and insulin resistance, which is a hallmark of T2DM. Both T1DM and T2DM have loss of β-cell and of its functions (Cerf, 2013).

In T2DM, the initial manifestation of the abnormal insulin secretion is the loss of the first-phase, the fasting state, because of increased hepatic glucose production which explains the occurrence of hyperglycemia (Skyler, 2004), as well as hyperinsulinemia (Aronoff *et al*., 2004), (**Figure 6**). Besides, it is also observed a delayed response of insulin, as well as abnormal oscillations of insulin release throughout the day (Bilous & Donnelly, 2010). The initiating mechanisms that can cause this impairment are proposed to be the activation of FOXO1 in the liver and the disruption of GLUT4 translocation in the muscle (Czech, 2017). Moreover, elevated levels of serum retinol-binding protein 4 (RBP4) cause insulin resistance and impair insulin signaling. (Zemany *et al*., 2015)

Furthermore, amyloid deposits in the pancreatic islets is also considered to be a pathological characteristic of T2DM, since it is known that it limits the glucose-stimulated insulin secretion, although this mechanism is not fully understood (Cernea & Dobreanu, 2013).

T2DM has, in fact, many metabolic abnormalities and despite the main mechanism remaining poorly unknown, it is known that this disease leads to microvascular complications that affect many organs, namely the brain.

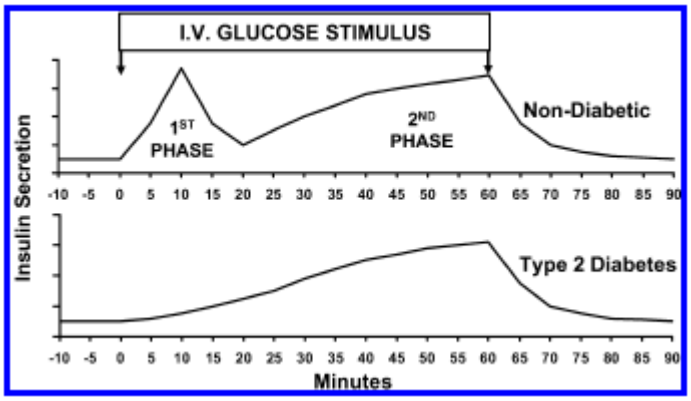

Figure 6 - Phasic insulin response in non-diabetic and diabetic patients (Skyler, 2004).

2.2.2. Brain metabolism impairments

In individuals with T2DM, the rate of hepatic glucose production (HGP) is increased under basal physiological conditions and insulin-dependent suppression of HGP is impaired, which represents hepatic insulin resistance (Petersen *et al*., 2017), at the brain level and periphery (Li *et al*., 2015), since insulin acts in multiple brain regions influencing many

pathways, such as regulation of food intake, neuronal growth, differentiation by regulating neurotransmitter (Das, 2002).

This impaired function of insulin alters glucose homeostasis in the brain, consequently, impacting energy metabolism, which can lead to a cognitive decline, that affects the brain through several mechanisms that include, for example, glucotoxicity and lipotoxiciy, that affect the vasculature of the brain, by impairing synaptic plasticity, as well as the metabolism of amyloid-β peptide (Aβ) and Tau protein and, consequently, accelerating cognitive decline (learning and memory) and dementia (Duarte, 2015).

Understanding pathways of glucose metabolism of healthy humans may help to clarify metabolic alterations that occur in diabetes mellitus (Adeva-Andany *et al*., 2016) and also in Alzheimer's Disease (AD), since in this disease, characterized by a decline of the cognitive function, metabolic alterations such as insulin resistance and hyperglycemia (Arieta-Cruz *et al*., 2016) also occur.

3. Alzheimer's Disease (AD)

AD is one of the most common neurodegenerative disease, first described by Dr. Alois Alzheimer, and accounts for more than 80% of the dementia cases worldwide in older people, usually older than 65 years of age (Kumar *et al*., 2015). According to Alzheimer's Association, the symptoms of the disease vary among individuals, because, like other common chronic diseases, AD develops as a result of multiple factors, being age the major one, rather than a single cause. It is expected that by 2050, one new case of AD develops every 33 seconds, resulting in nearly 1 million new cases per year (Alzheimer's Association, 2015). Due to that fact, is one of the most intensively studied disease, giving rise to several hypothesis about its pathogenesis, for example, Aβ and tau hyperphosphorytation hypothesis, (Chen & Zhong, 2013) despite until now the etiological mechanisms underlying the neuropathology still remain unclear.

3.1. Genetics

The majority of patients only start developing clinical symptoms at age older than 65 years (late-onset AD), despite 2-10% of them have an earlier onset of disease (early-onset AD) or autosomal dominant familial AD (FAD). The search for the genetic factors contributing to AD has evolved massively throughout the years, starting with the discovery of mutations that are involved as a cause of FAD, namely amyloid precursor protein (APP), presenilin 1 and

2 (PSEN1 and PSEN2) (Van Cauwenberghe *et al*., 2016). Mutations in these genes might result in alteration of Aβ production, consequently leading to neuronal death and dementia (Bagyinszky *et al.*, 2014) (**Figure 7**).

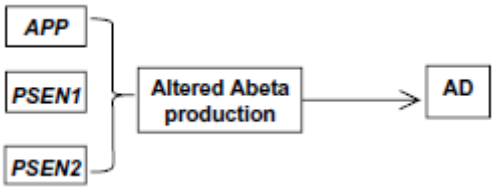

Figure 7 - The amyloid precursor (APP), PSEN1 and PSEN2 genes.

In the late-onset AD, the major risk factor is in apoliprotein E (ApoE) gene, but also non genetic factors may be involved. ApoE gene is located on chromosome 19q13 that encodes three alleles, namely ε2 (8%), ε3 (77%) and ε4 (15%) (Bagyinszky *et al*., 2014; Karch & Goate 2015), it is expressed in liver, brain, macrophages and monocytes (Van Cauwenberghe *et al*., 2016) and is responsible for the transport and metabolism of lipids (Imbimbo, Lombard, and Pomara 2005). Among the three different isoforms, the presence of ApoE ε4 allele increases the risk by 12-fold, for AD development. Conversely, the ApoE ε2 is associated with decreased risk for AD and later age at onset (Imbimbo *et al*., 2005; Karch & Goate 2015).

3.2. Pathophysiology

3.2.1. Neuropathology

Auguste Deter, was the first AD patient and when she was autopsied, changes in the brain were observed that, by the time, were associated with dementia and general cortical atrophy, but no prior descriptions existed (Stelzmann *et al*., 1995). Then, when senile dementia started to increase in proportion to life expectancy, Kartzman assumed that *"AD and senile dementia are a single process and should, therefore, be considered a single disease"*. In fact, structural studies back then, observed neurofibrillary tangles (NTFs) originated from intracellular aggregates of hyperphosphorylated tau protein and senile plaques (SP) caused by Aβ accumulation and degenerating neurites in the cerebral cortex (Katzman, 1976), besides the visible atrophy of the brain, macroscopically (Tomlinson *et al*., 1970).

3.2.1.1. Neurofibrillary tangles

Since the first description of two microscopic structures by Alois Alzheimer in 1907, previously mentioned, that senile plaques and neurofibrillary tangles became hallmarks of AD (Kumar *et al*., 2015). The NTFs are fibrils with ~10 nm of diameter that form pairs with helical 3D conformation, named paired helical filaments (PHFs) (Serrano-Pozo *et al*., 2011) and are composed of the microtubule-associated protein tau (Selkoe, 2001), although other proteins, such as, ubiquitin, Aβ, can also be associated to NTFs (Perl, 2010). When tau is hyperphosphorylated, it results in a loss of function, resulting in its dissociation from the microtubule and, consequently, formation of NTFs, leading to neurons' apoptosis (Brion & Brion, 1998).

These microscopic structures have been descried to have three distinguished morphological stages: pretangle or diffuse NTFs, mostly present in the somatodendritic compartment; mature or intraneuronal NTFs, which appear in soma and dendrites (Brion & Brion, 1998; Serrano-Pozo *et al*., 2011); and the extraneuronal, the classical NTFs that can be observed in the layer II neurons of the entorhinal cortex, the CA1 and subicular regions of the hippocampus, the amygdala, and the deep layers (layers III, V, and superficial IV) of the neocortex (Perl, 2010; Selkoe, 2001; Serrano-Pozo *et al*., 2011) (**Figure 8**).

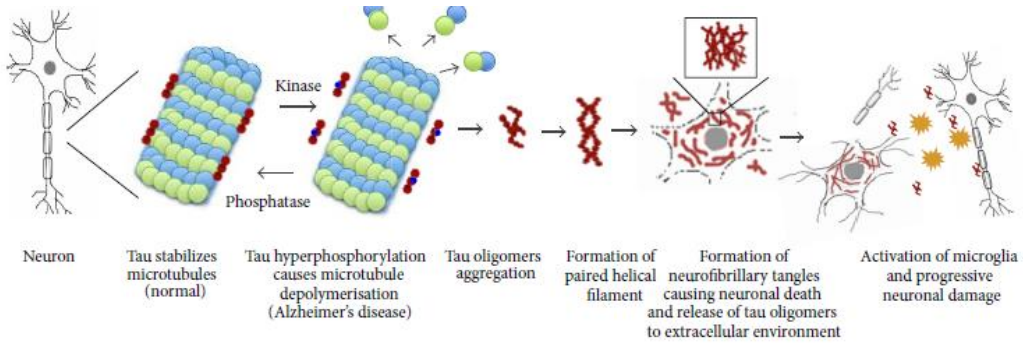

Figure 8 - Representation of NTFs formation (Mokhtar *et al*., 2013).

Moreover, AD is not the only disease associated with neuropathological lesion, there are many others, namely, Parkinsonism, Pick's disease, posttraumatic dementia, and many others (Armstrong, 2009; K. Iqbal *et al*., 2010; Perl, 2010).

3.2.1.2. Senile plaques

The other lesion observed in brains of AD patients are the senile plaques (SP) (**Figure 9**), also known as neuritic plaques, which are extracellular amyloid deposits, whose chemical structure was identified by George Glenner (Sweatt, 2010), but can be also a characteristic of aging (Cras *et al*., 1991; Tomlinson *et al*., 1970). This plaques, found in cortex and hippocampus, have a variable size and morphology, thus being divided in different subtypes, such as diffuse SP (pre-amyloid), primitive (neuritic), classic (dense-cored) and compact-type (burnt-out) (Armstrong, 2009). Moreover, this plaques are regionally dependent, in other words, mostly found in limbic and association cortices, and often associated with microglia *(Cras et al.*, 1991; Selkoe, 2001). The dense-cored subtype is associated with increased neurite curvature, dystrophic neurites, synaptic loss, neuron loss, and recruitment and activation of astrocytes and microglia (Serrano-Pozo *et al.*, 2011). Importantly the main component of these structures is Aβ.

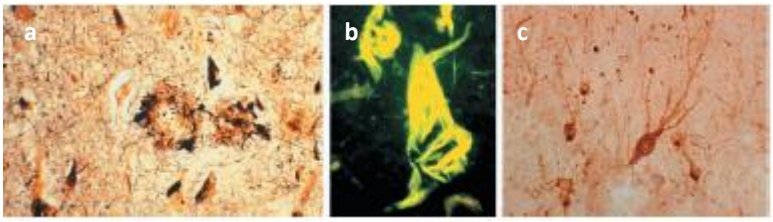

Figure 9 - Representation of microscopic hallmarks of AD. (a) senile plaques; (b) neurofibrillary tangles; (c) phosphorylated tau (Bossy-Wetze *et al*., 2004).

3.3. Aβ Biochemistry

As previously mentioned, Aβ is the main component of senile plaques in AD. This small peptide, with approximately 4 kDa, is the product of the proteolytic processing of the type I transmembrane protein – APP – important for normal brain (Shariati & De Strooper, 2013). Glenner and Wong were the first ones to isolate and sequence Aβ, that was found to be present also in the serum, brain and CSF (Glenner *et al*., 1984).

Since the initial identification of Aβ, that it has been seen as a neurotoxic peptide, but not all of its physiological functions have negative effects, for instance, it has been shown to be an antimicrobial peptide (D. Kumar *et al*., 2016), as ion channel modulation for neuronal survival (Pearson & Peers, 2006), transcriptional factor and cholesterol transport regulator (Nhan, 2015).

APP is a protein abundant in the brain and there is great genetic evidence that altered cellular processing of APP is causative of many diseases (Di Carlo, 2010), namely early onset-AD with cerebral amyloid angiopathy (Thinakaran & Koo, 2008). APP can be processed

through two different pathways: nonamyloidogenic and amyloidogenic pathways, producing afterwards Aβ peptide (**Figure 10**).

3.3.1. The APP processing and the production of Aβ: non-amyloidogenic and amyloidogenic pathways

The proteolytic processing of APP, by a family of secretases, has two different pathways: nonamyloidogenic and amyloidogenic pathways, which results in the release of secreted derivatives into vesicle lumens and the extracellular space with different functions (Selkoe, 2001; Sweatt, 2010).

In the nonamyloidogenic pathway, that is in the normal physiological conditions, APP is cleaved by two enzymes: the α-secretase, an enzyme of ADAM family (Swerdlow, 2007) that generates a large soluble fragment (sAPPα), responsible for synaptic plasticity, learning, memory, neuronal survival; and the retention of C83 carboxyl-terminal (or αCTF) (Hernández-zimbrón *et al*., 2016). Then, γ-secretase cleaves the C83 resulting in the release of p3 peptide (~3 kDa) (Thinakaran & Koo, 2008) and an APP intracellular domain (AICD) fragment. This process prevents the deposition of β-amyloid in plaques, thus contributing, as previously mentioned, to neuronal survival (Hernández-zimbrón *et al*., 2016).

On the contrary, in the amyloidogenic pathway, instead of being cleaved by α-secretase, APP is cleaved by β-secretase, termed BACE-1, which generates a smaller soluble fragment sAPPβ and a retaining 99-residue fragment CTF (C99 – 12 kDA) in the membrane (Imbimbo, Lombard, and Pomara 2005; Selkoe 2001). This last fragment is then cleaved by γ-secretase, which leads to the production of Aβ peptides (~4 kDa), with 38-43 amino acids (Sweatt, 2010; Swerdlow, 2007). Consequently, this Aβ peptides can spontaneously aggregate into soluble oligomers and form the amyloid plaques (A. Kumar *et al*., 2015), which can have a neurotoxic effect in the brain, namely disruption of calcium homeostasis, mitochondrial oxidative stress, impaired energy metabolism and abnormal glucose regulation and, consequently, neuronal death (Cell Signalling, 2009) (**Figure 10**). However studies indicate that Aβ peptide is a normal metabolic event and it is detected also in CSF and plasma of healthy subjects throughout life (Selkoe, 2001), so how does Aβ peptide affect the brain by causing AD?

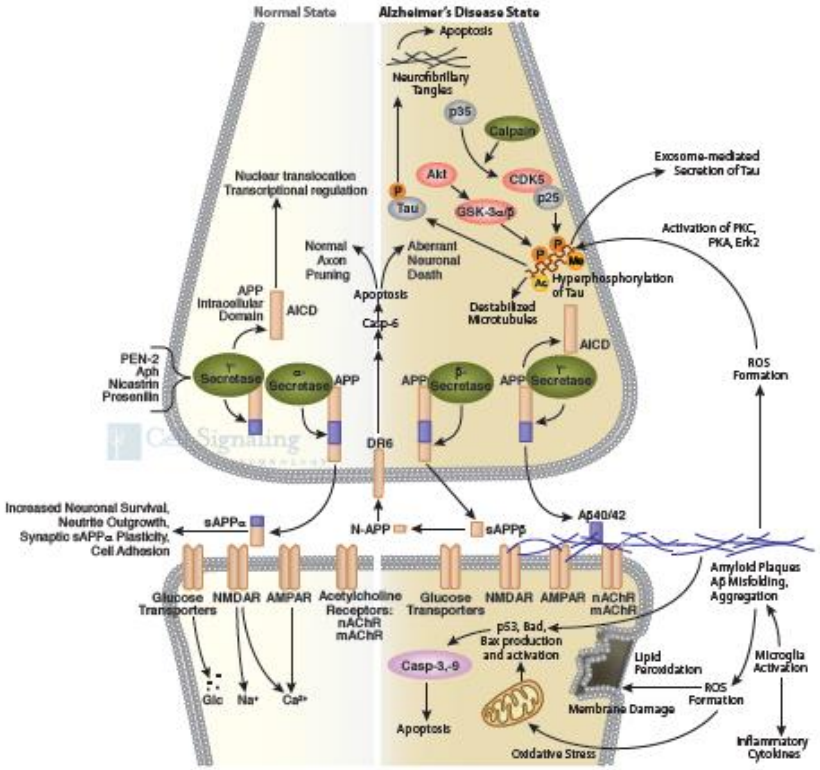

Figure 10 - Amyloid plaque and neurofibrillary tangle formation in AD (Cell Signalling, 2009).

3.3.2. The amyloid cascade hypothesis

AD still remains one of the most intriguing diseases, since its etiology remains a question mark and throughout the years many theories have been developed in order to explain this disorder, based in different molecular mechanisms.

Among these theories, the amyloid cascade hypothesis, formalized by Hardy and Higgins (1992), was, and still is, the most influential in the academic and pharmacological research (John & Gerald, 1992). This theory postulates that the neurodegeneration in AD is caused by abnormal accumulation of Aβ plaques (oligomers) in various areas of the brain (Barage & Sonawane, 2015; John & Gerald, 1992) leading, over time, to a number of negative effects (**Figure 11**). For example, neuronal and vascular damage (Haass *et al.*, 2012), toxic effects on synapses and mitochondria, altered ionic homeostasis (A. Kumar & Singh, 2015), which consequently leads to neuronal dysfunction and eventually cell death (Cell Signalling, 2009; Haass *et al*., 2012; Sweatt, 2010).

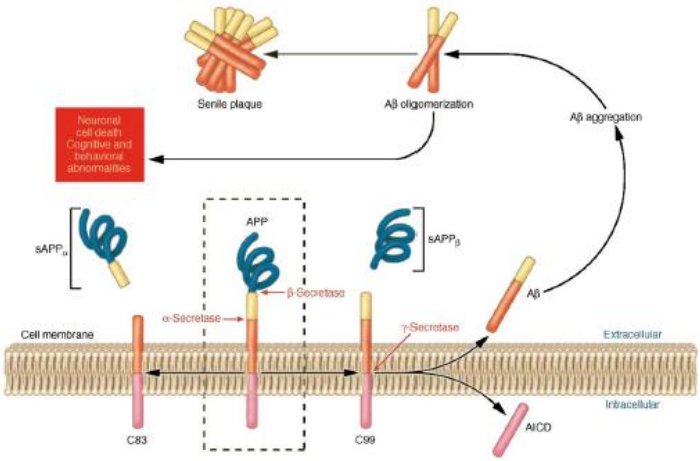

Figure 11 - Schematic representation of APP processing and Aβ accumulation (Imbimbo *et al*., 2005).

3.3.3. Transportation and degradation of amyloid-β peptide

Since the first description of the amyloid cascade, the effort has been towards the study of Aβ in order to discover a disease modifying therapy to reduce the production or to avoid the aggregation of the peptide. Yet, sporadic AD patients don't show an increase production of this peptide.

Firstly, it has been shown that extracellular Aβ42 can be internalized by neurons, followed by the marked generation of Aβ within neurons, which means that extracellular Aβ can influence intracellular Aβ and vice versa (Takahashi *et al*., 2017). In normal human CSF and plasma, Aβ40 levels are greater than Aβ42, which is the toxic isoform (Deane *et al*., 2009). However, in the disease state, this equilibrium can be compromised and, consequently, leading to an imbalance between Aβ production and clearance, that is determinant for Aβ accumulation. Therefore, major efforts have been done to understand the mechanisms behind Aβ clearance, since its dysfunction is a crucial step in the disease process (D. S. Wang *et al*., 2006).

There are three main different pathways responsible for Aβ clearance: receptor-mediated transport across brain barriers, enzyme-mediated degradation and immunotherapy, using anti- Aβ autoantibodies.

The clearance of Aβ can occur via active transport at the blood-brain barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB) (Alemi *et al*., 2016). This mechanism happens in both directions: brain-to-blood, where Aβ binds to many protein, such as, ApoE, ApoJ, Transthyretin (TTR) and a soluble form of the low-density lipoprotein receptor-related protein 1 (sLRP1) (Alemi *et al*., 2017), located on the cerebral endothelium (Deane *et al*., 2009), but also in the liver and astrocytes (Yoon & Jo, 2012); and blood-to-brain, mediated by RAGE,

which is located on the luminal membrane of the endothelium (Y. J. Wang *et al*., 2006) (**Figure 12**).

Besides that, Aβ can be also degraded by several peptidases, also known as Aβ-degrading enzymes (ADE), for example, neprilysin (NEP), an enzyme located in the brain, and insulin-degrading enzyme (IDE) (Y. J. Wang *et al*., 2006), either at a single site or at multiple sites within Aβ, which produce less neurotoxic and more easily cleared fragments. In fact, these ADE were validated *in vivo*, studies in Tg AD mice model showed a reduction in Aβ levels in the brain and improved cognitive function (D. S. Wang *et al*., 2006; Yoon & Jo, 2012). For example, in NEP gene KO in a human APP (hAPP) mouse model, NEP reduction contributes to the accumulation of Aβ and impaired hippocampal synaptic plasticity (Huang *et al*., 2006; Madani *et al*. 2006).

At last, the immunotherapy, using anti- Aβ autoantibodies has been extensively studied, since they have higher specificity for particular antigenic targets, therefore improving the selectivity for Aβ (Saido & Leissring 2012) and studies in mouse models of AD have been shown a reduced Aβ accumulation (Deane *et al*., 2009) in CSF, which improved the cognitive performance. Nevertheless, this is a mechanism that needs further investigation, since the levels of these autoantibodies are very low in the serum (Y. J. Wang *et al*., 2006).

To conclude, the equilibrium between the Aβ influx and efflux is important to maintain its normal levels in the brain, because if any alterations occur in one of these mechanisms it can compromise Aβ clearance and, consequently, leading to all the negative effects described in the amyloid cascade hypothesis.

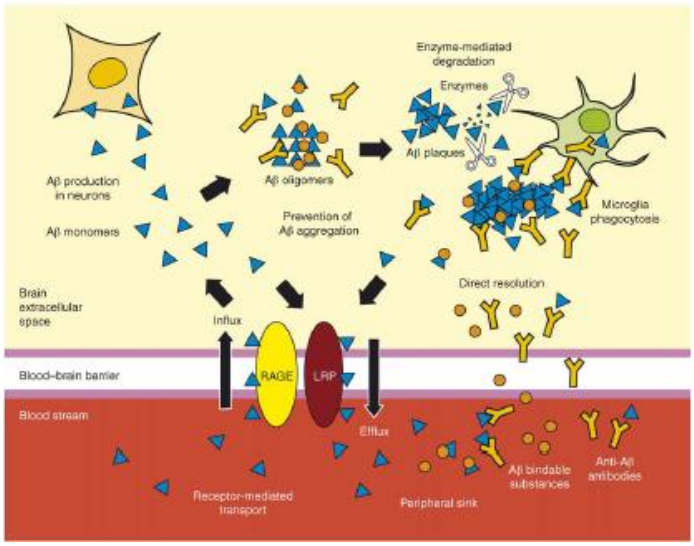

Figure 12 - Schematic representation of the mechanisms of Aβ clearance.

4. AD and DM

In the recent years, new hypothesis and theories regarding AD pathogenesis heavily implicate brain insulin resistance as a key factor (Mullins *et al*., 2017). Hyperinsulinemia and insulin resistance, prevalent in T2DM were shown to damage cerebral vasculature and increase Aβ accumulation in AD brain (Swaminathan *et al*., 2017). Besides, in case of insulin resistance it can lead to further implications such as inflammation, mitochondrial dysfunction, tau and Aβ pathologies (A. *et al*., 2004; Yin *et al*., 2016), which are some signs of AD. On the other hand, there's also an association between AD and glucose metabolism/transport, since AD patients demonstrate hypometabolism (Khalid Iqbal & Grundke-Iqbal, 2005). Several other studies also have shown decreased levels of GLUT1 and 3 and increased levels of GLUT2 in the brain of AD patients, thus reducing glucose uptake in the brain (Y. Liu *et al*., 2008; Mark *et al*., 1997). Importantly, it has been shown that patients with DM have about 65% increased risk of developing AD (A. *et al*., 2004).

The first clue for this correlation came from a study that showed that Aβ oligomers bind to hippocampal neurons triggering the removal of dendritic IRs. Importantly, insulin and IGF-1 inhibited Aβ production, as well as prevented its intracellular accumulation by promoting the transport of Aβ-binding carrier proteins, namely TTR, and increasing the extracellular secretion in the brain (Xiaohua Li *et al*., 2015; Vieira *et al*., 2015).

Studies in animal models of diabetes have shown deficits in hippocampal-based memory performance, as well as a reduction of hippocampal volume, similar of what happens in AD (Kamal *et al*., 2000; Pennanen *et al*., 2004). It also has been shown a spatial correlation between the distribution of regional glucose metabolism via glycolysis and Aβ deposition, in normal adults and AD patients, respectively, which suggests also a link between both diseases (Mullins *et al*., 2017) and an *in vitro* study demonstrated that the high glucose increased significantly the APP levels, consequently, Aβ production (Yang *et al*., 2013).

Furthermore, regarding glycolysis, regional lactate production was also linked to Aβ levels (Mullins *et al*., 2017). Lactate, as mentioned before, is one of the glucose products, when converted by astrocytes and it is used as an alternate neuronal energy substrate in anaerobic conditions (Zawiślak *et a*l., 2017).

Therefore, although AD is mainly seen as a brain disease it also has alterations in the periphery.

5. Transthyretin

5.1. Structure and functions

The protein transthyretin, also known as TTR, was described, in 1942, for the first time as “prealbumin”, due to its ability to migrate faster than the albumin on an electrophoresis gel of plasma samples (Alshehri *et al*., 2015), being mainly synthesized by the liver and choroid plexus and being secreted into the serum and CSF, respectively (McLean *et al*., 2017). After the first X-ray crystal structure this protein, (human) TTR was found to be an homotetrameric protein, composed of four identical subunits (Richardson, 2007), which originates a ~55 kDa protein with 127 amino acids per monomer (McLean *et al*., 2017). TTR is found in many species, since its amino acid sequences or homologous have been determined from species like amphibians, reptiles, fish, mammals, which demonstrates a highly conserved structure throughout vertebrate evolution (Richardson, 2007).

TTR is a protein extensively studied, although its catabolism is not fully understood, yet, it is known that the liver and kidney are the most active organs of TTR catabolism and its internalization is receptor-mediated, for example by megalin, which is expressed in the epithelium of renal proximal tubes, and LRP2 (Vieira & Saraiva, 2014).

The main known physiological role of TTR is the transport of thyroid hormone thyroxine (T_4) (Gouvea *et al*., 2013), as well as retinol (vitamin A) that is bound to retinol binding protein (RBP) (Berry *et al*., 2012). Among the three types of free THs, synthetized by thyroid, T_4 is the most abundant and, in plasma, this hormone binds to three types of transporters, namely thyroxin-binding globulin, albumin and, of interest, TTR, which is responsible for transporting 15% of the hormone. On the other hand, in the CSF, approximately, 80% of the hormone is transported by TTR (Vieira & Saraiva, 2014). Despite its role as a transporter and involvement in thyroid hormone homeostasis, suggested for a long time, recent studies on TTR null mice did not show alterations in T_4 or retinol metabolism, suggesting that this protein is not necessary for thyroid hormones to be normally distributed (Palha, 2002; Sousa *et al*., 2005). However, is important to highlight, that this strain of mice presents signs of increased anxiety, memory and sensorimotor impairment, decreased capacity of regeneration, since TTR plays a role in nerves regeneration (Vieira & Saraiva, 2014).

Furthermore, TTR has been described as a protease, due to its capability of cleaving Apolipoprotein A1 (ApoA1), *in vivo*, and subsequently, impact the cholesterol efflux, by reducing it, as observed in cultured cells (Liz *et al*., 2010; Liz *et al*., 2004). Moreover, TTR has another proteolytic activity, at least in vitro, which is the cleavage of Aβ peptide, suggesting that this protein could be involved in the protective mechanism of AD (Liz *et al*., 2012). Besides, NPY was also shown to be cleaved by TTR, according with Liz et al study. All together, these

studies suggest that TTR has natural substrates in the nervous system and might have, that remain unknown (Gouvea *et al*., 2013; Liz *et al.*, 2010).

Additional data also demonstrates that TTR has neuroprotective effects in other diseases from the CNS namely ischemia (Gomes *et al*., 2016; Santos *et al*., 2010), nerve regeneration (Fleming *et al*., 2009; Santos *et al*., 2010) and memory *(Sousa et al.*, 2007).

5.2. TTR as a cause of disease

Regarding the negative conformational changes that TTR can undergo, which means being able to change from a normally soluble form to an insoluble fibril, it can be related as cause of the most common types of amyloidosis: the senile systemic amyloidosis (SSA) (Ueda & Ando, 2014), which is derived from normal-sequence TTR, and familial amyloidotic polyneuropathy (FAP) which is derived from variant-TTR sequence (Alshehri *et al*., 2015).

FAP, which was described for the first time in 1952, in the north of Portugal (Alshehri *et al*., 2015; Andrade, 1952) and is characterized by misfolded and fibrillary aggregated TTR, which most commonly starts with autonomic and peripheral nervous system symptoms, over the age of 20, and eventually other organs are affected, as it progresses (Hund, 2012).

Among the many different pathogenic TTR mutations (Mazzeo *et al*., 2015), that give rise to an heterogeneity of clinical phenotypes, V30M was the first to be identified (Ueda and Ando 2014), a substitution of methionine for valine at position 30, but L55P is the most aggressive mutation (Sousa & Saraiva, 2003). It is suggested that the disruption of β-structure of TTR under such conditions enable self-assembly of the amyloidogenic intermediate and, consequently, causing formation of amyloid fibrils (K. Liu *et al.*, 2000).

5.2.1. TTR and DM

Studies on the TTR role in glucose metabolism seem very new and, sometimes, controversial. Although only a few studies focused on TTR role in glucose metabolism, in T2DM, TTR is found in higher levels (Zemany *et al*., 2015). In fact, studies in insulin-resistant *ob/ob* mice (leptin-deficient obese diabetic animal model, monogenic, with genetic induced obesity or diet induced) have shown elevated levels of serum TTR (Mody *et al*., 2009), as well as high levels of RBP4, indicating that TTR, by preventing the glomerular filtration of RBP, contributes to its increased levels and therefore, to disease development (Wei *et al.*, 1995); and in some insulin-resistant humans. For instance, a recent study by Xiong *et al*., evidenced elevated levels of TTR in pregnant women, which, consequently, leads to GDM (Xiong *et al*., 2017).

On the other hand, in T1DM TTR levels were found to be decreased (Itoh *et al*., 1992). A recent study showed that TTR KO mice show impaired recovery of blood glucose and glucagon levels, suggesting that TTR may have an important role in glucose homeostasis (Su *et al*., 2012). However, other study by Marques and co-workers reported no alterations in glucose metabolism in TTR KO mice, comparing with WT TTR (Marques *et al*., 2007). However, the effect of insufficient TTR, which would mimic more closely the human situation, was not analyzed.

Furthermore, it also has been shown that TTR promotes insulin release to protect pancreatic β-cells against death (Refai *et al*., 2005) and to bind to glucose-regulated proteins, such as Grp78, Grp94, and Grp170, besides the GLUTs, that were previously mentioned. These proteins are molecular chaperons of the heat shock protein family, also known as stress proteins located in the endoplasmatic reticulum (ER) and induced by oxidative stress, defective Ca^{2+} homeostasis and glucose deprivation (Dekki *et al.,* 2012). This process facilitates the internalization of TTR in pancreatic β-cells, as well as hepatomas cells and astrocytes (Divino & Schussler, 1990).

Moreover, TTR can a be a possible biomarker for dyslipidemia (Zemany *et al*., 2015). Therefore, in a recent study TTR-antisense oligonucleotides (ASO), where shown to decrease LDL cholesterol in high-fat diet-fed mice, leading to improvements in insulin-glucose homeostasis, such as increased levels of glucose uptake and insulin signaling (Zemany *et al.*, 2015).

Regarding the CNS, TTR expression was shown to be elevated in the dorsomedial hypothalamus of rats with exercise-induced anorexia, suggesting a possible role of TTR in the modulation of food intake and energy balance, since ICV administration of TTR in normal growing rats, decreased NPY levels and, consequently, food intake and body weight (Zheng *et al*., 2016).

Very recently, neuron-derived TTR has been shown to stimulate glycolytic enzymes of astrocytes, such as pyruvate kinase M1/2 isoforms (PKM1/2), which is an enzyme responsible for turning glucose into pyruvate, in the glycolysis pathway (**Figure 13**) suggesting that TTR is involved in the formation of the astrocytes glycogen-neuronal mitochondria metabolic pathway of energy production, which is required for memory formation (Zawiślak *et al*., 2017).

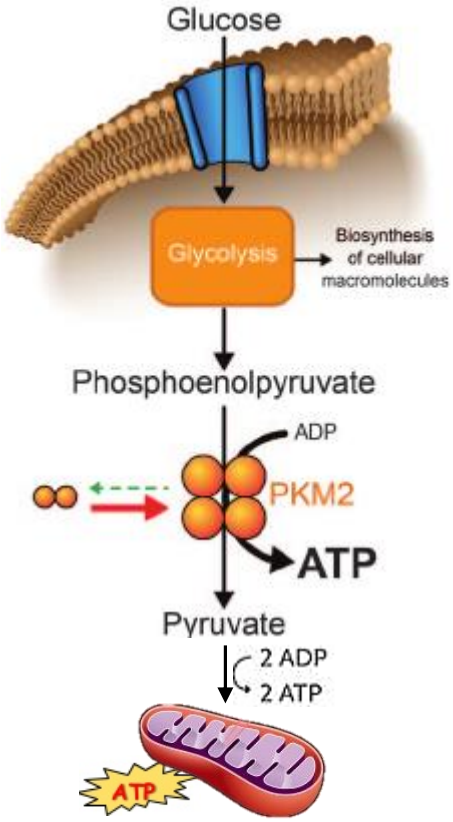

Figure 13 - Schematic representation of glycolysis pathway.

5.2.2. TTR and AD

The main features of AD are the depositions of aggregated Aβ in the brain and cerebrovasculature, but because the proportion of aggregation found in extracellular fluid of AD patients compared with the proportion found in CSF, serum and *in vitro* cultures was inversely proportional, Schwarzman and his colleagues hypothesized that Aβ was sequestered by extracellular protein, being the first group to find a relation between TTR and Aβ, showing that TTR is the major sequestering protein in CSF, suggesting that TTR could have a neuroprotective role in AD (Schwarzman *et al*., 1994). Therefore, several studies have been done, throughout the years, in order to understand this relationship. In fact, this was corroborated by the observation of AD patients brains where TTR levels where decreased in CSF (Gloeckner *et al*., 2008). Importantly, TTR levels correlate with disease state, meaning that TTR could be a biomarker (Gloeckner *et al*., 2008).

Studies in vivo obtained from AD transgenic mice established in different TTR genetic backgrounds demonstrated that the genetic reduction of TTR resulted in increased Aβ clearance and total Aβ brain levels (Buxbaum *et al*., 2008; Costa *et al*., 2008).

In previous study, Costa *et al*. (2008) observed that variants of TTR bind differently to Aβ peptide. For example, L55P TTR, which is the less stable and most amyloidogenic, had less affinity to bind do Aβ. On the contrary, T119M TTR showed the highest affinity among all tested. Because T119M TTR is the non-amyloidogenic variant known to attenuate the symptoms of FAP patients, it is suggested that the conformational molecular changes are important to the binding to Aβ and thereby its clearance (Costa *et al*., 2008).

Furthermore, Alemi *et al*. (2016), using an *in vitro* BBB model, human cerebral microvascular endothelial cell line (hCMEC/D3), also observed Aβ efflux promoted by TTR at the BBB, but only in the brain-to-blood direction, indicating that TTR is only responsible for the removal of the peptide from the brain and not for his entry back into the brain, corroborating the neuroprotective role of TTR in AD (Alemi *et al*., 2016).

Objectives

Although some reports attribute some roles to TTR in glucose metabolism, the underlying mechanism(s) are not completely known. Additionally, if and how this function relates to the neuroprotective role of TTR in AD, is also not understood.

Thus, this project aimed at clarifying the mechanisms by which TTR is involved in glucose metabolism, both at the liver and brain, and at providing some clues on how this might be important in AD.

Therefore, to pursue with this project, the following experiments were performed:

1. Measurement of glucose levels in different cellular models with different levels of TTR, to assess TTR effect on glucose levels:
   1.1. In plasma of mice with different TTR genetic backgrounds (TTR+/+, TTR+/- and TTR-/-), under fasting conditions.
   1.2. In media of primary hepatocytes derived from TTR+/+, TTR+/- and TTR-/- mice.
   1.3. In media of $HepG_2$ cells treated and non-treated with TTR.
   1.4. In media of hCMEC/D3 cells treated and non-treated with TTR.

2. Measurement of TTR levels under different concentrations of glucose, to assess effect of glucose on TTR levels:
   2.1. In culture media of primary hepatocytes (TTR+/+ or TTR+/-) treated with different concentrations of glucose.

3. Investigation of the effect of TTR on glucose transporters by measuring levels of GLUT1 in:
   3.1. Primary hepatocytes derived from TTR+/+, TTR+/- and TTR-/- mice and in TTR-/- hepatocytes treated with TTR.
   3.2. $HepG_2$ cell line treated with or without TTR.
   3.3. hCMEC/D3 cells treated with or without TTR.
   3.4. In brains and livers of mice TTR+/+, TTR+/- and TTR-/-.

4. Address the impact of TTR involvement in glucose metabolism in the context of glucose signaling cascade by assessing the effect of TTR in the gene expression of PKM1/2 at the transcript level using the qRT-PCR approach, in hCMEC/D3 and $HepG_2$ cell lines.

5. Measurement of the levels of glucose in the absence or presence of Aβ in primary hepatocytes derived from mice with different TTR backgrounds, to link TTR, glucose metabolism and AD.

Material and Methods

1. Human recombinant TTR production

Escherichia coli BL21 was used as a bacterial expression system to produce Human recombinant TTR (hTTR), using pET plasmids (Furuya *et al*., 1991) and the purification was performed as previously described (Almeida et al. 1997). In general, after growing the bacteria, the protein of interest was isolated and purified by ion-exchange chromatography and isolated afterwards, through gel electrophoresis. The protein concentration was determined using the Bradford Method (Bio-Rad), using bovine serum albumin (BSA) as standard.

2. Animals

The model used for this purpose was a mouse model (SV129 background) with different backgrounds of TTR: TTR-wild type (TTR+/+), TTR-heterozygous as a model for TTR insufficiency (TTR+/-) and TTR-Knock out as a model for TTR deficiency (TTR-/-). Animals were housed in a controlled environment (12h light/dark cycles, temperature between 22-24 ºC, humidity between 45-65% and 15-20 air changes/hour), with freely available food and water.

Furthermore, all these procedures were done with extreme caution and always taking into account the less or absence of pain and stress in the animal; all animals experiments were carried out in accordance with the European Communities Council Directive, as well as the recommendations of the Federation for Laboratory Animal Science Association (FELASA) and approved by the National Authority for Animal Health (DGAV; Lisbon, Portugal).

3. Blood and organ collection

After 7h of fasting - in which mice are deprived of food during 7h in the day, but have access to water - mice were anesthetized with a mix of anesthetics (Ketamine 75 mg/kg and Medetomidine 1 mg/kg) intraperitoneally (IP) and after evaluating the anesthetic depth by caudal and paw withdrawal reflexes, mice were cut in the skin and subcutaneous tissues, the intestine and liver were displaced and blood was collected from vena cava with syringes with EDTA (as an anticoagulant), in order to obtain plasma, which was followed by centrifugation at 14000 rpm for 10 min at 4 ºC. Plasma were collected and frozen at -80 ºC until used.

Then, a catheter cannula was inserted in the heart of the animal, specifically in the ventricular zone, then the inner needle of the cannula was removed and the connected peristaltic pump was turned on, letting the perfusion medium, which was PBS at 37 °C, perfuse through the heart to the whole body, in order to eliminate any traces of blood in the tissues,

and then the right atrium was immediately cut. After 4 minutes of perfusion, the liver, kidneys and brain were carefully collected. The brains were bisected longitudinally and fixed for 24h at 4 ºC in 10% formalin. Then, they were washed with PBS and finally transferred to a 30% sucrose solution for cryoprotection before cryostat sectioning and immunohistochemical analyses.

4. Immunohistochemistry

To investigate GLUT1 expression in brains from mice under fasting conditions, 30 µm-thick coronal brain sections of mice were washed with PBS and dried O/N at RT on APES-precoated slides. Then, sections were permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT, blocked with 5% BSA in PBS for 1h at RT and incubated with primary antibody mouse GLUT1 (1:400, Abcam) in 1% BSA in PBS overnight at 4 ºC. Next, sections were washed with PBS and incubated with Alexa Fluor-488 donkey anti-rabbit IgG antibody (1:2000) for 1h at RT. To remove tissue autofluorescence, Sudan Black B solution (0.3% Sudan Black B in 70% ethanol) was applied for 5 min at RT, followed by washing one time with water and after 8 times with PBS. Then, brain sections were mounted with FluoroshieldTM with DAPI (Sigma-Aldrich) and visualization was done with the Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR3.0 camera and Axivision 4.9.1 software.

5. Human cell lines and primary cultures of mouse hepatocytes

Two different cell lines were used, one as a BBB model: immortalized human cerebral microvascular endothelial cell line (hCMEC/D3 cell line), purchased at Tebu-Bio, and the other one as a model of the liver: immortalized human liver carcinoma cell line ($HepG_2$ cell line), available in the lab. Both cell types were cultured according to the available cell line data sheet.

hCMEC/D3, used between passages (25-35), were seeded in flasks or plates coated with rat tail collagen type I (Sigma) at a concentration of 150 µg/mL during 2h at 37º C or O/N. The growing medium for hCMEC/D3 consists of EBM-2 medium (Lonza) supplemented with 5% Fetal Bovine Serum (FBS) (Gibco), 1% Penicillin-Streptomycin (Lonza), 1.4 µM of Hydrocortisone (Sigma), 5 µg/mL of Acid Ascorbic (Sigma), 1% of Chemically Defined Lipid Concentrate (Gibco), 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco) and 1 ng/mL of bFGF (Sigma).

$HepG_2$ cells were used between passages 21 and 24, seeded in flasks or plates with no coating surfaces, were grown in Dulbecco's Modified Eagle Medium (DMEM) (1x) (Lonza) and Ham's F-12 media (Gibco), supplemented with 10% FBS (Gibco), 100 U/mL penicillin-

streptomycin (Gibco), 2 mM L-Glutamine (Gibco) and 1x Non-essential amino acid solution (Lonza).

To obtain the primary hepatocytes, firstly, the liver was perfused, that is, the abdomen was opened, the organs displaced and the vena cava and portal vein were detected. Then, a catheter cannula was inserted inside the portal vein and wash perfusion medium (HBSS 1x medium containing 0.025 M HEPES and 2 mM EDTA) was allowed to perfuse through the liver, for 10 min and the vena cava was cut immediately. After that time, the perfusion medium was changed to digestion medium containing collagenase type V ((Williams E medium (WE, Gibco), containing 10% FBS, 3 mM $CaCl_2$, 0.01 M HEPES and 0.25 mg/mL Collagenase type V (sigma-Aldrich)) for another 10 minutes. At the end, the liver was removed to a Petri dish containing isolation medium (WE medium containing 10% FBS, 2 mM EDTA and 0.01 M HEPES), ready to proceed to the next steps: centrifugation, counting live cells and seeding cells, with attachment media (WE medium containing 10% FBS and 0.01 M HEPES) for 3 h.

After 3h, the cells were washed with warm PBS so that unattached cells dissociate and incubated with stimulation medium, containing 2x penicillin-streptomycin, 0.01 M HEPES, 0.04% Fungizone, 0.05 mM Dexamethazone, 1 µM Insulin and 0.05 M 2 Mercapto-ethanol. After 24h, the cells were washed again with warm PBS and new stimulation medium was added. After 48h, the experiments were performed.

6. Protein Extraction

Proteins from hCMEC/D3, $HepG_2$ and primary hepatocytes cells were extracted in lysis buffer (20 mM MOPS pH 7.0, 2 mM EGTA; 5 mM EDTA, 30 mM sodium fluoride, 60 mM β -glycerophosphate pH 7.2, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% triton X-100) by doing up and down with the pipette, followed by incubation for 20 min on ice. Then, the extracts were centrifuged at 14000 rpm for 20 min, at 4º C, and the supernatants were collected and further used for protein analysis. The total protein concentration was quantified in all extracts by the Bradford Method (Bio-Rad), using BSA as standard.

7. Glucose measurement in mice plasmas and supernatant media of primary hepatocytes

Glucose levels in plasmas (mg/dL) from fasted mice and in supernatants from cultures of primary hepatocytes, previously incubated for 24h, with or without additional exogenous hTTR (0.5 µM), as well as supernatants from cultures of hCMEC/D3 and $HepG_2$ cells, previously incubated for 7h, with or without additional exogenous hTTR (0.2 µM and 0.5 µM),

were measured using a glucose assay kit (Abcam) following the manufacturer's instructions. Proteins from cells were also extracted, quantified by Bradford and used to normalize the levels of glucose in the supernatants. Results are expressed as glucose levels (mg/dL) per mg of protein.

8. Quantification of mouse TTR by ELISA

Mouse TTR in the supernatants of primary cultures of hepatocytes derived from TTR+/+ and TTR+/- mice, control or treated with glucose (20 mM and 35 mM, for 1h, 6h and 24h), were quantified using Mouse Prealbumin ELISA Kit (MyBioSource) according to the manufacturer's instructions. Data were expressed in mg/L.

9. Immunocytochemistry

hCMEC/D3 and $HepG_2$ cells were grown on glass coverslips (Thermo Fisher Scientific) previously autoclaved. Cells were grown until reach 80% of confluence and then incubated with their respective media in the absence or presence of TTR variants.

To study GLUTs levels, in particular GLUT1, hCMEC/D3 were incubated with 0 µM; 0.2 µM; 0.5 µM of hTTR for 7h. To study GLUT1, $HepG_2$ cells were incubated with the same concentrations of hTTR, for 30 min, 1h and 7h. Then, cells were washed 3 times with PBS and fixed with acetone for 7 min at RT. Following fixation, cells were washed again 3 times with PBS, the last wash for 5 min at RT. Next, blocking was performed with 5% BSA in PBS for 1h, followed by incubation with primary antibody mouse GLUT1 (1:33.3, Abcam), O/N, at 4º C. After being washed 3 times with PBS for 5 min each, cells were incubated with the Alexa Fluor-488 anti-mouse IgG antibody (Invitrogen 1:2000) for 1h at RT. Coverslips were mounted with FluoroshieldTM with DAPI (Sigma-Aldrich) and visualization was done with Zeiss Axio Imager Z1 microscope. The images visualization were performed with Axiovision 4.9.1 software and quantification was done using ImageJ software.

10. Western Blot (WB) analysis

The expression of glucose transporter GLUT1 in hCMEC/D3, $HepG_2$ cells and primary hepatocytes were studied by Western Blot (WB) analysis.

Protein extract samples (50 µg), partially denatured after 15min incubations at 37 ºC, were separated (amperage of 20 mA) in 10% SDS-PAGE gels. Then, the proteins were transferred for 90 min (voltage of 100 V and 400 mA) to nitrocellulose membrane, previously

treated with distillated H_2O (dH_2O) (15 min) and Transfer Buffer (10 min), using a wet system (Bio-Rad Criterion Blotter). Then, the membranes were dyed with Ponceau, washed with dH_2O, followed by PBS containing 0.05% Tween-20 (PBS-T) and blocked 1h at RT with 5% of milk in PBS-T. After blocking, membranes were incubated with primary antibody against mouse GLUT1 (1:500 / 54 kDa, Abcam) overnight at 4 ºC, or with antibody against mouse β-actin (1:5000 / 47 kDa, Sigma) as reference protein, for 1h at RT in 3% milk/PBS-T. Then, after three washes in PBS-T, membranes were incubated 1h at RT with anti-mouse HRP (1:2500) in 3% milk/PBS-T. The blots were developed using Clarity™ Western ECL substrate (Bio-Rad) and proteins were detected and visualized using chemiluminescence detection system (ChemiDoc, Bio-Rad). When necessary, membranes were stripped for 10 min at RT with, also, gentle shaking, using commercial stripping buffer (Restore™ Western Blot stripping buffer; Thermo scientific) for re-utilization of the membranes, according to the manufacturer's instructions.

11. Gene expression- qRT-PCR

For the Real-Time Polymerase Chain Reaction (qRT-PCR) analysis, hCMEC/D3 and $HepG_2$ cell lines were used and incubated in the presence or absence of hTTR (0.5 µM) for 16h (O/N) and total RNA was then isolated from cells using Trizol reagent (Invitrogen) following the instructions available in the product datasheet. RNA concentration and purity were quantified by reading the absorbance at 260 nm and the A_{260}/A_{280} ratio, respectively, with NanoDrop photometer (Thermo Fisher Scientific), and RNA integrity was determined by Experion. For qRT-PCR, the primers were designed, according to their sequence available in the National Center for Biotechnology Information (NCBI) and using the functionality Beacon Designer 7 provided by CCGene lab from i3S described in **Table 2**. Human β-actin was used as an internal control for normalization. The reaction mix was done with iTaq SYBR green (iQ SYBR green supermix, BioRad) following the manufacturer's instructions. PCR primers and conditions used for all analyzed genes are also shown in **Table 2**. The qRT-PCR was performed using the iQ5 BioRad Detection System (BioRad). The relative quantification was performed according to the comparative method ($2^{-\Delta\Delta ct}$).

Table 2. Sequences of the "in-house designed" primers. PCR condition was the same for all primers.

Gene	Primer Sequence	PCR Condition
Human PKM1/2	Forward:5' CAGCCAAAGGGACTATCCT 3' Reverse: 5' GAGGCTCGCACAAGTTCTTC 3'	Initial denaturation: 95ºC, 3 minutes Denaturation, annealing and extension (40 cycles): 95ºC, 15 seconds 60ºC, 30 seconds
Human β-actin	Forward: 5' CCAACCGCGAGAAGATGA 3' Reverse: 5' TCCATCACGATGCCAGTG 3'	

12. Statistical analysis

Data following a Gaussian distribution, was analyzed by one-way ANOVA and the comparisons between groups made with Student's t-test, when p values lower than 0.05, they were considered statistically significant. All data was expressed as mean±SEM.

Results

1. TTR affects glucose levels

1.1. Insufficient TTR in plasma of mice with only one copy of the TTR gene presents elevated glucose levels

As previously described, TTR is found in higher levels in plasma of T2DM and in lower levels in T1DM and in the plasma and CSF of AD patients. To assess the influence of TTR on the regulation of glucose metabolism, as a first approach, we measured the glucose levels in mice plasma with different backgrounds of TTR, under fasting conditions.

As it can be seen in **Figure 14**, mice with insufficient TTR (TTR+/-) showed increased plasma glucose levels. However, mice deficient in TTR (TTR-/-) showed no significant differences compared to TTR+/+ mice, confirming previous results reported by Marques and co-workers (Marques *et al.,* 2007), which may be explained by compensation mechanisms developed to overcome the total absence of TTR, at all stages of develoment of the animals.

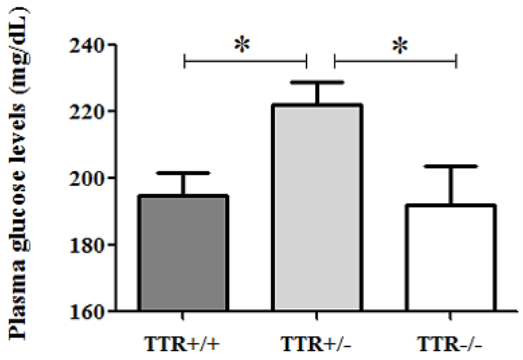

Figure 14 - Plasma glucose levels (mg/dL) in fasted mice, illustrating the higher glucose levels in plasma from TTR+/- mice as compared to TTR+/+ and TTR-/- animals (n=4 for TTR+/+; n=11 for TTR+/-; n=7 for TTR-/-). Data are expressed as mean ±SEM. p< 0,05 (*).

1.2. Insufficient TTR results in elevated levels of glucose in the media of cultured hepatocytes

Given the importance of the liver, as described in the introduction, being the major site of production and catabolism of TTR, as well as the main responsible organ in the maintenance of normal glucose homeostasis of the body, we then analyzed the glucose levels in supernatant of hepatoma cells ($HepG_2$) incubated in the absence or presence of hTTR.

As depicted in **Figure 15 (A)**, there are no alterations in the glucose levels in the supernatants of $HepG_2$ cells incubated in the absence of hTTR, which could be due to the fact that cell lines, sometimes, don't reproduce the phenotype observed *in vivo*.

Therefore, and aiming at bringing the model closer to the *in vivo* situation, we analyzed the same parameters in primary hepatocytes derived from mice with different TTR genetic

backgrounds. The results are displayed in **Figure 15 (B)** and confirm that insufficient TTR in TTR+/- mice significantly increased the glucose levels in supernatant of primary hepatocytes. Further, addition of hTTR (0.5 µM) to the media of the TTR+/- primary hepatocytes, partially rescued the phenotype and significantly decreased the glucose levels. We also observed that glucose in the supernatants of TTR-/- hepatocytes was higher than in the TTR+/+, differently from the measurements in plasmas. Nevertheless, these levels were lower than in the TTR+/- cultures and, importantly, addition of hTTR did not alter glucose concentration, again indicating that other mechanisms are activated.

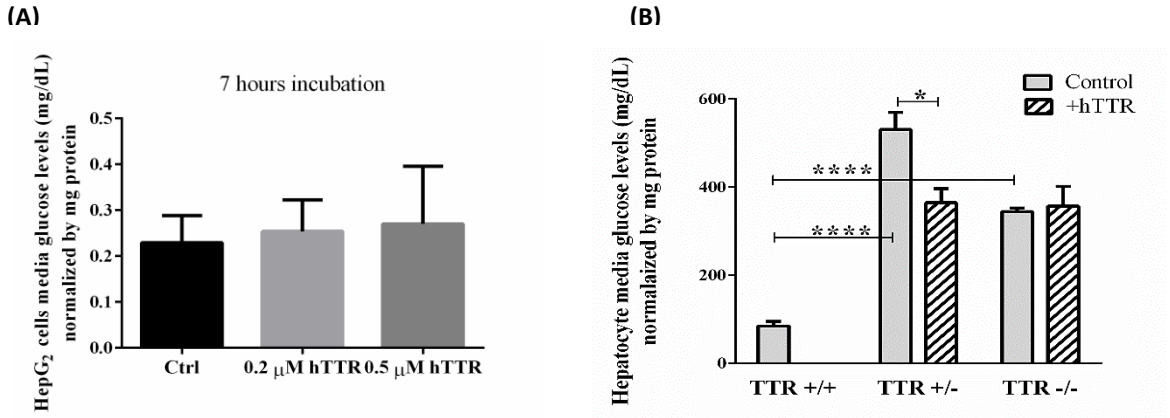

Figure 15 - Glucose levels (mg/dL) in the supernatants of cultured hepatocytes, normalized by mg of protein. (A) In $HepG_2$ cells, the presence or absence of hTTR (0.2 and 0.5 µM) did not alter the levels of glucose (n=5 for each condition). (B) Supernatants from primary hepatocytes derived from TTR+/- and from TTR-/- mice presented higher glucose levels, compared to those from TTR+/+; addition of hTTR (0.5 µM) only rescued the phenotype from TTR+/- cultures, resulting in decreased glucose concentration (n=3 for each condition). Data are expressed as mean ±SEM. p< 0,05 (*); p<0,0001 (****).

1.3. Insufficient TTR results in elevated levels of glucose in the media of a BBB cellular model

Furthermore, regarding the constant need of the brain to be supplied with glucose through the BBB, which is used to transduce energy through glycolysis and mitochondrial oxidative phosphorylation to support synaptic transmission (Yin *et al*., 2016), we also investigated the effect of TTR in glucose levels in the BBB model used in our study (the hCMEC/D3 cell line).

For that, glucose levels were measured in the media of hCMEC/D3 cells, incubated in the absence or presence of hTTR (0.2 µM and 0.5 µM). As presented in **Figure 16,** result show a significant decrease in glucose levels in the supernatant of these cells in the presence of hTTR.

Taken together, our results suggest that insufficiency of TTR impairs glucose metabolism, both in the periphery and at the BBB.

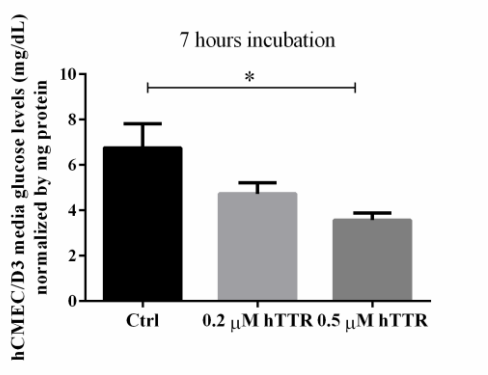

Figure 16 - Glucose levels (mg/dL) in the supernatants of cultured hCMEC/D3, normalized by mg of total protein, showing that the presence of hTTR (0.5 μM) significantly decreased the concentration of glucose in the media (n=3 for each condition). Data are expressed as mean ±SEM. p< 0,05 (*).

2. Glucose does not affect TTR expression in primary hepatocytes

To understand if the observed decreased glucose metabolism was a consequence or a cause of the TTR insufficiency, we evaluated the effect of different concentrations of glucose in TTR levels. For that, we incubated primary hepatocytes derived from TTR+/+ and TTR+/- mice, with different glucose concentrations (11.11 mM (Ctrl media), 20 mM and 35 mM) at three different time-points (1h, 6h and 24h) and measured the levels of TTR by ELISA. Our results showed no alterations in the TTR levels for any of the glucose concentrations, in any of the incubation times (**Figure 17**), which indicates that glucose alterations do not precede TTR insufficiency, but instead, probably it is the other way around: alterations in TTR are prior to the changes in glucose levels.

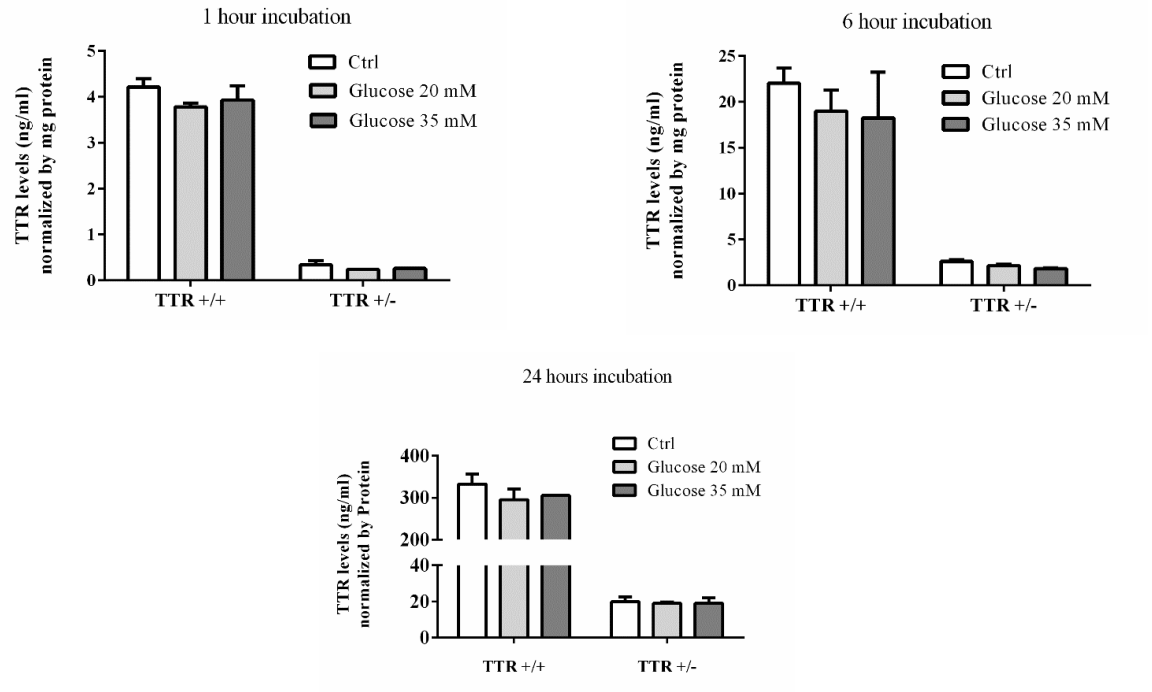

Figure 17 - Analysis of TTR in the supernatants of cultured primary hepatocytes derived from TTR+/+ or TTR+/- mice, showing that different glucose concentrations (11.11 mM (Ctrl), 20 mM and 35 mM at 1h, 6h and 24h) did not affect the levels of this protein (n=2 for each condition). Data are expressed as mean ±SEM.

3. Transthyretin modulates the expression of GLUT1:

3.1. In liver models

A previous study demonstrated that TTR binds to glucose-regulated proteins (Dekki *et al.*, 2012) and glucose transporters are the main family mediating glucose uptake in tissues. Since GLUT1 is expressed in most cells, in the case of hepatocytes it is mainly expressed on the sinusoidal membrane, and is responsible for basal glucose uptake, therefore we decided to investigate the effect of TTR on this glucose transporter in the livers of mice with different TTR backgrounds by immunohistochemistry. However, we could not draw conclusions from those experiments, since the primary antibody used produced nonspecific binding.

To partially overcome this drawback, we proceeded to immunocytochemistry analysis using $HepG_2$ cells, incubated in the absence or presence of hTTR and our results showed a significant increase in GLUT1 expression in the presence of hTTR, after O/N incubation (**Figure 18 (A)**). To corroborate these results, we also performed a WB, and despite the images are technically not very good, a significant increase in GLUT1 expression was observed in the presence of hTTR (**Figure 18 (B)**).

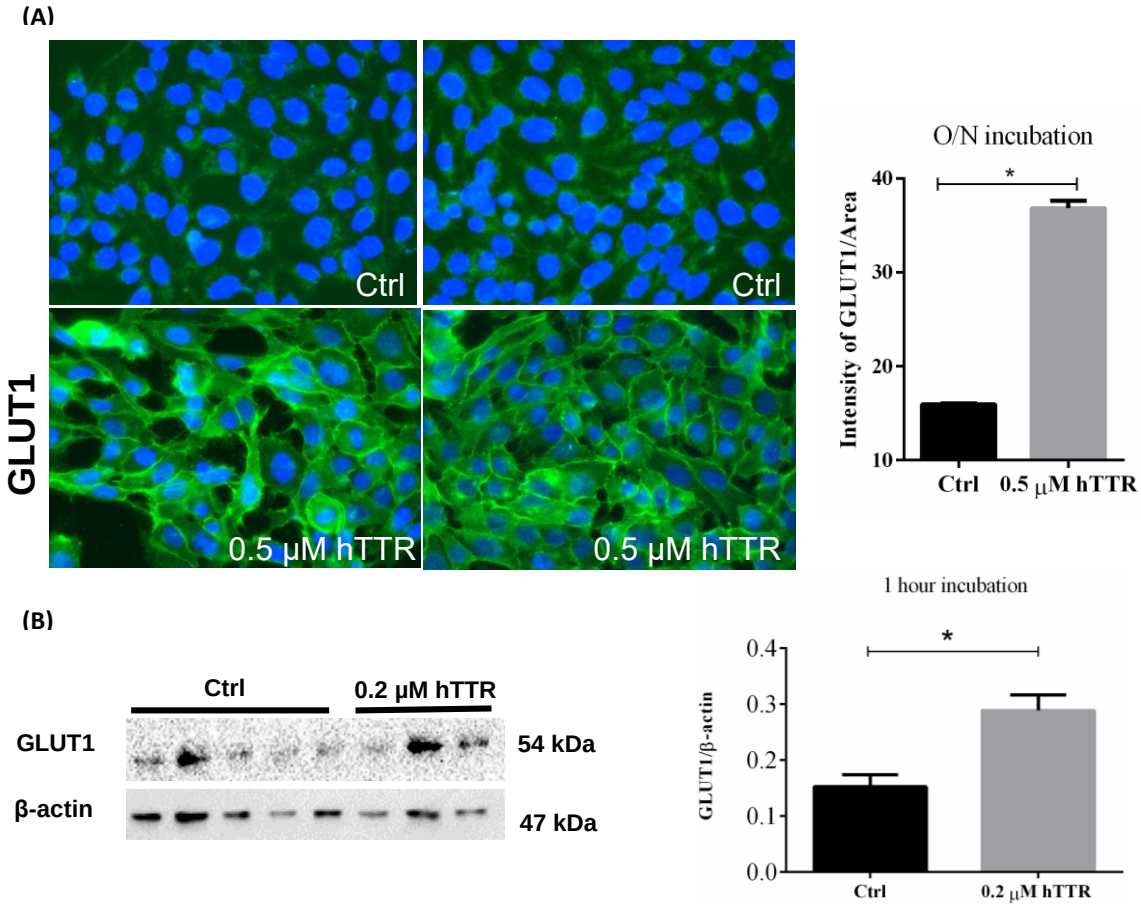

Figure 18 - Analysis of GLUT1 expression in $HepG_2$ cells without or with incubation with hTTR (0.5 µM). (A) Immunofluorescence analysis of GLUT1 expression in $HepG_2$ cells stained with an antibody against the protein (green). Nucleus of cells are stained with DAPI (blue). (B) WB analysis for GLUT1 expression in the absence or presence of hTTR (0.2 µM) (n=7 for each condition). Data are expressed as mean ±SEM. p< 0,05 (*).

Also, GLUT1 levels in primary hepatocytes from TTR+/+ and TTR-/- mice were analyzed. TTR+/+ hepatocytes and TTR-/- hepatocytes treated with hTTR (0.5 µM) to the TTR-/- showed a trend for increased GLUT1 expression compared to TTR-/- hepatocytes incubated with no hTTR (**Figure 19**). The lack of statistical significance may be related to the low number of replicas.

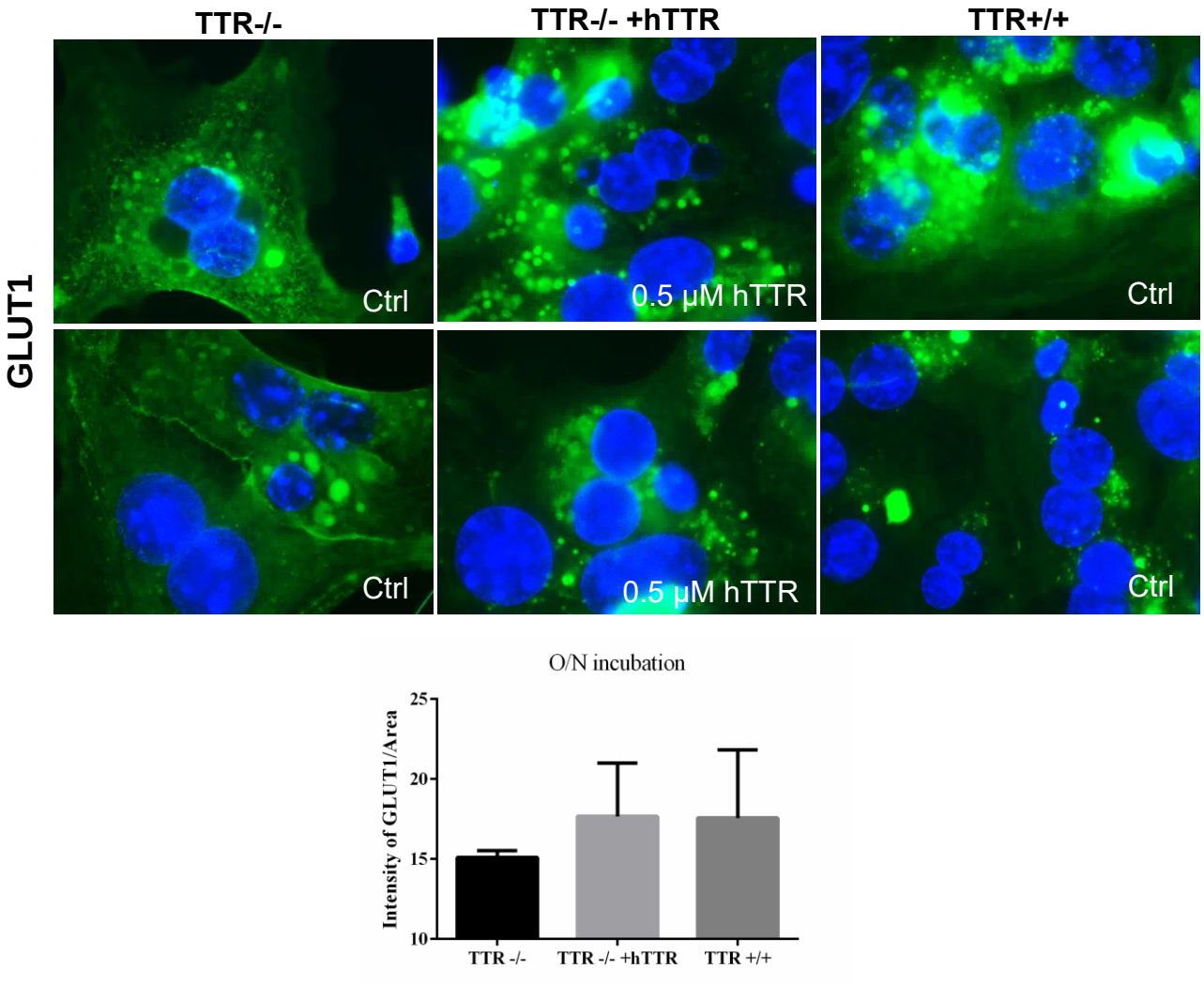

Figure 19 - Analysis of GLUT1 expression in primary hepatocytes cells without or with incubation of hTTR (0.5 µM) O/N. Immunofluorescence analysis of GLUT1 and expression in primary hepatocytes cells stained with an antibody against the protein (green). Nucleus of cells are stained with DAPI (blue) (n=8 for each condition). Data are expressed as mean ±SEM.

3.2. In the BBB model

GLUT1 is the major glucose transporter in the BBB, providing a homeostatic control for glucose into the brain by preventing glucose accumulation in the brain interstitial fluid (Zlokovic, 2008). Thus, we also investigated the expression of this transporter in mice brains by immunohistochemistry, but again, we encountered technical problems related to the primary antibody.

Therefore, we proceeded again to immunocytochemistry analysis, using hCMEC/D3 incubated in the absence or presence of hTTR (0.5 µM) for 7 hours. Our results showed no significant difference in the expression of GLUT1 by hCMEC/D3, under the experimental conditions, and contrarily to the liver models, the presence of hTTR tended to induce a decrease in GLUT1 (**Figure 20**).

Our results are not in concordance with previous studies that have shown that brain glucose uptake correlates with GLUT1 levels at the BBB (Zeller *et al*., 1997), so we questioned what other mechanism involved in glucose transport at the BBB could be affected by TTR and explain the lower glucose levels in the supernatants of hCMEC/D3 TTR-treated cells.

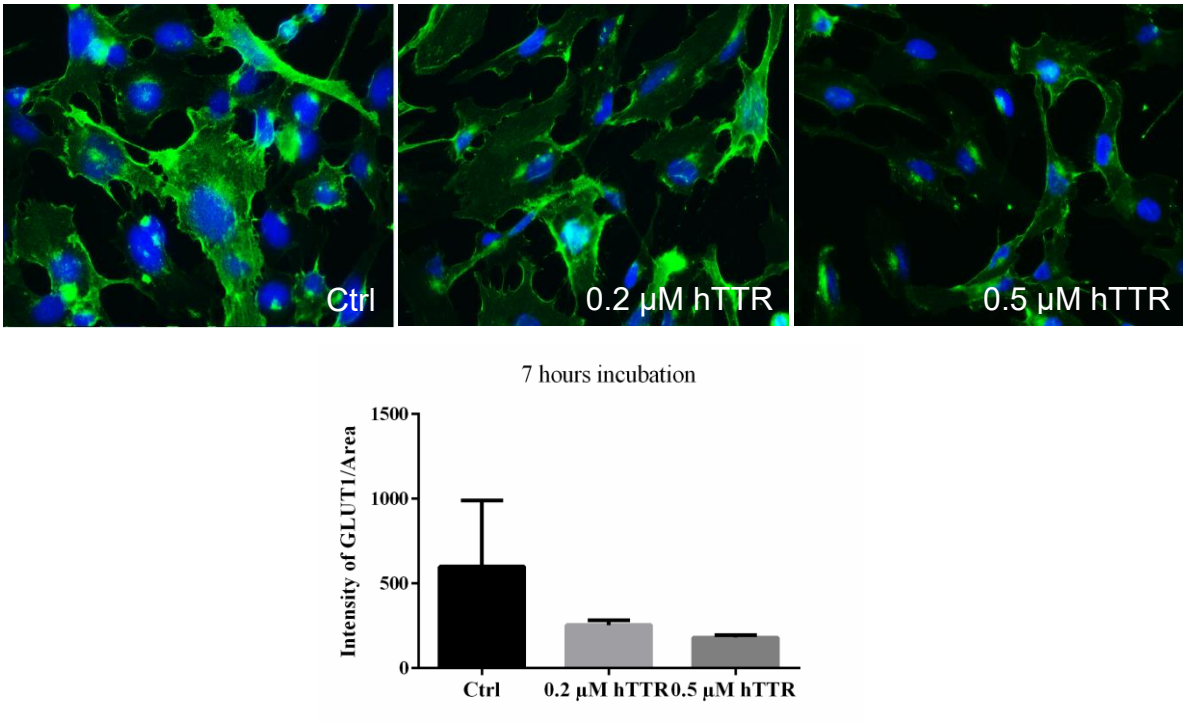

Figure 20 - Analysis of protein expression in hCMEC/D3 without or with incubation of hTTR (0.2 and 0.5 µM). Immunofluorescence analysis of GLUT1 expression in hCMEC/D3 stained with an antibody against each protein (green). Nucleus of cells are stained with DAPI (blue) (n= 7 for each condition). Data are expressed as mean ±SEM.

4. Impact of TTR involvement in glucose metabolism in the context of glucose signaling cascade in the expression of PKM at the transcript level

Recently, a study evidenced that neuron-derived TTR stimulates expression of glycolytic enzymes, namely pyruvate kinase M1/2 (PKM1/2), in astrocytes (Zawiślak *et al*., 2017). In order to investigate if TTR also affects this pathway in our models, we incubated hCMEC and $HepG_2$ cells in the absence or presence of hTTR and performed a qRT-PCR to determine the relative gene expression of PKM1/2.

Our results show a trend for increased expression of PKM in the presence of hTTR in hCMEC/D3 (**Figure 21 (A)**) and the opposite trend in HepG2 cells (**Figure 21 (B)**), although both did not reach the statistical significance

Altogether, the results referring to the effect of TTR in GLUT1 and in the PKM1/2 gene suggest that different mechanisms are influenced by TTR, in different organs.

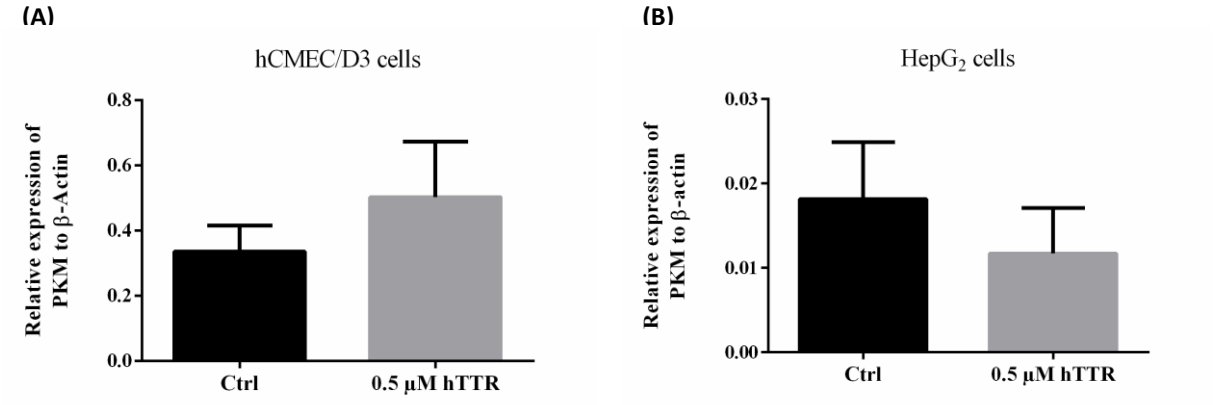

Figure 21 - qRT-PCR analysis of PKM gene at the transcript levels in a BBB and hepatocyte cell line models without or with incubation of hTTR (0.5 µM). (A) hCMEC/D3 and (B) $HepG_2$ cells data are expressed as mean ±SEM (n=3 for each condition).

5. Effect of Aβ in glucose metabolism at the liver

Finally, to study the link between TTR, glucose metabolism and AD, we incubated primary hepatocytes derived from mice with different TTR genetic backgrounds in the absence or presence of Aβ (2 μM) and showed that the presence of Aβ significantly increases glucose levels in the supernatants of cultures hepatocytes, within each genotype (**Figure 22**). Further studies are necessary to explore these preliminary results.

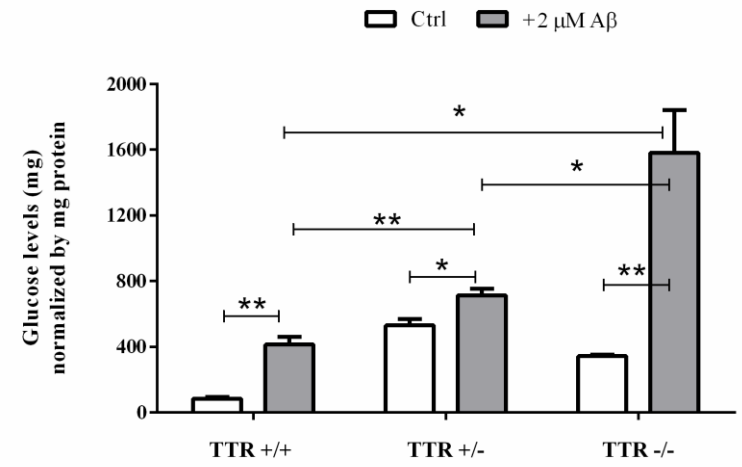

Figure 22 - Analysis of glucose levels (mg) normalized by mg of protein in primary hepatocytes with different backgrounds of TTR treated in the absence or presence of Aβ (2 μM) (n=3 for each condition). Data are expressed as mean ±SEM. p<0,05 (*); p<0,01 (**).

Discussion & Future Perspectives

Glucose is an important fuel for our body and mainly for the brain which has to be constantly supplied through the BBB to maintain its normal function. This glucose is provided through food intake or produced in the liver, a crucial organ that is responsible for the maintenance of normal glucose homeostasis, by regulation of glycolysis and gluconeogenesis.

Transthyretin, also known as prealbumin, is as previously described, a ~55 kDa homotetrameric protein, responsible for the transport of the thyroid hormone thyroxine (T_4) (Gouvea *et al*., 2013) and retinol, in the blood and CSF (Berry *et al*., 2012). Throughout the years, many studies focused on the neuroprotective role of TTR, including its binding to Aβ peptide and impact in AD. Our group has previously shown that TTR promotes Aβ transport from the brain to the blood (Alemi *et al*., 2016) and facilitates its degradation in the liver, contributing to its neuroprotective role in AD (Alemi *et al.*, 2017). However, in AD patients TTR is found to be decreased in the plasma and CSF (Ribeiro *et al*., 2012), which can be related to the impaired Aβ clearance, attributed to the development of Sporadic AD, which constitutes 90-95% of all AD cases.

Recent studies show evidence that abnormalities in glucose transport and insulin signaling are associated with AD, since it is reported that patients with DM have about 65% increased risk of developing AD (Arvanitakis *et al*., 2004), and also that AD patients show reduced glucose metabolism. For instance, previous studies have shown decreased levels of GLUT1 (Mark *et al*., 1997) and, thus, reduced glucose uptake in the brain (Khalid Iqbal & Grundke-Iqbal, 2005).

Although, the role of TTR in glucose metabolism is still not extensively studied and still controversial, according to some studies, TTR is found in higher levels in both T2DM (Zemany *et al.*, 2015) and GDM (Xiong *et al*., 2017), and in lower levels in T1DM (Itoh et al. 1992). Besides, TTR was also found to promote insulin release to protect pancreatic cells against death (Refai *et al*., 2005) and its absence can even impair recovery of blood glucose and glucagon levels, as shown in a study in TTR KO mice (Su *et al.*, 2012).

Therefore, this work started by assessing the effect of not only the deficiency but also the insufficiency of TTR in glucose metabolism, by measuring mice plasma glucose levels. As previously reported by Marques and colleagues (Marques *et al*., 2007), TTR KO mice did not show alterations in the basal or fast-induced circulating levels of glucose, which is in agreement with our results, since we did not observe any differences between TTR+/+ and TTR-/- mice. However, in our work, differences between TTR+/+ and TTR+/- were evident, as mice with insufficient TTR had significantly higher glucose plasma levels. These results indicate that TTR is important in the context of glucose metabolism and that insufficient amount of this protein impairs uptake of glucose from blood. Also, it is likely that TTR-/- animals activated mechanism(s) of compensation, overcoming the absence of TTR. The presence of compensation mechanisms related to TTR and AD has been previously reported; AD/TTR+/-

but not AD/TTR-/- mice present a more severe form of AD, compared to AD/TTR+/+ (Oliveira *et al*., 2011). In the future, effort should be made to produce and perform studies in conditional models, where expression of TTR can be active even before birth and only blocked at a certain age, providing a closer model to the human events in AD.

To gain further insights into the importance of TTR in glucose metabolism, we aimed at evaluating this effect both in the brain and at in the liver. As for the liver models, upon incubation, primary cultures of TTR+/- hepatocytes showed significantly higher glucose in the supernatant, compared to TTR+/+, and addition of hTTR to those cultures, partially rescue the phenotype, decreasing the amount of glucose in the supernatant. In this experiments, the TTR-/- cultures presented glucose levels higher than the TTR+/+ cells (although lower than the TTR+/-), indicating that: 1) even primary cultures do not totally reproduce the *in vivo* events and, for instance, these cells may have active mechanism(s), which are inactive *in vivo*, and/or even 2) that plasma glucose is the product of several factors and organs, which are not all contemplated in the cellular model, namely the pancreas/insulin system.

To mimic the BBB, through which glucose can enter into the brain, we used the hCMEC/D3 cellular model, showing that increasing the concentration of hTTR, resulted in decreased glucose in the supernatant of the cultures. Although we did not assess TTR expression by hCMEC/D3 cells, it is important to refer that TTR synthesized by the choroid plexus and meninges can be transported to other cells in the brain. For instance, in cases of brain ischemia, CSF TTR can control neuronal death and inflammation (Santos *et al*., 2010), meaning that TTR is transported from the CSF to other brain areas via receptor-mediated endocytosis (Liz *et al*., 2010) and, as previously shown, it participates in Aβ clearance (Alemi *et al.,* 2017). Further, the confinement of TTR expression in the brain to the choroid plexus (and meninges) is controversial, and for instance, it has been shown by quantitative PCR and WB, that primary neurons from APP23 mice transcribe TTR mRNA and that these cells synthesize and secrete TTR (Xinyi Li & Buxbaum 2011; X. Wang *et al*., 2014). Another work, reports that TTR expression is induced by the APP fragment, AICD, in SH-SY5Y cells, resulting in decreased total cellular Aβ levels (Kerridge *et al*., 2014).

Because these first results showed a decrease glucose metabolism, a question arose whether if this would be a cause or consequence of TTR insufficiency. Aiming to answer this question, we evaluated the effect of different concentrations of glucose in TTR levels, and our results showed no alterations in TTR levels, indicating that alterations in TTR are prior to the changes in glucose levels.

TTR was found to be able to be internalized by the pancreatic β-cells (Refai *et al*., 2005) and hepatoma cells (Sousa & Saraiva, 2001), which are cells involved in the glucose metabolism. Moreover, according to a previous study by Dekki *et al*., TTR was also found to bind to glucose-regulated proteins (Dekki *et al*., 2012). Regarding these mechanisms and

aiming to explain the increased internalization of glucose, in other words the decreased levels of glucose in the supernatants and plasma in the presence of TTR, we further investigate the mechanism(s) underlying TTR participation in glucose metabolism, by evaluating the expression of GLUT1 transporter, which has been described to be expressed on the sinusoidal membrane of hepatocytes (Shinoda *et al*., 2001). Our results showed, as expected, an increase in GLUT1 expression in our liver models in the presence of hTTR. In hCMEC/D3, however, TTR tend to decrease the GLUT1 expression, suggesting that in these cells there is another mechanism involving the decreased levels of glucose in the supernatants of these cells. Previous studies, evidenced a down-regulation of GLUT1 in the BBB, besides other transporters, in hyperglycemic animals, and they thought that the glycolytic flux could be explained by the levels of lactate, which could be influencing other transporters (Duelli *et al*., 2000). However, in other studies in hyperglycemic rats, an increase in GLUT1 mRNA expression was observed, but no changes were observed in the glucose transport (Simpson et al. 1999), which makes it controversial, and further insight in this correlation GLUT-glucose at the BBB should be investigated.

Regarding the CNS, TTR was described to be involved in cognition, behavior (Sousa *et al*., 2004), neuropeptide maturation (Nunes *et al*., 2006), nerve regeneration (Fleming *et al*., 2009; Fleming *et al*., 2007; Groop & Pociot, 2014) and in ischemia (Santos *et al*., 2010) but most importantly, in a recent study neuron-derived transthyretin (TTR) was shown to stimulates expression of glycolytic enzymes, namely pyruvate kinase M1/2 (PKM1/2), in astrocytes (Zawiślak *et al*., 2017), which provides energy required for memory formation. In order to confirm what mechanism could be involved in the BBB cell model, and to investigate the effect of TTR in downstream pathways of glucose signaling cascade, we determined the relative gene expression of PKM in the absence and presence of hTTR, using hCMEC/D3 and $HepG_2$ cell lines. Here we showed that PKM1/2 tended to increase in the presence of hTTR in hCMEC/D3, which could explain the decreased glucose levels, in spite of the observed decrease in GLUT1. In the $HepG_2$ cells, however, we saw the opposite trend, PKM1/2 tended to decrease in these cells, in spite of the observed increase expression of GLUT1, suggesting that TTR modulates differently the liver and the BBB.

To further link TTR, glucose metabolism and AD, we evaluated glucose levels in the presence of Aβ peptide, using primary hepatocytes. Aβ deposition is found to induce lipid peroxidation, which has been demonstrated to decrease glucose transport and metabolism in hippocampal and cortical neurons (Mark *et al*., 1997). Besides, Aβ deposition can also cause disruption of ion homeostasis and apoptosis (Mark *et al*., 1997). Importantly, IGF-1 was found to influence Aβ clearance from the brain, by promoting Aβ transport across the BBB (Freude *et al*., 2009) but Aβ can also compete with insulin transporters, affecting insulin response to high glucose levels. In our results, we saw an increase of glucose levels in the supernatants

of cultures hepatocytes in the presence of Aβ within each genotypes (TTR+/+; TTR+/- and TTR-/-), but further studies are necessary to explore these preliminary results.

Altogether, our results indicate that TTR is involved in glucose metabolism and modulates both the BBB and the periphery. Regarding the levels of glucose in the plasma, and in the supernatants of BBB and liver cells, TTR decreased those levels, suggesting that TTR influences the internalization of glucose, but probably through different mechanisms. On one hand, in the BBB, TTR decreased GLUT1 expression, but increased PKM1/2 expression, which can be the reason behind the decrease in glucose levels in the supernatants, suggesting TTR may act downstream in the glucose cascade signaling. On the other hand, in the $HepG_2$ cells, TTR increased the levels of GLUT1 expression, and decreased the PKM1/2 levels, which suggests that TTR may affect more significantly on upstream proteins such as glucose transporters.

In the future, it would be interesting to perform an experiment using the hCMEC/D3 cells cultured in transwell inserts, inducing the BBB polarity, in order to understand TTR effect on GLUT1 levels on each side of the BBB and thus explain the observed increased glucose uptake in the presence of TTR. Besides, in order to address the downstream pathways of glucose metabolism, by qRT-PCR, instead of using only hCMEC/D3 cell line, as used in our work, primary cultures, namely cerebral endothelial cells, astrocytes or neurons, could be also used for a more detailed insight.

As for the upstream pathways of glucose signaling cascade, it would be interesting to study the other glucose transporters present in the liver, as well as the effect of TTR on insulin expression.

Furthermore, although our mouse model was already established with different backgrounds of TTR, it would be interesting to know the effect of TTR, by silencing the 2 copies of TTR gene expression in TTR+/+, instead of reproducing mice born already with no TTR.

Overall, our results have shown that TTR 1) influences the internalization of glucose, through 2) modulation of upstream proteins in the liver, such as glucose transporters and possibly 3) modulation of downstream pathways in the BBB. These results provide relevant information for TTR-based therapeutic strategies for AD, since TTR role in glucose metabolism seems to be neuroprotective. Alterations in glucose metabolism are thought to be related to AD and thus this work highlights the potential role of TTR as a protective protein and as a therapeutic target.

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