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# ADIPOSE TISSUE-TARGETED STEM CELL TRANSPLANTATION FOR INSULIN RESISTANCE-RELATED CNS DEFICITS

Settore scientifico-disciplinare BIO/17

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# INDEX

## ABBREVIATIONS

ABCA7 = ATP-binding cassette sub-family A member 7

ADP = Adenosine diphosphate

AGE = Advanced Glycation End-products

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPA-R = AMPA-Receptor

AMPK = 5'-AMP-activated protein kinase

ApoE = Apolipoprotein E

APP = Amyloid-beta Precursor Protein

AS160 = Akt-substrate of 160 kDa

AT = Adipose Tissue

AT-MSC = Adipose Tissue-derived MSC

ATP = Adenosine triphosphate

Aβ = Amyloid-beta

BACE1 = Beta-Amyloid Cleaving Enzyme-1

BBB = Blood Brain Barrier

BM-MSC = Bone-Marrow-derived MSC

CA1 or CA3 = Cornu Ammonis Area 1 or 3

cAMP = cyclic adenosine monophosphate

CD = Cluster of Differentiation

CNS = Central Nervous System

CSF = cerebrospinal fluid

DAG = Diacylglycerol

DM = Diabetes Mellitus

DPP-4 = Dipeptidylpeptidase-4

ENPP1 = ectonucleotide pyrophosphatese/phosphodiesterase-1

ER = Endoplasmic Reticulum

fEPSP = field Excitatory Post-Synaptic Potential

FFA = Free Fatty Acids

FOXO = forkhead box protein O

GABA = gamma amminobutirric acid

GABA-R = GABA-Receptor

GD = Gestational Diabetes

GHb = Glycated hemoglobin

GLP-1 = Glucagone-Like Peptide-1

GLUT = Glucose Transporter

GSK = Glycogen Synthase Kinase

HFD = High-Fat Diet

HGF = Hepatic Growth Factor

HSL = Hormone Sensitive Lipase

IAPP = Islet Amyloid Poly-Peptide

ICV = intracerebroventricular

IDE = Insulin Degrading Enzyme

IDO = Indoleamine-2,3-dioxygenase

IGF = Insulin-like Growth Factor

IKK = IκB kinase

IL = interleukin

IR = Insulin Receptor

IRB = Institutional Review of Board

IRS = Insulin Receptor Substrate

IRSp53 = IR-tyrosine kinase Substrate p58/53

IV = Intravenous

JNK = c-Jun N-terminal kinases

$K_{ATP}$ = ATP-sensitive potassium channels

LIF = Leukemia Inhibiting Factor

LTD = Long-Term Depression

LTP = Long-Term Potentiation

MAPk = mitogen-activated protein kinase

MCP = Monocyte Chemotactic Protein

MSC = Mesenchymal Stem Cells

mTOR(C) = mammalian target of rapamycin (complex)

NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells

NMDA = N-Methyl-D-Aspartate

NMDA-R = NMDA-Receptor

NZO = New Zealand Obese (mice)

OLETF = Otsuka Long Evans Tokushima Fat (rats)

P70SK = p70 S6 kinase

PBS = Phosphate Buffer Solution

PDK = $PIP_3$ (phosphatidilinositol 3,4,5-triphosphate)-dependent kinase

PD-MSC = Placenta-derived MSC

PGC = PPAR gamma coactivator

PI3K = phosphoinositide 3 kinase

PKA = Protein Kinase A

PKB = Protein Kinase B (alternate name of Akt)

PKC = Protein Kinase C

PP1 = Protein Phosphatase 1

PP2A = Protein Phosphatase 2A

PSD-95 = Post-Synaptic Density-95

PTP1B = Protein-Tyrosone Phosphatase 1B

RAGE = Receptor for AGE

RNS = Reactive Nitrogen Species

ROS = Reactive Oxygen Species

SCAP = SREBP Cleavage-Activating Protein

Ser/Thr = Serine/Threonine

SNARE = SNAP (Soluble NSF Attachment Protein) Receptor

SREBP = sterol-regulatory element binding protein

Stat = signal transducer and activator of transcription

STZ = Streptozotocin

T1D = Type 1 Diabetes

T2D = Type 2 Diabetes

T3D = Type 3 Diabetes

TBC1D1 = (Tre-2/Bub2/Cdc16)1 – domain 1

TG = triglycerides

TIMP = Tissue Inhibitor of MetalloProteinases

TLR = Toll-Like Receptors

TNF-α = Tumor Necrosis Factor – alpha

Tyr = Tyrosine

UC-MSC = Umbilical Cord-derived MSC

UTMB = University of Texas Medical Branch

VEGF = Vascular Endothelial Growth Factor

WJ-MSC = Wharton's Jelly-derived MSC

ZDF = Zucker Diabetic Fatty (rats)

## ABSTRACT

Compelling evidence indicates that Type 2 Diabetes (T2D) and Alzheimer's Disease (AD) may possibly share a common pathological origin, but the underlying mechanisms remain poorly understood. T2D is a known risk factor for AD and insulin resistance (hallmark of T2D) has been extensively documented in AD patients. Notably, insulin is important for learning and memory due to its role in LTP and LTD modulation. Adipose tissue (AT) dysfunction is a risk factor for T2D, in fact elevated levels of free fatty acids are prodromal to insulin resistance and have been reported in AD brains, as well. In this study, I used a mouse model (AtENPP1Tg mouse) that recapitulates typical characteristics of human metabolic syndrome and insulin resistance, when nourished with a high-fat diet, but also shows hippocampal dysfunction and memory deficits, hence offering a unique chance to explore which mechanistic pathways connect diabetes with AD. In last decades, stem cell therapy has recently developed as potential therapeutic strategy for diabetes. Previous studies showed that a systemic administration of mesenchymal stem cells (MSC) improves peripheral insulin sensitivity and blood glucose levels as well as restores insulin signaling cascade. Interestingly, the pool of MSC in AT of diabetic patients is significantly reduced, with consequent decreased adipocytes' turnover. As a result, the adipocytes cannot be replaced, thus becoming immature, the fat cannot be stored anymore, consequently ectopic fat deposition occurs. A major limitation of a systemic stem cell transplantation is the scattered cell distribution throughout the body and the circulation (with high risk of vessel occlusions) which reduced presence in target organ. Here, I propose a novel approach aimed to deliver, directly into AT, via subcutaneous injection, human umbilical cord-derived Wharton's Jelly (WJ) MSCs. The overall aim was to restore diabetes-related CNS alterations through the correction of peripheral insulin sensitivity. The results show improvement of blood glucose levels and LTP response in hippocampus in transgenic transplanted mice compared to not–transplanted ones.

It is conceivable that the replenishment of MSCs within the AT may restore insulin signaling both in periphery and CNS, thus reestablishing both peripheral and CNS insulin sensitivity with a mechanism, likely mediated by MSC-released factors and supposedly delivered to CNS from the periphery. Further studies are needed to elucidate the potential protective mechanism provided by MSCs.

# INTRODUCTION

## *DIABETES MELLITUS*

**Diabetes Mellitus** (**DM**, referred to as **diabetes**) is a chronic metabolic disorder characterized by hyperglycemia due to inadequate insulin production or reduced insulin sensitivity. There are several forms of DM:

- **Type 1 Diabetes** (**T1D**), also known as juvenile-onset or insulin-dependent diabetes, characterized by the autoimmune destruction of pancreatic β-cells, which causes the lack of insulin.
- **Type 2 Diabetes** (**T2D**), also called adult-onset or non-insulin-dependent diabetes, the most common form of diabetes (about 90% of diabetes cases are T2D), mainly characterized by reduced tissue sensitivity to insulin, named **insulin resistance**, and in the majority of cases, by obesity and dyslipidemia. This form of diabetes will be discussed in details in this manuscript.
- **Gestational Diabetes** (**GD**), particular condition that may affect pregnant women, similar to T2D. Usually, it improves or disappears after the delivery[1,2].
- **Specific type of diabetes**, due to other causes, mostly genetic factors or excessive use of drugs: under this category fall **neonatal diabetes**, **mature-onset diabetes of the young (MODY)**, **drug/chemical-induced diabetes**[3].

According to 2017 United States National Diabetes Statistics Report, in 2015 people with diabetes were 30.3 million (9.4% of US population), 1.25 million of which had T1D, and 1.5 million new cases of diabetes are diagnosed every year; the percentage of adults with diabetes rises up to 25% among people aged 65 years old or older. Moreover, in USA, diabetes is still the 7$^{th}$ leading cause of death. As far as regards Italy, 3.2 million resulted affected in 2016 (5.2% of overall population 16.5% among people over 65 years old) even if the mortality rate has significantly decreased in last decade. Consequently, the financial impact on public health is quite significant, thus encouraging further studies aimed to reduce its impact to the population[4,5].

## *TYPE 2 DIABETES*

Type 2 Diabetes is a heterogeneous disease mainly characterized by **insulin resistance**, especially in skeletal muscle, liver and adipose tissue (AT): it is defined as reduced sensitivity to the hormone with consequent overproduction of insulin by pancreas, thus leading to hyperinsulinemia[1,2]. Other features of T2D are the decreased insulin secretion (in late stages of the pathology), particularly after a glucose stimulation and an increased hepatic gluconeogenesis[1]. Noteworthy, accelerated lipolysis by fat cells and central nervous system (CNS) dysregulation of metabolism may be considered as additional hallmarks of T2D[6]. T2D accounts for about 90% of all diabetes diagnosis worldwide[1] and is often accompanied by obesity[2]. It is also known as non-insulin dependent diabetes, indeed T2D does not require insulin administration at the beginning of the pathology, although the secretory capacity of the pancreas decreases with the development of the disease[1,2]. The reduced responsiveness to insulin leads to hyperglycemia, which further impairs β-cell response and insulin signaling, even though therapeutic treatments aimed to improve blood glucose levels seem to restore the insulin response by the organs. With the progression of the disease, the risk of complications becomes higher. Such complications include cardiovascular issues, retinopathies, kidney failure and CNS complications[2].

There are many major risk factors for T2D, as listed below[1]:

- Overweight (according to body mass index)
- Physical inactivity
- First-degree relative with diabetes
- Member of a high-risk ethnic population (e.g., African American, Latino, Native American, Asian American, Pacific Islander)
- Female with a history of delivering a baby weighing >9 lb or prior diagnosis of GDM
- Hypertension (≥140/90 mm Hg or on therapy for hypertension)
- HDL cholesterol level <35 mg/dL (0.90 mmol/L) or triglyceride level >250 mg/dL (2.82 mmol/L) or both
- Female with polycystic ovary syndrome
- Hemoglobin A1c ≥5.7%, impaired glucose tolerance, or impaired fasting glucose on previous testing

- Other clinical conditions associated with insulin resistance (e.g., severe obesity, acanthosis nigricans)
- History of cardiovascular disease
- Age over 45 years.

### *Insulin*

Insulin is a peptide hormone produced by pancreas, specifically in β-cells which are located in the islets of Langerhans[7]. It regulates primarily substrates metabolism in liver, muscle and fat by promoting their storage, through stimulation of lipogenesis, glycogen and protein synthesis as well as inhibition of lipolysis, gluconeogenesis and protein cleavage[7,8]. It also stimulates cell growth and differentiation[9]. Its crystallographic structure was first reported in 1926[10], the monomeric sequence was fully solved by Sanger et al in 1950s[11–14], while the 3D structure was determined by Hodgkin's laboratory in 1969[15,16]. Insulin is formed by two chain, A and B, bond by three disulphide linkages and tends to form hexamers around a $Zn^{++}$ ion inside the granules in which is packed by β-cells: once secreted, the hexamers break down, due to the low concentration outside the cell and for electrostatic repulsion[10,17–19]. The bioactive secreted peptide consists of 51 aminoacids but is synthetized as a 110-amioacids precursor, called preproinsulin. Preproinsulin contains a signal peptide, like other secreted proteins, which is needed for its translocation in endoplasmic reticulum (ER) before its release. In ER the signal peptide is cleaved, thus resulting in proinsulin. Proinsulin is then transported to Golgi apparatus where is further processed in insulin and C-peptide, and finally carried out in secretory vesicles, ready to be released[2,10,17]. Insulin biosynthesis is controlled by many factors, although the most important is blood glucose concentration: β-cells are able to sense glucose concentration thanks to their strategical position in islets of Langerhans, strictly connected to vasculature, which allows them to receive 10 times the amount of blood than surrounding cells[17]. In addition to glucose, some amino acids and fatty acids also regulate insulin secretion[20,21]. Glucose is taken up by β-cells through the GLUT2 transporters, constitutively expressed in pancreas and insulin-insensitive organs, with difference to GLUT4. After its entry, glucose is phosphorylated by the rate-limiting enzyme glucokinase[22]. The product of this phosphrylation, glucose-6-phosphate, enters in glycolysis pathway with consequent

production of pyruvate, which, in turn, falls into Krebs cycle ending up to the production of ATP, thus contributing to the rise of ATP/ADP ratio[2,17]. ATP is fundamental for the action of ATP-sensitive potassium ($K_{ATP}$) channel-dependent insulin release, which is finely regulated by intracellular calcium concentration[17]. The following steps are, finally, closure of $K_{ATP}$ channels, depolarization of plasma membrane, opening of voltage-dependent calcium channels with consequent increase of $Ca^{2+}$ concentration which in turn leads to exocytosis of insulin-containing vesicles[17]. Proteins of SNARE complex mediate the exocytosis of insulin vesicles, but their fusion is triggered by an increase of calcium concentration. Other important regulators of insulin secretion are free fatty acids (FFA), diacylglycerol (DAG) and aminoacids: for example, FFA can potentiate insulin secretion to compensate increased needs of the hormone in states of insulin resistance. β-cells express a receptor for FFA, thus allowing their entry in cells and their metabolism, which in turn leads to two other regulators of insulin secretion, DAG and long-chain acyl-CoA. These two metabolic products affect insulin secretion by interacting with some protein involved in exocytosis machinery[17]. Another known regulator of insulin secretion is leptin, AT-derived hormone: it inhibits insulin secretion; in fact, leptin deficiencies bring to hyperinsulinemia[23].

### *Insulin signaling*

Maintaining a steady glucose concentration is important for the organism. In order to accomplish this task, many players act a role, especially insulin, in consequence of the rising of blood glucose levels. Insulin inhibits glucose output from the liver and promotes the uptake of glucose from blood stream to skeletal muscle and adipose tissue through the induction of the GLUT4 trafficiking[24].
Insulin binds its own receptor and after this event, a cascade of phosphorylations and dephosphorylations and protein-protein interactions begins in almost every tissue[1]. The insulin receptor (IR) is a glycoprotein composed of two subunits α and two subunits β, linked by disulphide bonds, to form a tetramer[9,25,26]. Two isoforms of insulin receptor are known, isoform A and B, (the difference is due to an alternative splicing on exon 11 of IR gene, thus leading to a "longer" isoform) and their simultaneous existence suggests the possibility that each isoform has different roles[1,25,27]. The subunit α (135 kDa) is entirely extracellular and contains the binding site(s) for the hormone, while the β-subinit (95 kDa) has three components, extracellular,

transmembrane and cytosolic[25,26]. IR may bind more than one molecule of insulin; however, the binding of the first molecule is sufficient to stimulate downstream events and occurs with high affinity, while the other molecules of insulin bind α-subunit with lower affinity[27,28]. Therefore, at increased insulin concentrations, occupancy increases but affinity diminishes[27,28]. Upon insulin binding, the cytosolic portion of β-subunit undergoes auto-phosphorylation at tyrosine (Tyr) residues, conferring also tyrosine kinase activity: one β-subunit phosphorylates the other on specific tyrosine residues. Binding of insulin causes a conformational change that moves Tyr-1162 out of the kinase domain catalytic pocket, therefore it gets phosphorylated. Then, this tyrosine phosphorylates in turn the correspondent Tyr on the other subunit thus allowing a third Tyr-phosphorylation: these three phosphorylated tyrosine residues bring to the fully activated IR[25]. Insulin receptor can also undergo serine/threonine (Ser/Thr) phosphorylation, which has an inhibitory meaning on insulin signaling (it might decrease autophosphorylation ability) and, in fact, this type of phosphorylation results increased in insulin-resistant and diabetic patients[29]. Ser/Thr phosphorylation is mediated by several protein kinases C (PKC)[30] or cAMP-dependent kinase[31], whose levels are increased as well in diabetic patients[1,25].

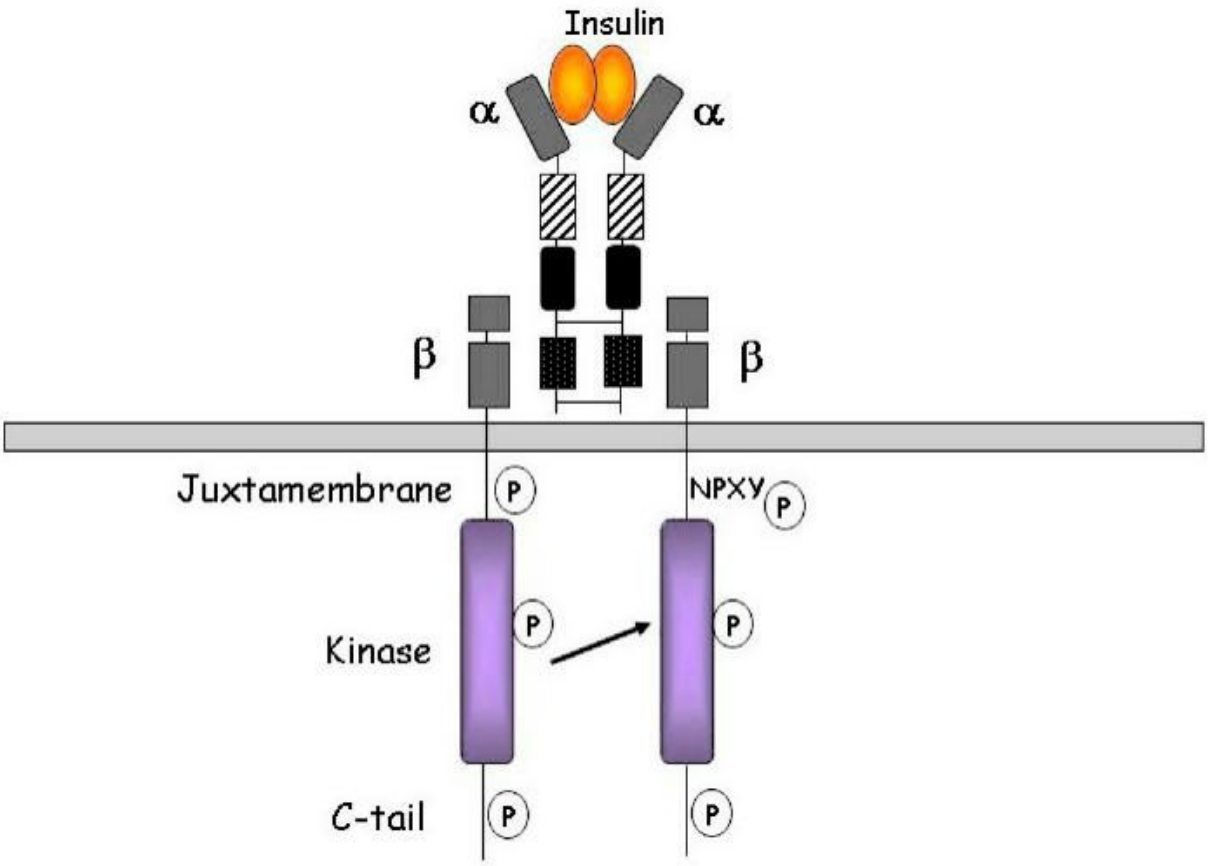


Figure 1: Structure of insulin receptor *(from Chang L, Chiang S, Saltiel AR,* Molecular Medicine*, 2004)*

Insulin signaling ends when the receptor is dephosphorylated and internalized: two phosphatases, PTP1B and LAR, participate in dephosphorylation processes, thus leading to IR internalization, which is followed by disruption of the complex and degradation of insulin in endosome/lysosome system[32]. Also, the protein ectonucleotide pyrophosphates/phosphodiesterase-1 (ENPP1) plays a role in the inhibition of insulin-induced Tyr-kinase activity[32,33]. The receptor number at the cell surface is subject to regulation and insulin itself mediates this process of down-regulation, called desensitization. Besides, chronic exposure to insulin induces desensitization and degradation processes[25,27].

IR autophosphorylation activates insulin receptor substrates (IRS) by Tyr phosphorylation, particularly IRS1 and IRS2 (IRS1 seems more related to glucose metabolism, IRS2 to lipid metabolism[32]), whose functional domains allow them to interact with other proteins in order to

diffuse insulin signaling inside the cell[34]. In addition, IRS can undergo Ser/Thr phosphorylation by mean of Akt, GSK3, several PKC isoforms or mTOR/S6 kinases with consequent reduction of insulin signaling[32,35,36]. Another important modification that contributes to the decrease of insulin signaling is the O-GlcNacylation of IRS1, which limits the possibility to activate other proteins by IRS[37,38]. Tyr-IRS phosphorylation leads to activation of phosphoinositide-3-kinase (PI3K), crucial for metabolic actions of insulin (PI3K inhibition blocks most metabolic actions of insulin[9]), by contributing to the separation of regulatory (p85) and catalytic (p110) subunits that compose PI3K[32]. PI3K generates 3,4,5-phosphoinositol which in turn activates several phosphatydilinositol 3,4,5-triphosphate ($PIP_3$)-dependent kinases (for example PDK1 and PDK2), which in turn activate Akt and PKC[1]. Akt (also known as protein kinase B, PKB) activation requires three steps, relocation to plasma membrane, then binding of $PIP_3$ in a specific domain which induces its activation, through phosphorylation at specific serine/threonine residues: these two phosphorylations are made by PDK1 (at Thr) and mTORC2 complex (at Ser)[1,24]. Akt activation is crucial for the activation of other proteins involved in lipid and cholesterol synthesis (SREBP-1), protein synthesis (mTOR and Rheb), glycogen synthesis (GSK3) and glucose transport[1,26,32]. Dysfunctions of Akt are related to diabetes and insulin resistance[1]. Insulin cascade ends with GLUT4 translocation to the membrane of adipocytes, myocytes and cardiomyocytes: Akt-substrate of 160 kDa (AS160) and TBC1D1 normally inhibit GLUT4 translocation, but when get phosphorylated they allow the vesicles containing GLUT4 to fuse with plasma membrane, thus exposing the transporter to extracellular fluids where glucose can be taken up[1,39]. Hepatic production of glucose is regulated by glucagon and insulin, which, respectively, activate or suppress this process: the suppressive action of insulin on gluconeogenesis is important to maintain a proper glucose tolerance and involves mTORC2, PDK1 together with the transcription factor Foxo1. Foxo1 is inhibited by mTORC2 and PDK1 resulting in suppression of the transcription of gluconeogenesis-related genes[26].

Noteworthy, insulin regulates cell growth by activating Ras/MAP kinase pathway[1,32], which will not be described in this manuscript.

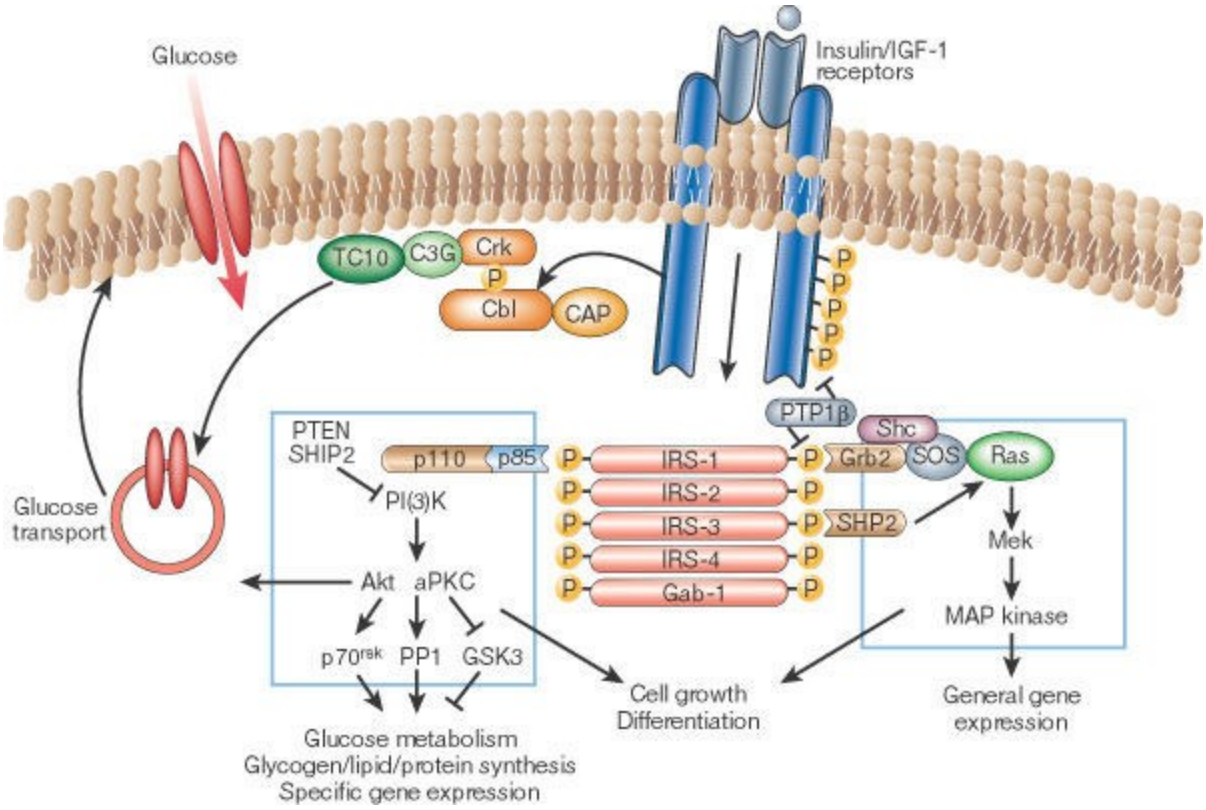


Figure 2 Schematic representation of insulin receptor cascade. *(from Saltiel AR and Kahn CR,* Nature*, 414, 799–806, 2001)*

Insulin, then, stimulates glycogen accumulation by increasing glucose transportation and glycogen synthesis[9]. Indeed, insulin promotes activation of glycogen synthase by dephosphorylation, due to inhibition of PKA or GSK3 and activation of protein phosphatase 1 (PP1)[9]. In details, upon PI3K gets activated, Akt is activated as well and promotes phosphorylation of GSK3, hence leading to its deactivation with consequent decrease of phosphorylation rate of glycogen synthase[9].

Insulin promotes lipid synthesis and suppresses lipolysis: these changes require changes in level of steroid regulatory element-binding protein (SREBP)-1c, whose activation is mediated by the pathway mTORC2-Akt[9,26]. In obese rodent models, the overexpression of SREBP-1c brings to increased FFA synthesis and gluconeogenesis, as occurs in insulin resistance and T2D[9]. Furthermore, in fat, insulin is able to inhibit lipolysis by acting directly on activation state of hormone sensitive lipase (HSL): this enzyme is activated by PKA-dependent phosphorylation and inhibited by activation of some phosphatases[9]. Since PKA is in turn dependent on cAMP levels,

insulin-dependent decreased levels of cAMP affect all PKA-downstream events, thus resulting in lipolysis inhibition[9].

In absence of insulin, GLUT4 vesicles are recycled slowly between plasma membrane and cytosol, while insulin action triggers the translocation of GLUT4-containing vesicles through exocytosis[39]. In the meantime, insulin reduces endocytosis, therefore the intake of glucose into muscle, fat and liver is regulated by GLUT4 concentration[9].

## *PATHOPHYSIOLOGY OF INSULIN RESISTANCE AND T2D*

Insulin resistance indicates a condition whereby the response to insulin results blunted either from an exogenous delivery or endogenous oversecretion of insulin[1]. During fasting states, insulin secretion lowers in favor to other hormones, primarily glucagon, which promotes glycogenolysis and lipolysis. The secretion of insulin (and other hormones) is tightly regulated, thereby any deviation from this firm control may provoke T2D[26]. Insulin resistance manifests as decreased glucose transport in fat and muscle, impaired lipolysis and increased hepatic gluconeogenesis. It is present years before the onset of diabetes even though reduced insulin response does not lead automatically to diabetes as long as β-pancreatic cells are functional and compensate for the increased needs[1].

AT role in metabolism regulation is important, as suggested by animal studies in which a knockout of GLUT4 in muscle does not affect glucose tolerance, while a knockout in AT induces a severe glucose intolerance which spreads to muscle and liver[9]. If nutrient intake exceeds expenditure, these excess is stored as fat, but if the storage ability of AT surpasses its capacity, the lipids and other nutrients are deposited in other departments (muscle, liver, vasculature) thus leading to adaptive responses which result in insulin resistance and diabetes[40]. It is known that the insulin sensitivity decreases as adiposity increases as well as it has been established that AT has a special role in insulin resistance, leading to the conclusion that overall body fat has an effect on insulin sensitivity[41]. In general, insulin effects on AT are fat cell differentiation, enhancement of glucose uptake, inhibition of lipolysis[26]. Likewise, AT can modulate insulin sensitivity, as well as food intake and nutrient homeostasis by the secretion of many factors, such as leptin, adiponectin, resistin, TNFα, IL6, (together called adipokines) that can affect insulin signaling in other organs. Leptin and adiponectin are considered “anti-diabetogenic” hormones because they can reduce

triglyceride synthesis, stimulate β-oxidation and glucose oxidation in both muscle and liver, through activation of 5'-AMP-activated protein kinase (AMPK) and peroxisome proliferator activated receptor (PPAR)-α. In insulin-resistant obese humans, leptin levels are increased while adiponectin decreases[42,43]. Besides, obesity causes a disbalance in adipokines secretion, by increasing pro-inflammatory cytokines release, thus contributing to insulin resistance[44,45]. For example, TNFα reduces IRS1 activation through JNK, causing insulin resistance[26,46,47].

As mentioned earlier, obesity may be present in a T2D patient, even if the association of obesity with diabetes varies among different ethnical groups[2]. AT is a dynamic organ that expands or relaxes in response, respectively, to overnutrition and energy deficit[48,49]. In general, lean people with a more peripheral fat distribution are more insulin sensitive than lean subjects who present a more abdominal fat distribution[43]. Different fat depots have different secretory properties: intra-abdominal fat secretes more adiponectin than the subcutaneous and seems to be more lipolitically active (also because does not respond to antilipolyitic effect of insulin). Therefore, it is conceivable that the relative proximity of intrabdominal fat to the liver induces hepatic insulin resistance, due to higher production of FFA, immediately loaded in portal circulation. Indeed, intra-abdominal adiposity is strictly linked to insulin resistance[1,43].

Interestingly, there are people, named metabolically healthy obese, with excessive fat but no sign of any metabolic issue, included insulin resistance[50,51]. Conversely, defects in maturation of adipocytes characterize people, normal in weight but metabolically obese, hyperinsulinemic and insulin resistant[51]. Diabetic, pre-diabetic persons and lean metabolically obese insulin-resistant persons, display larger adipocytes and impaired adipogenesis: these immature adipocytes are no longer able to deposit fat with consequent FFAs spillover[51–54]. FFA excess impairs both cognitive activity and hypothalamic regulation of metabolism due to their ability to cross blood brain barrier (BBB) and accumulate in brain, thus suggesting an inhibitory action on insulin in CNS[55–58].

**All the conditions that pave the way to peripheral insulin resistance are likely prodromal to CNS deficits as well, therefore an approach aimed to restore insulin sensitivity both in periphery and brain, by reestablishing a proper communication between AT and CNS, might prevent neurodegeneration.**

Increased FFA release from adipocytes are common in states of reduced insulin sensitivity[9]. Excessive caloric intake enhances FFA release, resulting in ectopic lipid deposition; high levels of

FFA can inhibit insulin signaling in other organs, thus contributing to insulin resistance[2,9,26]. Indeed, dysfunction of AT leads to unregulated lipolysis, hence high levels of FFA are released, thus impairing glucose uptake by muscle and removing inhibition of hepatic gluconeogenesis by insulin[59,60]. Moreover, high levels of FFA seem to reduce IRS1 phosphorylation and PI3K activation[9,27,43].

As further confirmation of the heterogeneity of this disease, other elements might contribute to the development of insulin resistance. As described above, AT may release, among adipokines, pro-inflammatory cytokines, such as TNFα and IL6, whose release rises as fat storage and release of FFA (or NEFA, non-essential fatty acids) increases[2,42,43]. The pro-inflammatory molecules summon macrophages, which further contribute to the release of such cytokines, thus resulting in chronic local inflammation and insulin resistance[2,43]: one of the molecules that recalls macrophages in AT seems to be monocyte chemotactic protein-1 (MCP-1)[42]. The inflammatory state, along with the increased FFA release, induces insulin resistance and inflammation in the other insulin-sensitive organs (skeletal muscle, liver and pancreas), as well[2]. A role has been attributed to NFκB and IKKβ pathway and JNK signaling, since their secretion increases with high-fat feeding, leading to hepatic insulin resistance and secretion of pro-inflammatory cytokines[42,43].

Lipid-induced hepatic insulin resistance is due to the accumulation of lipids in the liver as consequence of β-oxidation impairment. Insulin, in addition, inhibits β-oxidation by blocking PGC-1α (peroxisome proliferator-activated receptor (PPAR)-γ co-activator-1α), therefore these changes lead to an accumulation of lipids in the liver (thus paving the way to hepatic steatosis), even during insulin resistance states[42,61,62]. Loss of insulin inhibition towards the gluconeogenesis involves loss of Foxo1 phosphorylation by Akt: in T2D and insulin resistance, the pathway IRS1-PI3K-Akt is no longer able to keep Foxo1 phosphorylated, resulting in increased transcription of genes regulating gluconeogenesis[26,63].

The same condition occurs in skeletal muscle even if less evidence has been demonstrated for this organ. However, recent studies have showed that by-products of mitochondrial β-oxidation may have a role in developing insulin resistance, because the higher influx of lipid may not be compensated by an upregulation of mitochondrial enzymatic machinery for β-oxidation, therefore lipid metabolism could not be complete, leading to potentially toxic metabolites in mitochondria.

In studies with obese insulin resistant rats, it has been demonstrated that their muscle mitochondria were jammed with incomplete oxidized lipids, compared to lean rats[42,64].

In summary, the impairment of mitochondrial fatty acid oxidation both in liver and muscle, along with the accumulation in cytosol and ER has an important role in developing insulin resistance. This impairment is apparently owed to a missing upregulation of mitochondrial enzymatic machinery when an overload of nutrients occurs: the consequence is the accumulation of β-oxidation by-products and incomplete oxidized fats in mitochondria. Moreover, there is a decrease in expression of PGC1α, important transcriptional regulator of biogenesis. Whether muscle or hepatic mitochondrial impairment of lipid metabolism drives a distress signal, delivered to other departments, is still unclear[42].

The metabolic overload caused by impaired lipid metabolism has dramatic consequences on insulin signaling: PKCs isoforms, DAG-dependent enzymes, are of course implicated and their upregulation brings to increased Ser-phosphorylation of IRS, thus turning off insulin signaling. For example, PKCθ seems implicated in fatty acid-induced insulin resistance in skeletal muscle[42,43]. Other reports indicate that metabolic changes due to overnutrition may trigger endoplasmic reticulum stress with consequent involvement of Ser-kinases which interfere with downstream factors of insulin signaling[42].

A role of CNS insulin resistance in causing obesity has been described when an intracerebroventricular injection of insulin decreased both food intake and hepatic gluconeogenesis, whereas the administration of PI3K inhibitors blocked this effect. Moreover, a deletion of brain IRS2 results in hyperglycemia and obesity, compared to IRS1 deletion which did not cause neither disruption of glucose homeostasis nor obesity onset. Insulin-induced decreased food intake is similar to that caused by leptin, even if each of these hormones act on different pathways: insulin activates PI3K via IRS2, while leptin stimulates the Jak/Stat3 pathway[26].

T2D may be characterized by a cluster of abnormalities, such as hyperinsulinemia (consequence of insulin resistance), impaired glucose tolerance, spillover of lipids in plasma and hypertension, which are collectively part of the same syndrome, called metabolic syndrome, or syndrome X (also known as insulin resistance syndrome or the deadly quartet)[1,26,27].

Finally, hyperinsulinemia itself can induce insulin resistance, as summarized by Guo, in several fashions[26]:

1. Prolonged insulin treatment prevents acute insulin action, Foxo1 phosphorylation and GLUT4 translocation in adipocytes and myocardium.
2. Patients with hyperinsulinemia and T2D have low levels of IRS1 and IRS2.
3. P38/MAPk activation following extended insulin treatment in cardiomyocytes resulted in insulin resistance by increased Ser-phosphorylation of IRS1 and IRS2
4. P38/MAPk also mediates release of inflammatory cytokines that promote insulin resistance.
5. In high-fat diet (HFD), IRS are extensively Ser-phosphorylated by mean of MAPk or JNK, thus resulting in insulin resistance in liver and other tissues. This result was confirmed by deletion of JNK, which led to improvement of insulin resistance.

β-cells secrete a precise amount of insulin, depending on the nature of the stimulus and, most importantly, on glucose levels, in order to maintain blood glucose level within a specific narrow range. In obesity, insulin sensitivity of β-cells is decreased even though obese people show greater insulin responses but lower hepatic insulin clearance than non-obese subjects. It is worthwhile to underline that insulin sensitivity is strictly regulated, hence any change in sensitivity must be followed by a change in circulating hormone levels: if this control fails, glucose tolerance is lost and diabetes may develop[43]. This strict control between insulin sensitivity and circulating levels probably involves increased glucose metabolism and FFA release: in obesity glucose metabolism is increased, thereby ATP:ADP ratio is higher, thus leading to closure of $K_{ATP}$ channels and insulin release through exocytosis[43,65].

Obesity does not lead automatically to T2D. Only a portion of obese insulin-resistant individuals develops T2D, most likely due to failure of β-pancreatic cells[42]. It has been suggested that T2D occurs when pancreas loses 75% of its full functionality, thus leading to insufficient levels of insulin[43]. In mammals, the secretion of insulin after a meal is biphasic and regulated by many factors (secretagogues), with glucose that remains the primary one: FFA, aminoacids, the hormone glucagon-like peptide-1 (GLP-1)[42]. However, when pancreatic islets are exposed to chronic levels of nutrients, these cells become dysfunctional and can even die. Hyperglycemia leads to elevated insulin levels but suppresses glucose-stimulated insulin secretion[42,66], while chronic high levels of FFA impair insulin secretion stimulated by glucose and stimulate hepatic gluconeogenesis[43]. Conversely, chronic exposure of rodent islets to high levels of FFA causes a decrease in glucose

oxidation which in turn provokes a reduction of ATP:ADP ratio, thus inhibiting glucose-stimulated insulin release.

Another remarkable mechanism that has been linked to β-cell failure is the deposit of amyloid fibrils, composed of amylin, also known as islet amyloid polypeptide (IAPP). Amylin tends to aggregate in humans and non-human primates, but not in rodents. Some studies demonstrated that the overexpression of IAPP leads to reduced first phase insulin secretion, as well as to β-cell apoptosis and reduced mass, with consequent onset of diabetes[67–69].

## *BRAIN INSULIN SIGNALING*

Brain was considered for a long time an insulin-independent organ, given that the supply of glucose to the brain is continuously guaranteed and mediated by non-insulin-sensitive transporters (neurons express GLUT3)[70,71]. However, insulin presence in brain has been detected for the first time in late 1970s in rodents; it was also reported that insulin content in brain was independent from the peripheral concentration of the hormone[72,73]. Besides, there are some evidence of a local production of insulin in CNS and, also, it has been observed that postsynaptic excitatory potentials decreased when extracellular glucose concentration raise[74]. However, insulin can reach brain because is able to cross BBB through a saturable transport system, which may involve IRs, even though hypothalamus lacks an effective barrier, thereby it might be more sensitive to insulin compared to other brain regions[70,75,76]. In insulin resistance states, the transport of insulin to the brain results impaired, thus lessening insulin concentration in CNS[77]. Noteworthy, insulin in CSF is 10-20 fold less than plasma in healthy individuals, while in obese subjects this gradient becomes even higher. A similar behavior is showed by leptin, therefore it is important to understand whether an impaired transport of insulin across BBB impairs feeding behavior as well as hepatic and AT metabolism, thus paving the way to cognitive decline[78,79].

It has also been suggested that insulin released by peripheral departments, as well as leptin, sends a chronic signal to the brain in order to report energy reserves, therefore there must be a correlation between adiposity, plasma insulin levels and CNS insulin[77,80,81].

In brain, insulin shows a broad pattern of activities, such as neuronal development, energy expenditure, control of food intake, glucose homeostasis, learning and memory processes[82,83]. Most of the activities related to metabolic control involve hypothalamic nuclei (arcuate,

ventromedial and paraventricular)[84]. Moreover, intracerebroventricular (ICV) injection of insulin as well as intranasal administration of the hormone did decrease food intake, thus suggesting a direct role of brain, mediated by insulin, in feeding behavior[70,85]. Insulin decreases the release of orexigenic peptides as well as increases the expression of anorexigenic neuropeptides in hypothalamus: this effect involves PI3K-dependent activation of $K_{ATP}$ channels, whose activation hyperpolarizes orexigenic neurons with consequent inhibition of orexigenic peptides. In addition, this regulation of feeding behavior is abolished in streptozotocin (STZ)-treated diabetic mice-model[86,87]. Indeed, peripheral actions of insulin, such as hepatic inhibition of gluconeogenesis, lipolysis inhibition and de novo lipogenesis stimulation, are regulated by neurons of arcuate nucleus in the hypothalamus, which are enriched in IRs, as demonstrated after ICV administration of insulin in rats[70,82,83,88–90].

Brain usually uses ketone bodies during starvation, even if glucose is its main nutrient and is permanently supplied to CNS[82,91]. Interestingly, brain shows two populations of glucose-senstive neurons: glucose-excited (GE) and glucose-inhibited (GI) which are involved in control of feeding, energy expenditure and glucose homeostasis. The "sensor" of glucose concentration in glucose-sensitive neurons is glucokinase, thus facilitating the role of these cells in control of food intake[92]. As previously mentioned, the transport of glucose to the brain is insulin-independent even though the presence of GLUT4 is known in selective areas, such as olfactory bulb, dentate gyrus of the hippocampus, hypothalamus, thus suggesting the existence of a neuronal GLUT4 translocation mechanism triggered by elevated insulin levels[93]. Both GE and GI neurons express GLUT4, glucokinase and IR even if glucose uptake is not regulated by insulin signaling[82], therefore it is conceivable that brain insulin signaling plays another important role in glucose homeostasis. For example, an inhibition of this signaling in hypothalamus unleashed hepatic gluconeogenesis in rats[90], thus leading to the idea that a reduced insulin sensitivity in hypothalamus might induce decreased sensitivity in periphery, specifically in liver, and consequently cause hyperglycemia[90,94]. Insulin might activate $K_{ATP}$ channels which induce depolarization of neuronal plasma membrane and consequent amelioration of glucose sensitivity: the signals processed by hypothalamus are then transmitted to vagus nerve, that delivers the information to the liver[82].

Insulin is released by neurons similarly to the mechanism that occurs in periphery, therefore the activation of $K_{ATP}$ channels induces depolarization that in turn, with the presence of calcium, leads

to the release of insulin granules[82,95]. Interestingly, synaptosomes isolated from rats increase their insulin secretion when exposed to high glucose concentration[82].

Insulin in CNS regulates metabolism, food intake and hepatic glucose output by binding its own receptor, which is distributed in many brain regions. For example, in mouse IR is highly expressed in olfactory bulb, cortex, hippocampus, hypothalamus and cerebellum[85,96–98]. In particular, the activation of hippocampal IR increases cognitive function in both human and rodent models[56,99]. The IR isoform expressed in brain is the shorter one (IR-A), contrary to what is expressed in periphery (IR-B) and are not subjected to downregulation[82,85,100,101]. The binding of insulin triggers the same pathways already described for the periphery, but likely with another biological meaning. The activation of PI3K/Akt pathway leads to activation of mTORC1, known to be involved in protein synthesis, therefore in CNS important for the synaptic plasticity[102], while GSK3β (inhibited by insulin signaling when phosphorylated by Akt), whose involvement in Tau phosphorylation and therefore in AD pathogenesis has been extensively documented, regulates cell proliferation and neuroplasticity[103]. The expression of IRs occurs also in glial cells, especially astrocytes that are known to supply nutrients to neurons. Insulin signaling in astrocytes stimulates cell proliferation, expression of glutamatergic receptors as well as cholesterol biosynthesis[104,105].

As earlier described, the inhibition of hepatic gluconeogenesis by insulin is carried out in both brain and liver, thereby an inhibition of either liver or brain IR removes the inhibition of gluconeogenesis in liver[90,106]. The biochemical cascade that brings to the inhibition of glucose output by liver is initiated by the phosphorylation of hepatic Stat3 triggered by insulin[107] that stimulates IL-6 production, which in turn halts gluconeogenesis[90]. If insulin is administered in CNS, the liver becomes more sensitive and the processes of lipogenesis and fat accumulation are promoted[108].

Insulin is associated with alterations in cognitive function, especially in T2D subjects[109] and either T1D or T2D show smaller hippocampi and changes in connectivity between different areas of brain[110–113]. Interestingly, in a STZ-treated models of diabetes-induced cognitive decline, the infusion of insulin significantly retrieves memory[114,115]. Insulin can act directly on certain receptor and neuron populations: it regulates NMDA glutamatergic hippocampal receptors by phosphorylation of specific tyrosine residues located in subunits NR2A and NR2B[116], resulting in increased recruitment of the receptor to the membrane[117], hence in enhanced long term potentiation

(LTP)[118]. Conversely, insulin modulates long term depression (LTD) by phosphorylating AMPA receptors via PI3K-PKC pathway[119]: this regulation on hippocampal CA1 neurons is fundamental for insulin-induced LTD, essential for memory consolidation[120]. AMPA-receptor phosphorylation (at GluR2 subunit) induces its endocytosis, hence a decrease of post-synaptic excitatory ability[82]. In addition, insulin is involved in synaptic plasticity, modulating synapse number, dendritic plasticity[121] and scaffold protein PSD-95 expression, which is essential for the formation of post-synaptic terminals[122]. As far as regards the involvement of insulin in synaptogenesis, it has been demonstrated the co-expression of the protein IR-tyrosine kinase substrate p58/53, also known as IRSp53 in cultured hippocampal neurons, thus suggesting an insulin-dependent mechanism mediated by IRSp53 on neurite outgrowth and on synaptogenesis that may be affected when insulin signaling is no longer working[123,124].

Insulin has also a trophic role on neurons manifested especially during development: proliferation, differentiation, neurite growth[82]. The number of IRs increases during the development and insulin signaling modulates proliferation, neurite outgrowth, axon regeneration through IRS2[82,125,126]. IRs act also on glial cells where both number and activity of IRS can be regulated oppositely depending on cell type. Moreover, insulin and IGF2 look like are necessary to stimulate NGF-mediated neurite outgrowth and this action is controlled by astrocytes[82,127,128]. Moreover, insulin seems to act directly on PSD-95 in hippocampal area CA1 through PI3K/mTOR, therefore insulin appears to be involved in modulation of synapses as well as in regulation of dendritic spine formation and development of hippocampal excitatory synapses[122,129].

Insulin is a strong neuroprotective agent able to halt apoptosis, reduce β–amyloid toxicity, oxidative stress and ischemia[82]. The antiapoptotic effect is carried out through the PI3K/Akt/mTOR by modulation of the protein p70SK[130,131]. Remarkably, insulin also prevents the formation of β-amyloid fibrils[132]. Insulin opposes also the oxidative stress that may reduce glucose uptake as well as GABA and glutamate reuptake due to damage of neurotransmitters or glucose transporters[131,133,134]. Indeed, insulin is able to modulate both concentration and function of neurotransmitters: for example, it modulates GABA receptor expression since this receptor can be phosphorylated by Akt, hence insulin can modulate number of GABA receptor at synapses. Insulin also modulates release and uptake of several neurotransmitters either by directly blocking their transporters or indirectly by enhancing calcium concentration, crucial for neurotransmitter release.

## *ALZHEIMER'S DISEASE*

Alzheimer's Disease (AD) is the most common form of dementia characterized by progressive loss of memory and cognitive decline[135,136]. From a neuropathological point of view two major hallmarks characterize this pathology: the deposition of extracellular amyloid-β-peptide plaques (which have been detected also in blood vessels walls) and neuronal hyperphosphorylated-Tau tangles[135,137]. Amyloid-β is one of the products derived from the cleavage of the amyloid precursor protein (APP) by the enzymes β-site cleavage enzyme-(BACE)-1 and γ-secretase complex[138]. The pathology is first detected in frontal and temporal lobe, affecting primarily cerebral cortex and hippocampus, then spreads in neocortex, finally in most of the other brain regions[135]. Among the several theories proposed for the pathogenesis of AD the most studied are the amyloid hypothesis and the Tau hypothesis. According to the amyloid hypothesis the initiating event is the aggregation of Aβ-peptides into soluble oligomers that in turn can arrange in β-sheet units to form fibrils, normally known as plaques. Aβ aggregation acts as a trigger, leading to the aggregation and deposit of hyperphosphorylated Tau, although this protein can act independently of Aβ leading to other types of dementia, normally referred to as tauopathies[139]. For this reason, in last decades the Tau hypothesis has been proposed as the starting mechanism by which AD pathology begins: briefly, according to this hypothesis, hyperphosphorylated Tau detaches from microtubules and begins to aggregate in oligomers, thus spreading the pathology throughout the brain and leading to synapse loss and neuronal death, years before the appearance of tangles[140]. In any case, the most toxic species are the soluble oligomers, whose toxic action on synapses is established, thus justifying the initial synapse loss that precedes both plaque deposition and neuronal death[138,139,141].

Increasing evidence has demonstrated that many factors can have a role in dementia and cognitive decline, the major of which are related to diet and metabolism, namely diabetes and insulin resistance (extensively described in next paragraph), obesity and lacking of physical activity[136,142,143]. Another important risk factor for the development of the disease is the presence of ApoEε4 allele: people who carry this allele have a 50% enhanced risk to develop AD. ApoE is a class of proteins involved in fat metabolism, since it is a fat-binding molecule mainly produced by liver and astrocytes: its main function is to carry cholesterol in brain[144]. In addition, ApoE are involved in Aβ clearance and the clearance efficiency is diminished when the isoform Eε4 is highly

expressed, compared to ApoEε3 and ApoEε2[141]. Therefore, an impairment in lipid and cholesterol metabolism might be at the basis of the development of cognitive deficits, as confirmed by a study in which the brain lipid transporter ABCA7, when mutated in a non-functional form, highly correlates with impaired lipid transport to apolipoproteins, thus promoting cognitive deficits[141,142].

## *DIABETES AND ALZHEIMER'S DISEASE: IS THERE A COMMON PATHOLOGICAL LINK?*

Compelling evidence (supported by epidemiologic and laboratory analyses, widely reviewed by Schilling in 2016[145]) suggests a link between T2D and AD, indeed they share many pathophysiological features, such as hyperglycemia, insulin resistance, oxidative stress, accumulation of advanced glycation end-products (AGE) and increased production of pro-inflammatory cytokines[146,147]. Recent reports from Biessels et al state that the risk for dementia results the 73% higher in people with diabetes than those non-diabetic, therefore T2D increases the risk to develop cognitive deficits[99,148]. Also, in many AD patients an impairment of CNS insulin signaling has been reported, thus suggesting an involvement of altered insulin signaling in the development of the neurodegeneration[85]. Therefore, insulin resistance and T2D can be considered major risk factors for the development of AD, even though many researchers have started to consider AD as a particular form of diabetes, called Type 3 Diabetes (T3D)[83,146,149]. In fact, one of the mechanisms that might underlie the link between T2D and AD is the alteration in brain glucose metabolism[55,150] or the decreased transport of insulin in brain[151,152], hence suggesting a particular condition of brain insulin resistance, independent of metabolic abnormalities that may occur in periphery.

Perturbed cerebral glucose metabolism characterizes AD brains and may contribute to the development of the disease, given that anomalies in glucose transporters and intracellular glucose catabolism have been reported in AD patients, even if not owed to reduced glucose transport expression but rather to increased lipid peroxidation which impairs glucose uptake by neurons and glial cells[153,154].

Noteworthy, obesity is a major risk factor for insulin resistance and T2D, which are in turn pathological condition that may induce AD, therefore obesity is a risk factor for AD too. In fact, Luchsinger has proposed to consider increased adiposity, hyperinsulinemia, glucose intolerance,

and T2D as a continuum, given that they often occur simultaneously: understanding this relationship is fundamental in the study of the role of adiposity, insulin resistance, and T2D in Alzheimer's disease[155]. The role of AT and whole body adiposity in overall insulin sensitivity has been described earlier as well as it is clear how dysfunctional AT may trigger both insulin resistance and AD. Higher release of FFA from AT not only affects peripheral organs but does also impair brain function: indeed, increased levels of saturated FFA are associated with brain insulin resistance and Toll-like receptors (TLR)-2 and -4 are involved in FFA-dependent inflammatory responses[89]. Aging is a high-risk factor for both AD and T2D even if T2D can be present in obese young people[82]. Conversely, it has been reported that past the age of 70 years, every 1% rise in body mass index increases the risk of AD by 36%, likewise a link between waist circumference and hippocampal atrophy has been demonstrated, indeed AD patients (known to show atrophic hippocampus) have larger waist circumferences and high levels of FFA and triglycerides (TG)[156].

Interestingly, in obesity CSF/plasma insulin ratio is decreased and CSF of insulin resistant people shows presence of typical AD markers such as soluble APP-β and Aβ42[152]. Furthermore, neural-origin plasma extracellular vesicles isolated from both AD and T2D patients show presence of Ser- and Tyr-phosphorylated IRS1, with AD patients that showed the higher rate, followed by T2D patients and then controls subjects, thus supporting the idea of a pathological link between the two diseases[152,157].

Some studies have demonstrated reduced expression of IRs in hippocampus and hypothalamus isolated from post-mortem brain samples, as well as increased Ser-phosphorylation of IRS-1, which leads to inhibition of insulin signaling[93], and decreased activation of IR and Akt[99,158,159]. Markedly, HF-fed mice showed increased Ser-phosphorylation of IRS1 in hippocampus[58]. Moreover, a functional insulin signaling prevents the aggregation of Aβ and hyperphosphorylation of Tau. Also, insulin increases Aβ trafficking and clearance via MAPK, pathway, thus reducing gliosis and cognitive deficits[160,161]. As far as regards hippocampus, the brain region committed to memory and learning processes, some researchers have reviewed all the changes that hippocampus undergoes during insulin resistant states and high-fat fed rodent models, such as decreased neurogenesis in dentate gyrus, decrease of dendritic spines, deficit of synaptogenesis, but also LTP deficits and spatial memory impairment[58,99]. Interestingly, some studies attempted to divide

brain/hippocampal insulin resistance from the peripheral one by mean of antisense strategies in brain in order to elicit only CNS insulin resistance: in this case, the insulin resistance does not spread peripherally although the animal model shows the same cognitive impairments described for systemic insulin resistant one. These observations implicate that peripheral metabolic derangements along with insulin resistance due to T2D are prodromal to CNS deficits, which in turn develops a hippocampal insulin resistance, thus resulting in structural and functional anomalies[99,159].

Impaired insulin signaling brain, especially of the axis PI3K/Akt, results in hyperactivation of GSK3β: this enzyme, no longer inhibited by Akt-dependent phosphorylation, can induce hyperphosphorylation of Tau at several Ser/Thr residues, thus leading to its misfolding and aggregation, first in oligomers, then in neurofibrillary tangles[138]. In addition, the phosphatase PP2A, which normally dephosphorylates Tau, is implicated in both T2D and AD, due to its reduced capacity to dephosphorylate Tau, hence causing its aggregation. The implication of GSK3β in the aggregation of hyperphosphorylated Tau might be triggered by Ser-phosphporylation if IRS-1, that halts insulin signaling with consequent lack of GSK3β-inhibition, because downstream factors of IRS-1 are no longer activated[152].

Increasing evidence shows an involvement of the enzyme insulin degrading enzyme (IDE) in the relationship between AD and T2D. IDE is a dimeric enzyme of 110 kDa[162]: specifically, it is a zinc-metallopeptidase able to degrade small peptides of 3-10 kDa, such as insulin, glucagon and Aβ[82,163]. IDE levels are reduced in AD patients with ApoEε4 allele, therefore since it is involved in Aβ degradation, any defect in either expression or activity may develop AD. Insulin signaling regulates IDE expression, probably via PI3K, therefore any impairment of the insulin pathway might have an impact also on IDE expression[164]. Also, FFA and high glucose concentration downregulate IDE activity[163]. IDE is actually ubiquitous and its main function is the degradation of small peptides in order to avoid their deposition[162]. In brain, IDE is secreted from both microglia and neurons and degrade Aβ extracellularly[163]. Some studies has taken into account the possible involvement of IDE at the crossroad between T2D and AD, thus concluding that low levels of IDE – in turn induced by defective insulin signaling or FFA excess - cause Aβ accumulation[138,145,162,163]. Moreover, IDE shows higher affinity to insulin than Aβ, when insulin levels in blood are

particularly high[156], therefore any rise in insulin concentration translates into higher degradation rate from the enzyme, thus provoking Aβ aggregation[165].

One of the possible link between AD and T2D is the impairment of cholesterol metabolism, given the impact of genetic variant ApoEε4 as major risk factor for AD[166]. As previously mentioned, CNS cholesterol biosynthesis occurs in astrocytes, then it is packaged in ApoE particles, released and taken up by neurons in order to increase synaptogenesis and allow vesicle formation[85]. It is known that rodent models of T2D show a decrease in brain levels of SREBP2, crucial regulator for cholesterol synthesis; furthermore, these mice show a decrease in SREBP cleavage-activating protein (SCAP), sterole-sensing molecule that is involved in missing maturation of SREBP2. ICV insulin delivery reverses this decrease[104]. Cholesterol is crucial for synaptogenesis and vesicle formation, therefore any situation that impairs its biosynthesis negatively affects long term potentiation and synaptic transmission. The ability of insulin to retrieve this metabolic pathway suggests that diabetes might promote neurodegeneration owing to diminished cholesterol formation in CNS[85,104].

Other possible mechanisms that link T2D and AD are the amyloidogenesis (aggregation of amyloid protein, which may occur also in pancreas), oxidative stress, inflammation, the mitochondrial dysfunction, lack of acetylcholine transmission and the production of advanced glycation end-products.

As mentioned earlier, the amyloid hypothesis suggests that Aβ aggregation is the triggering events for AD onset; also in diabetes, there is presence of islet amyloid polypeptide (IAPP or amylin) aggregates, whose structure is very close to the one of Aβ plaques in AD. IAPP aggregates are able to inhibit glucose-induced insulin secretion[67,68,138].

The oxidative stress might be even considered the driving force for insulin resistance in AD[138]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), whose production can be induced by hyperglycemia[138] can provoke detrimental effect on proteins, lipids, nucleic acids that may damage crucial molecules involved in insulin signaling. Since brain is rich in fatty acids and iron, it is highly susceptible to the presence of ROS/RNS, especially during aging, thereby any imbalance between ROS/RNS production and anti-oxidation defense could be deleterious for neuronal and synaptic function[137]. Besides, both T1D and T2D are characterized by oxidative stress mediating diabetic neuropathy, as well as amyloid plaque and neurofibrillary tangles

deposition is preceded by oxidative damage, thus suggesting that occurs upstream of aggregation events[138,152]. HFD can also induce lipid peroxidation which may contribute to neuronal damage exacerbated then by insulin resistance[58].

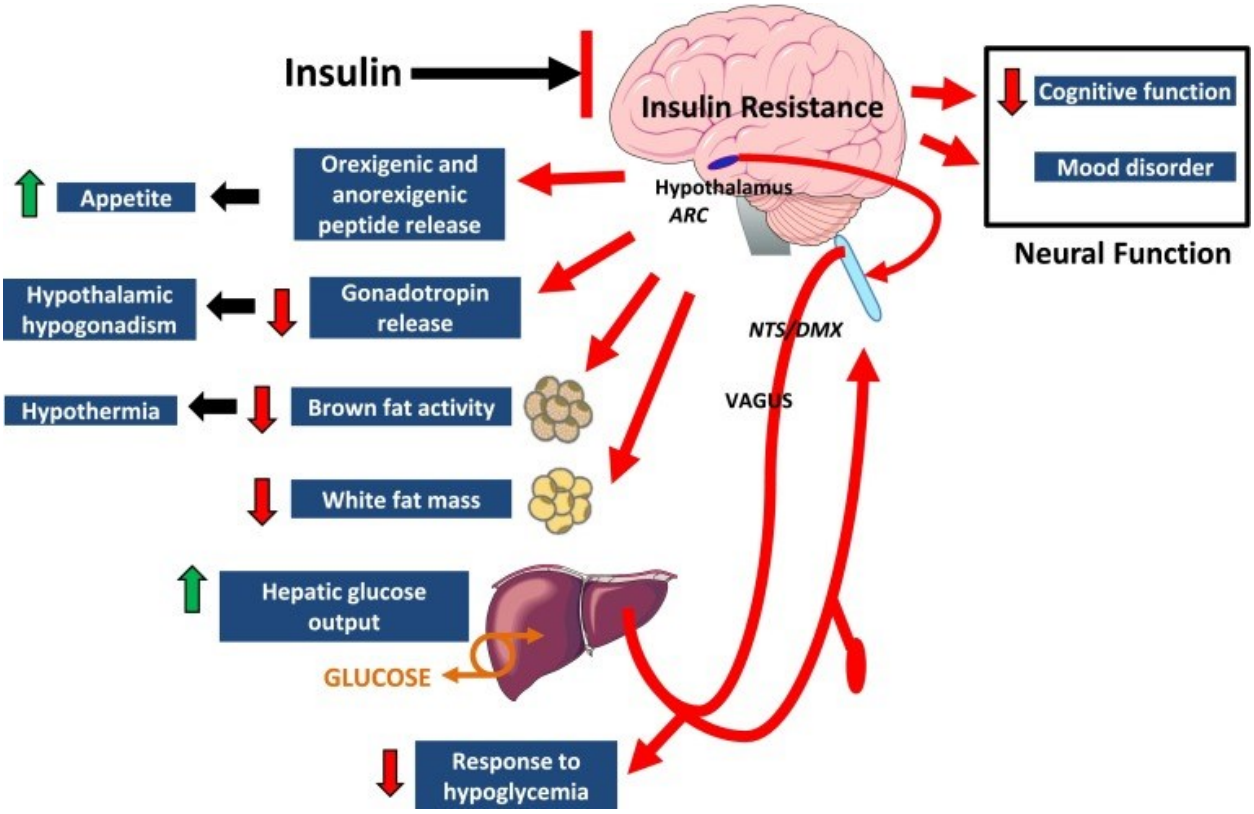


Figure 3: Effects of impaired brain insulin signaling on peripheral functions (from Kleinridders A, Ferris HA, Cai W, Kahn CR, Diabetes, 53; 2232-2243; 2014)

The mitochondrial dysfunction is correlated to the oxidative stress, since ROS damage may impact mitochondrial proteins or nucleic acids leading to disruption of metabolic pathways. This condition results unbearable for neurons, which rely on high levels of ATP produced by nutrient oxidation in mitochondria in order to obtain energy[138].

Another negative effect of oxidative stress is the formation of advanced glycation endproducts (AGE) that derive from the reaction between sugars and amino groups of proteins, lipids and nucleic acids. These AGE colocalize with both tangles and plaques, thus suggesting a possible involvement of these species in the aggregation and deposition of Aβ and Tau[138]. Along with the formation of AGE, a higher expression of their receptor, RAGE, occurs and their activation leads

to inflammation. AGE can also accumulate in vascular walls where they may trigger vascular remodeling that leads to another feature of both dementia and T2D: cerebral angiopathy[167].

Insulin resistance can induce inflammation both in periphery and CNS, as well as obesity and T2D show an important inflammatory component which can underlie higher risk to develop AD. Moreover, AD patients show high levels of IL-6 in AD, which is able to interact with Aβ (as well as TNFα), thus facilitating its deposition, whereas insulin seems to mediate antinflammatory activity[168]. High levels of pro-inflammatory cytokines alter hippocampal synaptic plasticity: obesity, more specifically increased adiposity, induces peripheral insulin resistance, manifested as increased macrophage infiltration in AT[58]. As such, AT starts to release pro-inflammatory cytokines and FFAs which may contribute to the development of T2D and to amyloid deposition because pro-inflammatory signals are delivered to the brain causing neuroinflammation[58,169–171].

In summary, since inflammation is triggered by obesity and HFD, and it drives the appearance of insulin resistance as well as the increase of pro-inflammatory cytokines has been demonstrated in AD, the neuroinflammatory component may most likely be at the crossroad between type 2 diabetes and cognitive decline.

The lack of acetylcholine is a common feature between T2D and AD; in fact, insulin resistance leads to reduced concentrations of acetylcholine as well as to increased concentration of enzyme acetylcholinesterase. Cholinergic transmission might have a role in suppression of inflammatory cytokines, therefore its reduction could pave the way to the development of neurodegeneration or insulin resistance[156,172].

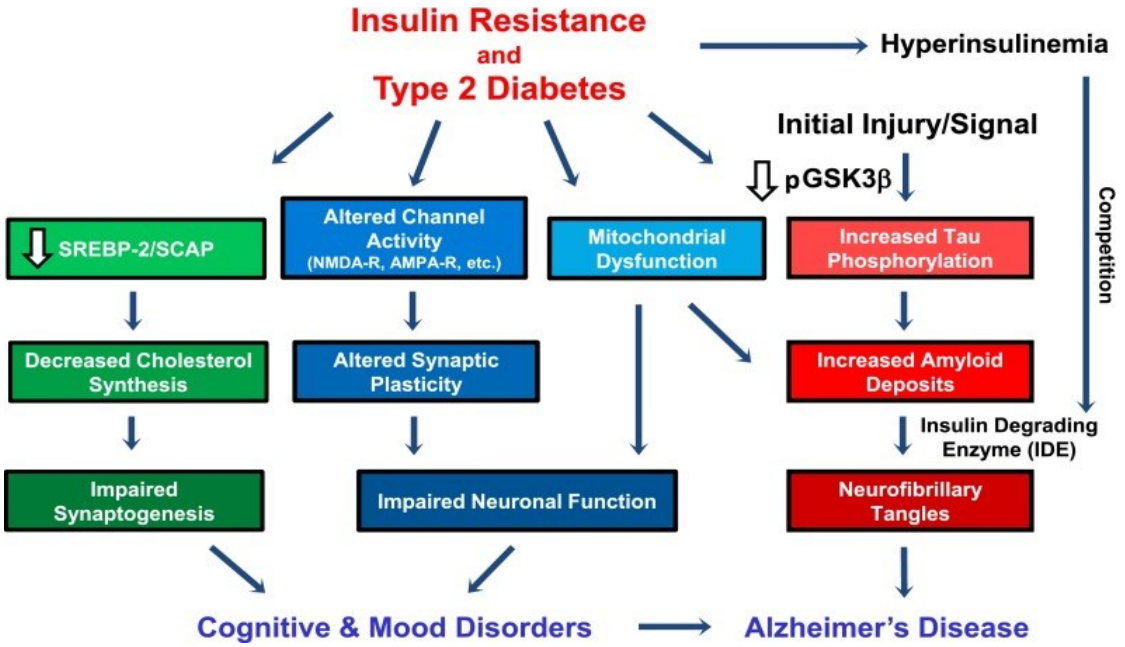


Figure 4: Brain insulin resistance along with induce a broad set of abnormalities that converge onto cognitive decline and most likely AD. (from Kleinridders A, Ferris HA, Cai W, Kahn CR, Diabetes, 53; 2232-2243; 2014)

## *ANIMAL MODELS OF T2D*

### *AtENPP1Tg mouse model*

In this project, I employed the AtENPP1Tg mouse model, which overexpresses the protein ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) in AT[173–175]. ENPP1 is an inhibitor of IR and it is known to cause insulin resistance when overexpressed: it physically interacts with α-subunit of IR and does not allow the conformational change necessary for the phosphorylation of β-subunit[33,176–178]. ENPP1 levels are elevated in insulin-resistant persons and contributes to adipocyte dysfunction and incomplete maturation (especially when overexpressed)[174], upregulation of genes involved in lipolysis, lipogenesis and inflammation, increased FFAs plasmatic levels and decreased adiponectin concentration[175,179]. One of the consequences of ENPP1 overexpression in AT is the diminished storage capacity, even when body weight is normal, as occurs in lypodistrophy and metabolic syndrome[173]. This model, when fed with HFD, summarizes features of human metabolic syndrome and insulin resistance. Indeed, it displays systemic insulin resistance, impairment of adipogenesis and enhanced adipocyte size, decreased

fat storage capacity, ectopic fat deposition (fatty liver), increased plasmatic FFAs, hyperglycemia and hyperinsulinemia. In particular, in liver there is a reduced phosphorylation of Akt, thus suggesting lessening of insulin signaling[173]. Further analyses revealed that this model shows CNS defects, specifically in the hippocampus: decreased basal synaptic transmission at Schaffer collaterals to CA1 synapses, altered membrane lipid composition, diminished phosphorylation of NMDA-R (subunit GluN1), crucial for LTP process underlying memory and learning, reduced expression of IRs, increased expression of FFA receptor and cerebral triglycerides content and decreased levels of phospholipids in brain[180]. To further support these alterations in synapses memory assays (Morris water maze) were performed on these mice and revealed that the HFD group of AtENPP1Tg mice show an exacerbated hippocampal-dependent memory impairment[181]. Such CNS deficiencies correlate with previous observations, above described, in which AT dysfunction and insulin resistance induce brain function impairments, for example increased FFA uptake and impaired glutamate transmission in the hippocampus. Therefore, this model gives a unique opportunity to understand the mechanisms underlying the link between peripheral insulin resistance, AT dysfunction and CNS deficits.

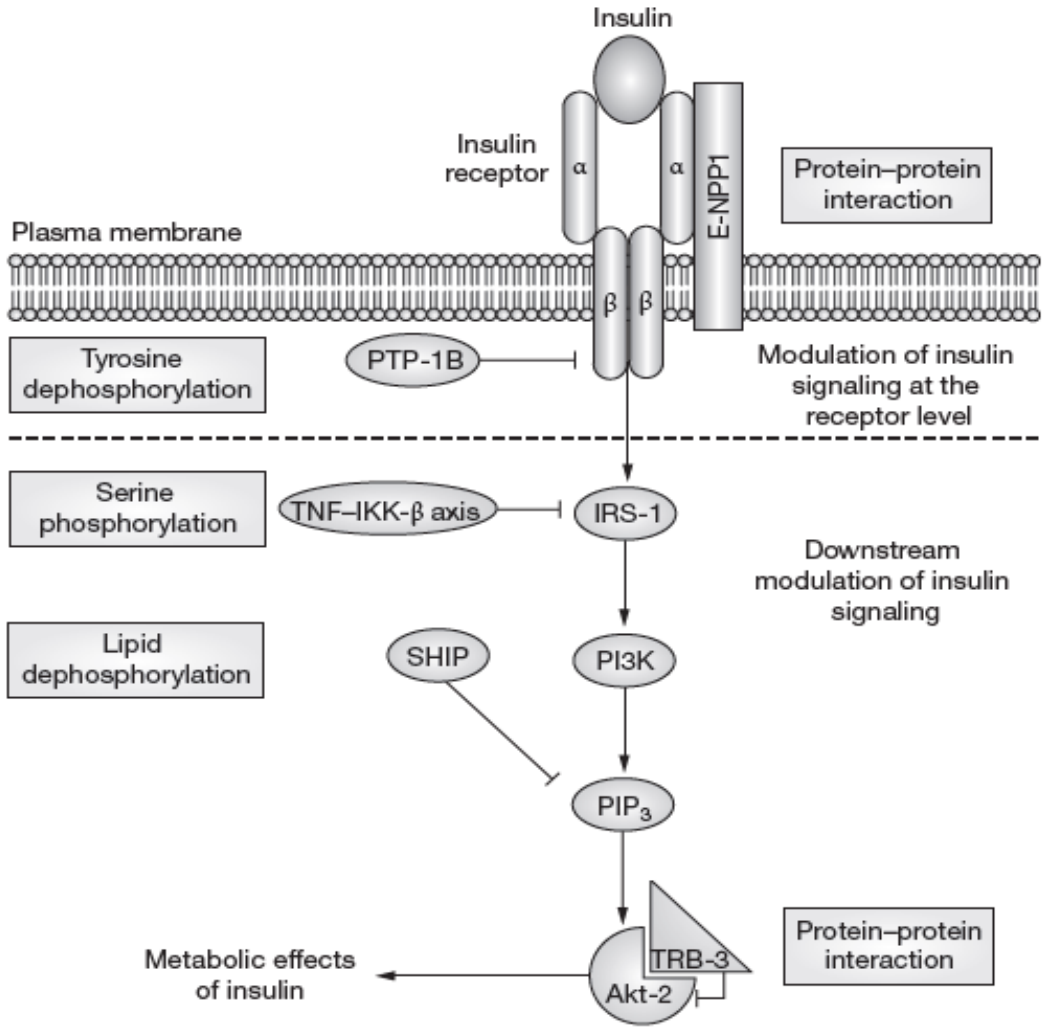


Figure 5: Schematic representation of insulin signaling inhibition. Of note, the presence of ENPP1 that inhibits IR phosphorylation by binding the receptor on extracellular α subunit. (from Abate N, Chandalia M, Di Paola R, Foster DW, Grundy SM, Trischitta V, Nat Clin Pract Endocrinol Metab. 2006 Dec;2(12):694-701.)

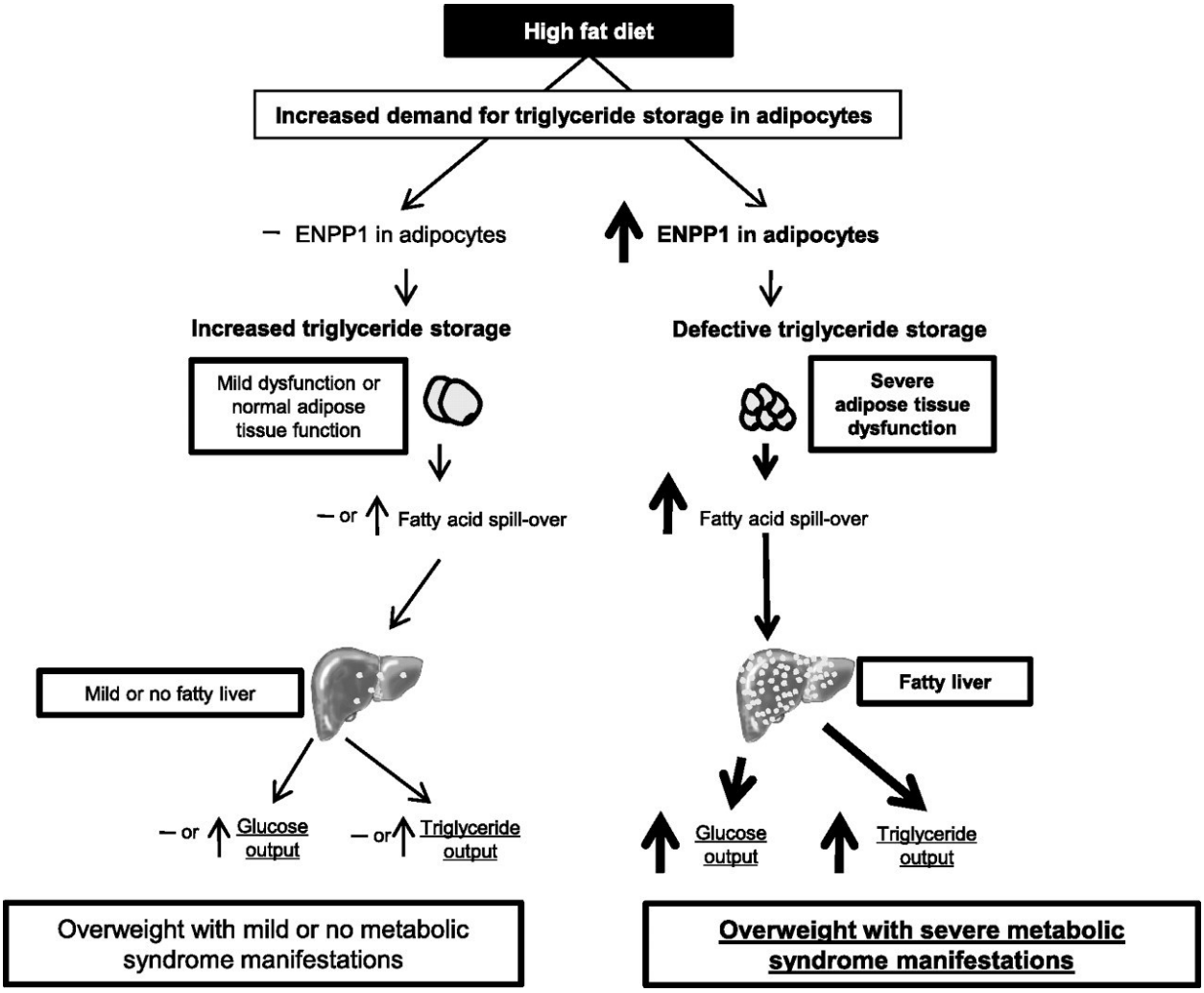


Figure 6: Schematic representation of the effects of ENPP1 on triglyceride storage in adipose tissue and its interaction with diet in the AtENPP1Tg mouse. (from Pan W, Ciociola E, Saraf M, Tumurbaatar B, Tuvdendorj D, Prasad S, Chandalia M, Abate N., Am J Physiol Endocrinol Metab. 2011 Nov;301(5):E901-11)

### *Other models of T2D and insulin resistance*

These models have been reviewed in [182], [183], [184], [185], [186]. Only recently, a growing body of studies has taken into account CNS deficits induced by either HFD or insulin resistance[187]. Below a list of the most common animal models used for T2D and insulin resistance studies.

**Table 1: List of the most commonly used rodent animal models to study T2D, obesity and insulin resistance**

| NAME | TYPE | FEATURES | NOTES |
|---|---|---|---|
| Fat-fed STZ-treated rat | Diet-induced | Insulin resistance and hyperinsulinemia after HFD. Hyperglycemia after STZ injection | Pancreas failure chemically-induced and not result of "natural" development of insulin resistance |
| $Lep^{ob/ob}$ mouse<br>$Lepr^{db/db}$ mouse<br>Zucker Diabetic Fatty (ZDF) rat | Monogenic | Mutation in leptin gene or receptor. Hyperinsulinemic, hyperglycemic, hyperphagic | Not relevant because of the rarity of these conditions in humans |
| NZO mice<br>KK mice<br>TallHo/Jng mice<br>OLETF rats | Polygenic | Severely hyperinsulinemic and sometimes hyperleptinemic and/or leptin-resistant. Pancreas progressively becomes hypertrophic and dysfunctional up to fibrotic (OLETF).<br>Increased adiposity and cholesterol, TG and FFA levels for TallHO/Jng | Although closer to human pathology they are highly sophisticated and gender-biased |
| HF-fed rodent models | Diet-induced | Obese, hyperinsulinemic, altered glucose tolerance | Sometimes HFD is combined to knock-out animals in order to exacerbate metabolic derangements |

## *THERAPEUTIC STRATEGIES FOR AD "BORROWED" FROM T2D*

The evidence that AD and T2D are somehow linked by common pathophysiological mechanisms has suggested the use of antidiabetic drugs with the aim to recover brain insulin signaling. Indeed, the few drugs (memantine – NMDAR agonist, tacrine, donepezil, galantamine and rivastigmine – cholinesterase inhibitors) approved for AD therapy can cure only symptomatic events and do not target insulin signaling, therefore tackling insulin resistance could result beneficial for both periphery and CNS[153]. Briefly, the most common proposed therapeutic approaches will be summarized in this manuscript.

Surely, one of the most attractive, non-invasive and innovative approaches is the intranasal administration of insulin which has led to promising results: indeed, a single dose already improves memory in people with AD or mild cognitive impairments, with the exception of people who carried ApoEε4 allele at low doses of insulin, and ameliorated also glucose uptake by neurons[188]. One drawback of this approach is that targeting insulin signaling in brain may lead to hyperinsulemic condition in brain that may promote insulin resistance[189]. Further studies are currently ongoing to ensure safety and effectiveness of this therapeutic approach, which, undoubtedly, seems promising.

Other drugs, normally used as insulin sensitizers are thiazolidinediones (TZD) that activate PPARγ, thus leading to increased translocation of GLUT4 to the membrane, due to preservation of insulin responsiveness[146]. Among TZDs, two drugs are commonly used, rosiglitazone and pioglitazone, although the use of rosiglitazone has been restricted due to its heavy side effect on cardiovascular function. Pioglitazone improved memory function, reduced amyloid-β concentration and reduced microglia activation and less extent of astrogliosis. Improvement of memory performances was instead reported for rosiglitazone, even if only for ApoEε4 negative. Unfortunately, the clinical trials developed for rosiglitazone failed, due to its poor capacity to cross BBB, thus failing to reestablish brain insulin signaling, even though its peripheral action still remains[82,137,153,189].

Another widely used drug for T2D is metformin, oral hypoglycemic that allows peripheral glucose uptake and block hepatic gluconeogenesis. Some preliminary studies on rodent models suggest that metformin crosses BBB thus sensitizing neurons to insulin, even though an increase of BACE1

and generation of Aβ has been reported[189], while other studies demonstrated enhanced hippocampal plasticity and increased synaptogenesis[99] as well as reduced Aβ and Tau burden but no improvement in memory and learning[137]. However, it is right to underline that each of the studies/trials with metformin were performed with different dosages and different treatment times[137].

Glucagon-like peptide (GLP)-1 agonists (exenatide and liraglutide) and dipeptidyl peptidase (DPP)-4 are another therapeutic strategy that has been recently proposed. GLP-1 is a hormone (incretin), secreted by intestinal epithelial L-cells in response to food intake, which lowers blood glucose levels, enhances insulin secretion, reduces glucagon release and activate several factors downstream of IR. GLP-1 is cleaved by DPP-4 but is expressed also in neurons where acts as a neurotransmitter[137]. Preclinical studies in vitro and mouse models of AD have shown promising results since these drugs promote synaptogenesis and neurogenesis and protect against oxidative stress, Most importantly, Aβ plaques load and activation of microglia were significantly reduced[82,189].

Finally, drugs that act on factors of insulin signaling (on phosphatases that dephosphprylate IR and IRS1, whose levels are increased in insulin resistant states and AD) are currently under investigation[189]. Interestingly, Takamatsu et al have proposed a combined immunotherapy with antidiabetic drugs in order to combine immunization against aggregates and reestablishment of insulin signaling: this approach might be more beneficial than the single therapies but is still under investigation[190].

However, some of these drugs showed harmful side effects while others failed because of rapid degradation, poor BBB penetrance and inefficient activity in vivo[137], suggesting to try different approaches, such as stem cell therapy with the aim to reduce Aβ burden in brain and recover insulin signaling. Though, the insulin unresponsiveness seems to start in periphery, then spreads out in all the organism, including brain, hence an approach aimed to correct peripheral deficits may benefit and even rescue CNS from developing memory deficits and neurodegeneration. For this reason, in this manuscript, I propose an approach that targets peripheral metabolic activity in order to halt in advance the progression of insulin resistance throughout the body.

## *MESENCHYMAL STEM CELLS*

Stem cells are able to differentiate in mature cell types, and this feature has made them subject of several studies, especially in the field of regenerative medicine.

Stem cells are able of long-term self-renewal, have an undifferentiated state, can generate various cellular types derived from all three germ layers and undergo asymmetric division: a stem cell replicates itself in one daughter cell that is still of a stem cell, while the other can differentiate into well-defined cellular type. Stem cells reside in "niches" in which they can maintain tissue homeostasis and eventually undergo differentiation, depending on signals derived from the surrounding microenvironment[191].

Stem cells are distinguished, generally, in *embryonic* and *somatic* (or *adult*). The main source of adult stem cells is bone marrow, from which *Bone Marrow-derived Mesenchymal Stem Cells, BM-MSCs* may be obtained. However bone marrow does not contain great amounts of MSCs, therefore alternative sources have been explored recently, such as extra-embryonic or perinatal tissues (placenta, amnion, umbilical cord)[192]. *Wharton's jelly (WJ)*, the umbilical cord mature mucous connective tissue[193] that protects umbilical vessels nourishing fetus[194] has raised as a reliable source of stem cells. Wharton's jelly contains two main cell types: fibroblast-like cells and myofibroblasts[195]: the latter are now referred to as *Wharton's Jelly derived Mesenchymal Stem Cells, WJ-MSC*[196].

WJ-MSC are able of self-renewal, grow easily in vitro and for long periods, may be easily cryopreserved and differentiated[197]. In order to be considered MSC, they must adhere to the standards defined by the International Society for Cell Therapy and later reviewed and readdressed by Dominici et al[198]. These criteria are:

- Adherence to plastic
- Expression of specific markers
- Multipotent differentiation potential, also known as trilineage differentiation potential: MSCs must be able to differentiate in adipocytes, chondrocytes, osteocytes, even though it is very well established that they can differentiate in many other cell types.

There is another criteria that is not included in Dominici's suggestions but has been proposed by Bianco et al, namely the ability to form cell clones (clonogenicity), namely the ability of a single cell to form colonies[199–202].

WJ-MSCs do adhere to plastic surfaces as well as other MSC. Furthermore, WJ-MSC express CD73, CD90 and CD105 as well as CD10, CD13, CD29, CD44, CD49e, CD117 (stem cell factor), CD166, nestin and HLA-A. Conversely, they do not express CD34, CD45 and HLA-DR as well as CD31 and von Willenbrand factor, typical markers of hematopoietic lineage[203]. As far as regards the multipotent differentiation potential and regardless the well-known ability of WJ-MSC to differentiate in several cell types, La Rocca et al observed that these cells are able to maintain the expression of immunomodulatory molecules even if they are committed to differentiate in a specific cell type. Likewise, the same group demonstrated that these cells are able to form colonies in a clonogenic assay[204,205].

It is worthwhile to highlight that the definition of mesenchymal stem cells has been under debate for a long period, since many researchers include under this classification also the stromal cells while others prefer to keep the latter separated from the mesenchymal stem cells. In this manuscript, the definition of MSCs will fall under the criteria described by Dominic et al and defined by the International Society for Cell Therapy, as earlier explained[198,205,206].

Interestingly, these cells do express neural markers like glial fibrillary acid protein (GFAP) and neuron specific enolase (NSE)[207] and markers of endodermal and mesodermal lineage, such as Gata-4, -5, -6, HNF4-α, vimentin, α-smooth muscle actin (α-SMA). In fact, these cells are able to differentiate in several cell types[208], for instance myocardyocytes[209], skeletal muscle cells[193,210], endothelial cells[211], insulin-producing cells[212–214], hepatocytes[215] and neuronal/glial cells[196,215–218].

MSC are easily obtained from bone marrow, AT and birth-related tissues (placenta, amniotic fluid, umbilical cord)[219,220]. The latter are considered an excellent source for isolating MSCs compared to others for two main reasons: the absence of any ethical issues for their isolation procedure and the prompt availability compared to adult sources; moreover, some groups reported other advantages, such as a higher proliferation rate, better differentiation ability and longer lifespan of birth-related MSCs than MSC derived from other sources[221].

It is well known that MSC do secrete a large number of secretion factors (interleukins, leukemia inhibitory factor (LIF), growth factors, tissue inhibitor of metalloproteinase (TIMP)-2), which can act in paracrine and autocrine fashion, therefore ensuring their hypoimmunogenicity, the evasion from host immune response (referred to as immunoprivilege[220]), the remodelling of the target organ microenvironment[220,222]. The immunomodulatory properties translates into the ability to

modulate T cell response thereby both contact-dependent and contact-independent action mediated by T cell is suppressed by MSC through the release of several molecules (for instance members of B7-H family[206], IDO, LIF, galectin-1) whereas the stimulation of regulatory T cells takes place[223]; other immunomodulatory mechanism range from the modulation of antigen-presenting cells to the modulation of B cells as well as the activity of NK cells: all these activities depends on inhibition of pro-inflammatory cytokines, such as TNFα, IL-10, IFNγ[223].

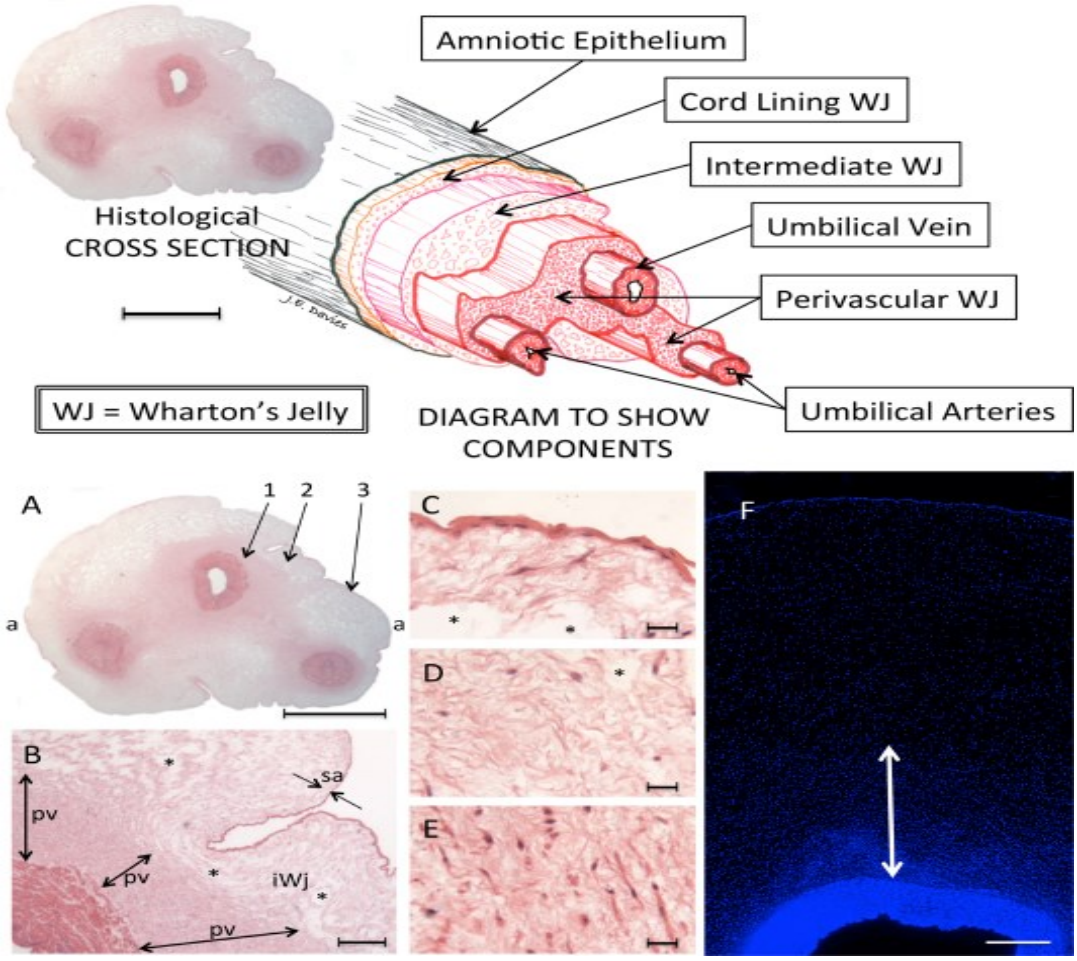


Figure 7: Three-dimensional structure of umbilical cord and paraffin embedded section of the umbilical cord. WJ = Wharton's jelly (from Davies JE, Walker JT, Keating A, Stem Cells Transl Med. 2017 Jul;6(7):1620-1630.)

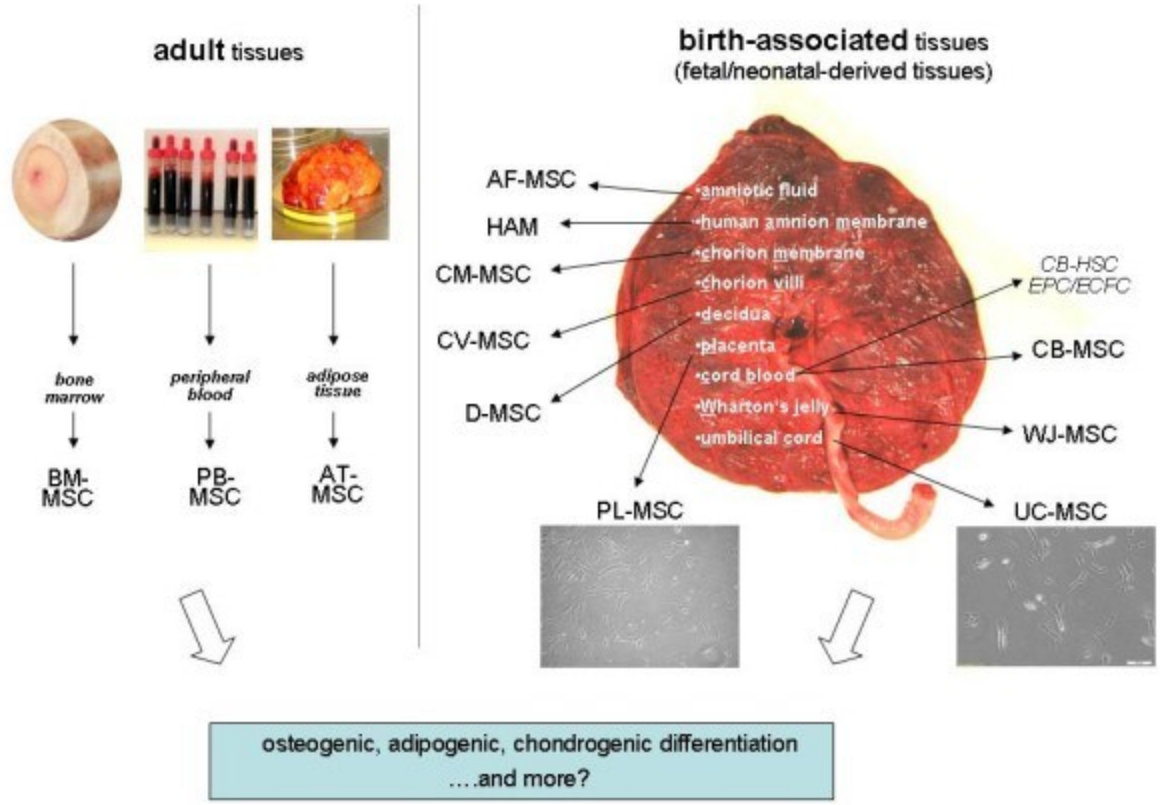


Figure 8: Major sources of mesenchymal stem cells. To the left cells from adult tissues, to the right birth-related MSC. (from Hass R, Kasper C, Böhm S, Jacobs R, Cell Commun Signal. 2011 May 14;9:12.)

These immunomodulatory characteristics allowed researchers to develop clinical trials for the therapy of T1D[223–225] and for the treatment of diabetes-related pathologies[226]. Several recent works have displayed the utility of MSC in improvement of insulin resistance and hyperglycemia in both animal models of T2D and human with T2D. Zang et al reviewed some of these works suggesting four main mechanisms of action by MSC: differentiation into insulin-producing cells (since these cells express critical transcription factors related to β-cells[227]), promotion of pancreatic β-cell regeneration since they are able to migrate in injured tissues[223], overall amelioration of insulin resistance, as seen in a rat model of T2D, in which has been demonstrated the activation of IRS-1 and the increased translocation of GLUT4 after MSC tranplantation[228]. In any case, these mechanisms are believed to work through the release of specific factors that may induce either differentiation of beta-cells or their protection or M2 macrophage phenotype (antinflammatory rather than M1 pro-inflammatory). The exploitation of released factors has also led researchers to test conditioned medium from cultured MSC in vitro with satisfactory outcomes[229]. In addition, MSC-derived exosomes have been tested in diabetes-related injuries such as nephropathies, retinopathies, cognitive impairments[230–233].

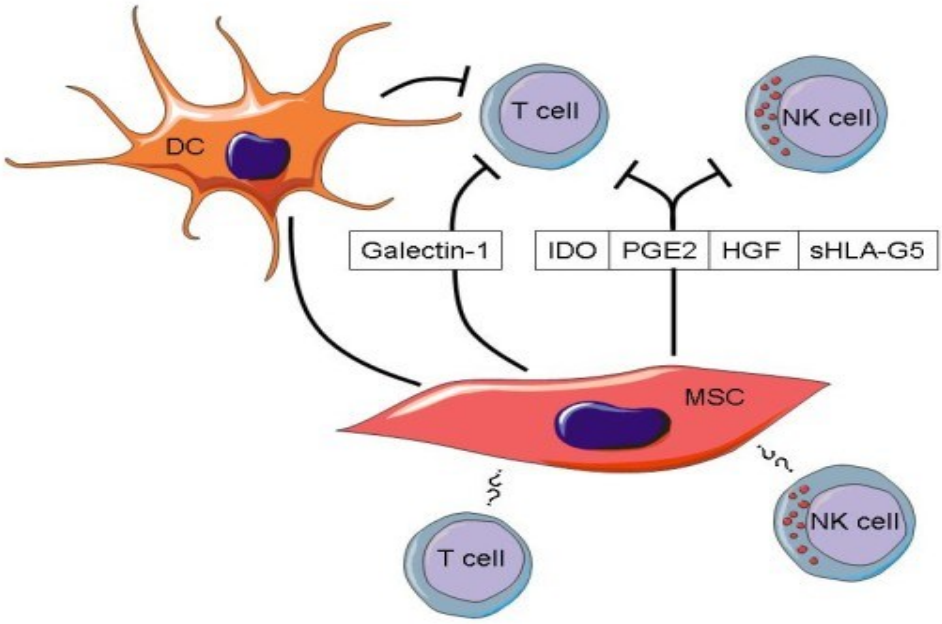

Figure 9: Mechanisms of immunomodulation by MSC. (from Hass R, Kasper C, Böhm S, Jacobs R, Cell Commun Signal. 2011 May 14;9:12.)

As far as regards specific studies on T2D models, a growing body of studies has emerged recently, proving that the technical and biological advantages beard by these cells are attractive features for the attempt to find novel, safer, tailored and more targeted therapies against pathologies that are continuously and sadly growing worldwide. These studies are listed in Table 2:

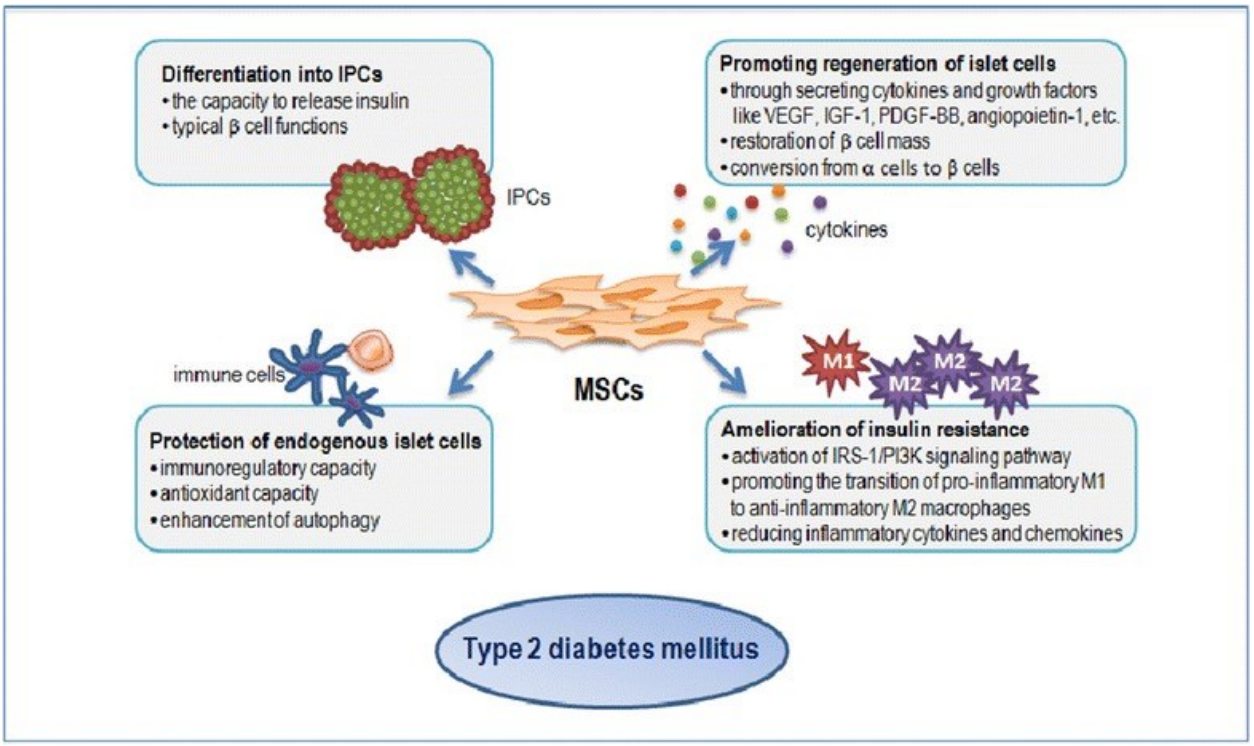


Figure 10: Schematic of the proposed mechanisms by which MSC ameliorate T2D and insulin resistance states. (from Zang L, Hao H, Liu J, Li Y, Han W, Mu Y., Diabetol Metab Syndr, 2017 May 15;9:36.)

| **Table 2*:* Summary of studies in which MSC have been used for T2D treatment. This table was made searching on Pubmed the terms "mesenchymal stem cells" "type 2 diabetes" "insulin resistance" without any limitation in dates or type of work.** | | | | | |
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| **TYPE OF MSC** | **MODEL** | **ROUTE OF INJECTION** | **OUTCOMES AFTER MSC TREATMENT** | **REF.** | **PROPOSED MECHANISM** |
| PD-MSC injected 3 times with one month interval between injections | Human T2D patiens | Intravenous (IV) | After treatment reduced insulin dosage, increased endogen insulin and C-peptide | Jiang et al.[234] | MSC differentiate in β-cells. Proposed a role of growth factors IGF, VEGF and HGF for differentiation. |
| BM-MSC injected twice | Fat-fed STZ-induced rats transplanted either 7 or 21 days after STZ | IV (tail vein) | Partial reconstruction of islet function; hyperglycemia ameliorated up to 4 weeks; improved insulin sensitivity. Higher expression of GLUT 4 in AT, muscle and liver but enhanced translocation only in AT and muscle.<br>Higher phosphorylation of IRS-1 and Akt, thus | Si et al[228] | Insulin resistance ameliorated in both early (7 days) and late (21 days) phase. β-cell function recovered only in early phase. Perhaps MSC restored GLUT 4 concentration via an insulin-independent pathway, since insulin levels were not increased. Restored insulin signaling might be due to a compensatory mechanism induced by increased GLUT4 |

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| | | | suggesting improvement of insulin sensitivity. | | levels. However, the improvement of glucose tolerance lasted only 4 weeks. |
| BM-MSC, single injection | Human T2D patients | In arteriae pancreatica dorsalis | Improvement of fasting glucose (reduced), insulin and c-peptide levels | Hu et al[235] | 3 years of follow-up and patients kept under strict medical control (diet, medications…). Proposed mechanism towards β-cells differentiation, through the release of growth factor by MSC |
| BM-MSC injected either once or multiple times | Fat-fed STZ-induced rats | IV | Ameliorated glycemia with multiple injection; restoration of pancreatic islets; decreased insulin levels | Hao et al[236] | Previous studies from the same group demonstrated that a single injection was able to lower glycemia to normal levels only for 4 weeks, therefore a multiple injection protocol was tried and was indeed successful. In a subset of rats was injected also BM-MSC conditioned medium with similar outcomes: this suggests that not only restoration of pancreatic function has a role in MSC-induced improved glycemia, but a plethora of groth factors and cytokines able to modulate insulin signaling and glucose |

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| | | | | | uptake in other tissues that most likely communicate to each other. |
| WJ-MSC injected twice in different sites | Human T2D patients | 1. Peripheral vein<br>2. Via splenic artery directed to pancreas | Increased levels of fasting c-peptide, decreased fasting glucose levels and 2h post prandial glucose as well as reduced needs of insulin treatment and oral hypoglycemic drugs 12 months after transplantation.<br>Reduced levels of pro-inflammatory cytokines and of $CD3^+$ and $CD4^+$ T lymphocytes | Liu et al[237] | Proposed WJ-MSC differentiation in β-cells supported by enhanced basal insulin secretion. Regulation of anti-inflammatory response by immune system restored by WJ-MSC which in turn can positively affect insulin signaling. First attempt aimed to combine different routes of injection in order to allow a better homing and distribution of stem cells only in affected organs: therefore,sassssQ the peripheral delivery would help recovery of peripheral insulin-sensitive organs, while the intrapancreatic would rescue pancreas from failure. |
| WJ-MSC | HFD-fed STZ-induced rats | IV | Ameliorated glucose levels in fasting conditions. Histological analyses on pancreas revealed | Hu et al[238] | The researchers believe that the differentiation of MSC into pancreatic cells is not the only one possible to explain why glycemia recovered as well as insulin-related parameters (GHb and C-peptide). It |

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| | | | recovered morphology after SC treatment | | is worthwhile to note that another group of rats treated with a combination of MSC and antidiabetic drug (sitagliptin) ended up to same outcomes. |
| UC-MSC single injection | Human type 2 patients | IV through cubital vein | Half of the patients did not need anymore insulin treatment; ameliorated glycemia only for insulin-independent group | Guan et al[239] | There was a small number of patients, half of which even suspended the insulin administration after MSC administration. All the mechanisms proposed so far have taken into account: differentiation in pancreatic β-cells and suppression of T-cell mediated immune response but the overall study is biased by the small number of the sample. It is not clear if MSC come from a specific zone of the umbilical cord. |
| AT-MSC single injection | HFD-induced obese mice | IV | Reduced blood glucose level and improved glucose disposal. Reduced serum TG and increased levels of HDL. Increased expression of IR and PPARγ. Reduced expression of TNFα, IL-6 | Cao et al[240] | The improvement of glucose and insulin sensitivity is due to suppressed inflammation in insulin-sensitive organs. MSC are thought to differentiate in β-cells thus allowing recovery of pancreas and |

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| | | | and F4/80. Reduced adipocyte size and preserved structure of β-cells. | | cytoprotection, which in turn leads to a better glucose uptake. |
| UC-MSC single injection | STZ-induced rats | IV | Reduced blood glucose levels, increased pancreatic islets area, enhanced insulin levels, increased levels of IGF-1, HGF, PDGFA. Activation of PI3K pathway, confirmed by assessment of Akt phosphorylation | Zhou et al[241] | WJ-MSC located in pancreatic islet led to an expansion of islet area afer transplantation. The duration of the follow-up was short (42 days), therefore better outcomes might have been obtained with longer observations. By the way, the increased levels of such growth factors might give an explanation about the ameliorated glycemia and the recovered pancreas functionality. It is not clear from which part of the umbilical cord the MSC were isolated. |
| AT-MSC single injection | HFD-fed STZ-induced rats | IV | Reduced blood glucose levels. In liver they found increased activation of PI3K/Akt pathway, as well | Xie et al[242] | Reduction of hyperglycemia might be due to an improved hepatic function, with consequent reduction of gluconeogenesis through activation of AMPK pathway and reduced expression of genes involved in |

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| | | | as higher activation of AMPK. | | gluconeogenesis and glycolysis. The results were produced 12 and 24 hours after transplantation, hence perhaps longer observation time could have led to improved outcomes. |
| WJ-MSC twice injected with a 4-week interval | Human T2D patients | IV (cubital vein) | Improved function of β-cells (increased C-peptide); ameliorated glycemia, since it is reduced in fasting plasma glucose assessment and postprandial glucose levels; reduced need of insulin therapy and/or hypoglycemic drugs | Hu et al[243] | 36 months of follow-up showed overall better glycemia control that even allowed termination of oral hypoglycemic drugs and insulin therapy in some patients. No T2D-related complication was shown in all follow-up period thus suggesting that WJ-MSC-mediated improvement was effective. |
| BM-MSC injected twice with 12-weeks interval | Human T2D patients | 1. Superior pancreaticoduodenal artery<br>2. Antecubital vein | Increased C-peptide, reduced dose of insulin therapy required. | Bhansali et al[244] | Proposed three mechanisms by which MSC improve β-cell function: secretion of growth factors that would allow angiogenesis and differentiation of MSC; transdifferentiation in β-cells and |

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| | | | | | upregulation of specific pancreatic genes in order to promote differentiation. |

Therefore, it is conceivable that an injection of "self"-produced MSC could improve glycemia and insulin sensitivity, but several studies have shown that MSC number and activity is decreased in diabetic people. For example, BM-MSC isolated from diabetic rats showed an altered profile of secreted molecules, shifted towards higher presence of proangiogenic factors compared to antiangiogenic, thus suggesting that T2D and insulin resistance induce disbalance of pro-/anti angiogenic factor, which is at the basis of diabetes-induced nephropathies and retinopathies. Conversely, genes involved in glucose metabolism were decreased compared to lean control[245]. Another in vitro study, performed with AT-MSC derived from either normal or diabetic people, demonstrated that diabetes alters MSC ability to differentiate in adipocytes, showing that a wide set of genes related to adipogenesis, adipocyte function and insulin signaling were dramatically downregulated in diabetic-derived AT-MSC[246]. Contrariwise, MSC co-culture with adipocytes isolated from a murine model of T2D demonstrated higher expression of GLUT4 and of the pathway PI3K/Akt, thus suggesting an improved insulin signaling, as well as reduced FFA secretion. IGF-1 secretion was detected, thus suggesting that this growth factor (also able to bind IR) may have a role in improving insulin sensitivity, due to its activity, similar to that of insulin. Further support of the improvement of insulin signaling comes from the observation that IRS-1 levels were increased in damaged adipocytes. However, no investigation on phosphorylation levels of these proteins was performed[247]. An interest study in which WJ-MSC transplanted in T2D-diabetic rats elicited macrophages to an anti-inflammatory phenotype (M2) demonstrated also higher expression of arginase-1 (Arg1) in AT, along with decreased expression of pro-inflammatory cytokines, suggesting that the improved insulin sensitivity after MSC injection might be due to an anti-inflammatory activity[248]. Another study evaluated the adipogenic and proangiogenc activity of AT-MSC from diabetic animal models: for the former, it has been demonstrated the increased ability form diabetic-derived stem cells to differentiate in adipocytes, although this translates into depletion of stem cells reservoir and failure of tissue turnover. The proangiogenic activity was blunted in diabetic rats compared to normal ones. Both activities might be regulated by Wnt/β-catenin pathway, which on one hand maintains pre-adipocytes in undifferentiated state, whereas on the other hand regulates angiogenesis and wound healing by promoting recruitment of stem cells in the site of injury. The inhibition of this pathway results in higher adipogenesis and inhibited angiogenesis, as well as reduced capacity of wound healing,

therefore the adipocyte turnover is impaired and the healing of wounds is delayed[249,250]. Similar results were obtained after a transplantation of diabetic mice-derived MSC to wild-type mice with hind-limb ischemia: the MSC failed to regenerate muscle tissue. MSC even underwent adipogenic differentiation with consequent adipocyte infiltration in injured muscle. The mechanism proposed for the impairment of reparative capacity by MSC was the activation of Nox, namely the activation of oxidant stress pathways. Moreover, MSC from diabetic individuals seem to lose their ability of multilineage differentiation[251].

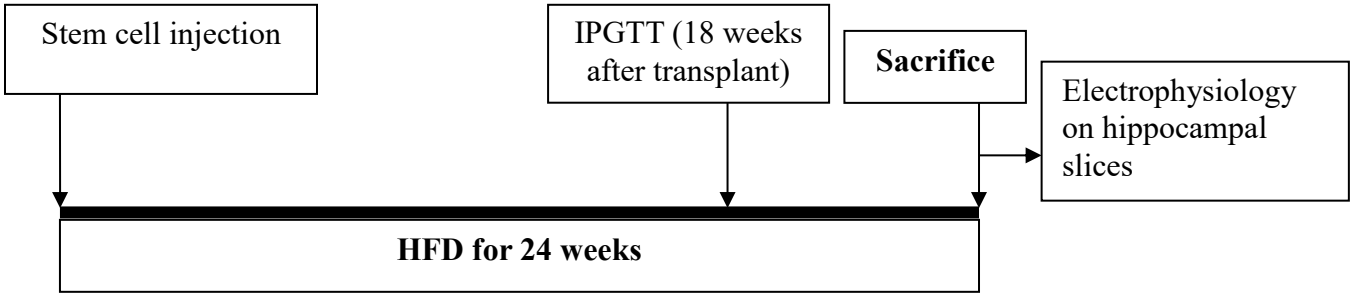
Therefore, in order to perform MSC treatments it is crucial to obtain stem cells from reliable and functional source, since the diseased microenvironment can affect the full functionality of stem cells, thus compromising the transplantation success.

## AIMS OF THE PROJECT

**Aim 1:** To determine if WJ-MSCs transplanted contextually to the beginning of the HFD correct peripheral and CNS deficits in high fat-fed AtENPP1-Tg mice (referred to as **contextual approach**).

**Aim 2:** To determine if WJ-MSCs transplanted after the beginning of HFD rescue peripheral and central deficits in AtENPP1-Tg mice (referred to as **delayed approach**).

**Contextual approach: stem cell transplantation simultaneously to beginning of high-fat diet**




**Delayed approach: stem cell transplantation after beginning of high-fat feeding**

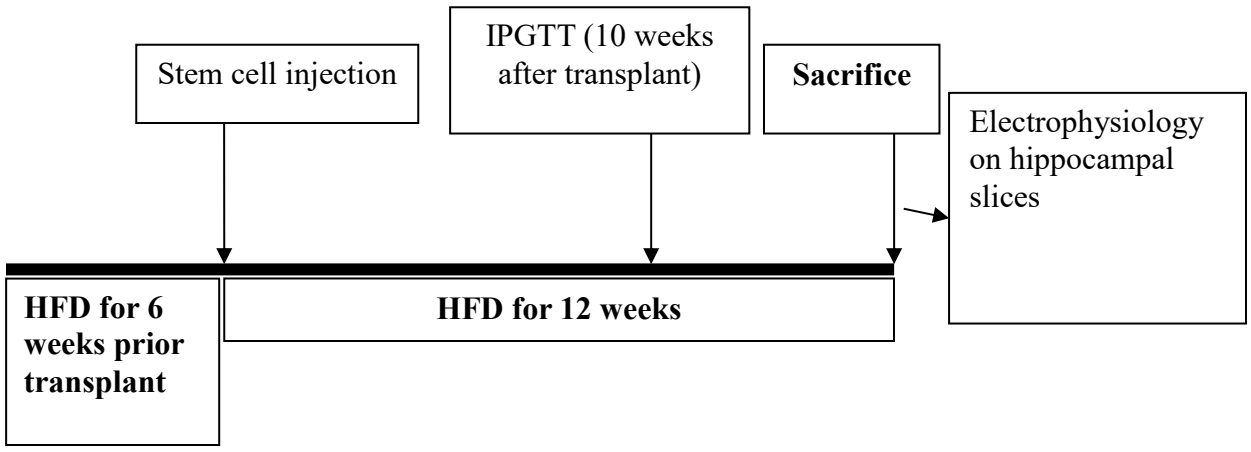



Figure 11: Experimental design:

Two different approaches have been used in this study.

3 animals/group in the contextual approach

4 animals/group in the delayed approach

# MATERIAL AND METHODS

**WJ-MSCs cell culture and characterization.**

In order to handle human samples, an authorization was obtained from the IRB (Institutional Review of Board) of UTMB (University of Texas Medical Branch), thus allowing the harvest of umbilical cords from the Department of Obstetrics and Gynecology of UTMB. The following criteria of inclusion/exclusion were followed: all mothers, regardless of age and ethnicity, giving full-term birth and $HIV^-$, $HBV^-$ and $HCV^-$ were included in the study, after giving their consent. The isolation procedure of WJ-MSC has been described in La Rocca et al[205]. Briefly, after the delivery the cord is cut from the newborn and kept in sterile conditions until dissection, following which MSC were isolated and grown in cell culture. This protocol is based on the natural migratory ability of WJ cells and, therefore, the use of potentially harmful enzymatic activities is not necessary. Each cord piece is cut longitudinally in order to expose Wharton's jelly under the amniotic membrane of the umbilical cord, and then each dissected piece of umbilical cord is moved in a 6/well plate, flipping it with the matrix facing the bottom of the well.

Each well is completely covered with culture medium supplemented with antibiotics, non-essential aminoacids and serum (Dulbecco Modified Eagle's Medium (DMEM, Gibco), supplemented with 1X Antibiotic/Antimicotic (A/A, Sigma-Aldrich), 1X Non-Essential Amino Acids (NEAA, Sigma-Aldrich) and 10% Fetal Bovine Serum (FBS, Gibco)). The cord pieces are left in culture for 15 days, changing the medium every second day: the slow degradation of the matrix allows growth factors and signaling molecules to exit from the cord with a continuous positive stimulation of the cultured cells. After 15 days of culture, the remnants of the cord fragments are removed from the wells and the cells attached to the wells are routinely cultured until confluence. When the cells reached the confluence, were detached with 1X TrypLE Select (Invitrogen), and replated in a new flask.

The characterization of the presence of MSC-markers was performed by mean of flow cytometry: the analysis aimed to detect the presence of the typical markers of mesenchymal stem cells lineage, CD29-44-90, and the absence of CD45-34.

For flow cytometry analyses, 1.5 million cells were pelleted and centrifugated twice in cold PBS. Then, 8 aliquots - 50 µl each – were incubated for 30 minutes in the dark with anti-human CD29-PE, CD90-FITC, CD44-PE, CD45-PE, CD34-PE, IgG-PE, IgG-FITC, PBS (w/o Ca and Mg). All

antibodies and immunoglobulines control were purchased from eBioscience/ThermoScientific®, while PBS is from Corning®. After few steps of washings and slow centrifugations, the cells were resuspended in paraformaldehyde and run in BD Aria Cell Sorter where side scattering, forward scattering and PE or FITC reactivity were measured.

**Animals and WJ-MSCs injection**

Three month old male AtENPP1-Tg (n=6) were included in the first study. Six month old male AtENPP1-Tg (n=8) were included in the second study. Animals were randomized to receive either 3x10^6 WJ-MSCs in PBS or PBS alone subcutaneously (SC). Mice underwent HFD (60% of calories from fat) for 24 weeks in the first study, whereas in the second study the mice were subjected to HFD for 6 weeks before receiving the stem cells, then the mice were kept in same conditions for 12 more weeks. For the transplantation, WJ-MSCs were detached with TrypLE as described above, then counted, resuspended in 1x PBS, and injected SC in the inguinal zone of each mice. Each mice was anesthetized with 5% isoflurane prior receiving the injection.

**Intraperitoneal glucose tolerance test (IPGTT)**

18 weeks after transplantation for the contextual approach and 10 following transplantation for the delayed approach, the mice were fasted for 5 hours and then challenged with an intraperitoneal (IP) injection of glucose (dose: animal weight*1ml/100 g glucose). Blood glucose level was assessed at 0, 15, 30, 60, 90, 120 minutes after glucose injection, as described in Pan et al[173]. Then, the values were analyzed with GraphPad 7.0 as well as the correspondent areas under the curve (AUC) from each group of animals.

**Electrophysiology analyses**

Electrophysiologic analyses were performed by Dr. Balaji Krishnan, PhD. Following IACUC-approved protocols, the mice were anesthetized with isoflurane, sacrificed by decapitation, and had their brains removed from the skull. 350 μm hippocampal slices in 2% agarose were prepared in NMDG-HEPES recovery solution (93mM N-Methyl D-Glucamine (NMDG), 93mM HCl, 2.5mM KCl, 1.2mM $NaH_2PO_4$, 30mM $NaHCO_3$, 20mM HEPES, 25mM Glucose, 5mM sodium ascorbate, 2mM Thiourea, 3mM sodium pyruvate, 20mM $MgSO_4.7H_2O$, 0.5mM $CaCl_2.2H_2O$:

300-310 mOsm pH=7.3-7.4) using a VF-300 Compresstome (Greenville, NC). After a < 12 min incubation in NMDG-ACSF at 33 °C, the hippocampal slices were incubated at room temperature under bubbled standard recording artificial cerebrospinal fluid (aCSF) (124mM NaCl, 2.5mM KCl, 1.2mM $NaH_2PO_4$, 24mM $NaHCO_3$, 5mM HEPES, 12.5mM Glucose, 2mM $MgSO_4.7H_2O$, 2mM $CaCl_2.2H_2O$, 300-310 mOsM, pH=7.3-7.4) before recording. Electrophysiological recording from a Multiclamp 700B (Molecular Devices, Sunnyvale, CA) was performed in a dual slice-recording chamber with a continuous 3 mL/min, room temperature (25 °C) bubbled aCSF. For each experiment, there were two slices (control and experimental) measured simultaneously. The stimulating electrode (Platinum/Iridium co-axial electrode) from FHC Electrodes (Framingham, MA) were placed in the CA3 region and glass-recording microelectrode filled with aCSF (3-4 MΩ) in the CA1 region to record CA3 to CA1 field excitatory postsynaptic potential (fEPSP). Stimulating pulses were provided by AMPI Master-9 (Israel) using Clampex 8.2 software (Molecular Devices, Sunnyvale, CA). fEPSPs were stimulated at 0.05 Hz at 300-1000 μA for 0.250 msec and baseline measurements were performed at 300 μA. Induction of LTP was accomplished by 3 high-frequency bursts (HFS) at 100 Hz with 2000 msec between each burst. The slope of each fEPSP was used to compare effects on LTP compared to baseline slope for determining % increase.

**Statistical analyses**

Paired t-tests statistical analyses were performed with GraphPad 7.0 with Mann-Whitney posthoc analysis in order to evaluate differences between groups for each experiment.

# RESULTS

**WJ-MSC characterization.**

As expected, the "stemness" markers CD29, CD90 and CD44 were present in the samples containing WJ-MSC, while CD45 and CD34 were absent. CD29, CD90 and CD44 are typical markers of MSC while CD45 and CD34 characterize mainly hematopoietic stem cells. The presence of the typical MSC markers confirms the high quality of the isolation technique employed in this study. Indeed, it is known that these cells do not express von Willebrand factor and other typical markers of hematopietic lineage. Moreover, these cells are known to express HLA-A and HLA-E, thus conferring their immunomodulatory properties and to secrete antiniflammatory cytokines (TGF-β, IL4 and IL10), that provide antinflammatory activity to MSC[198,204,205,252–254].

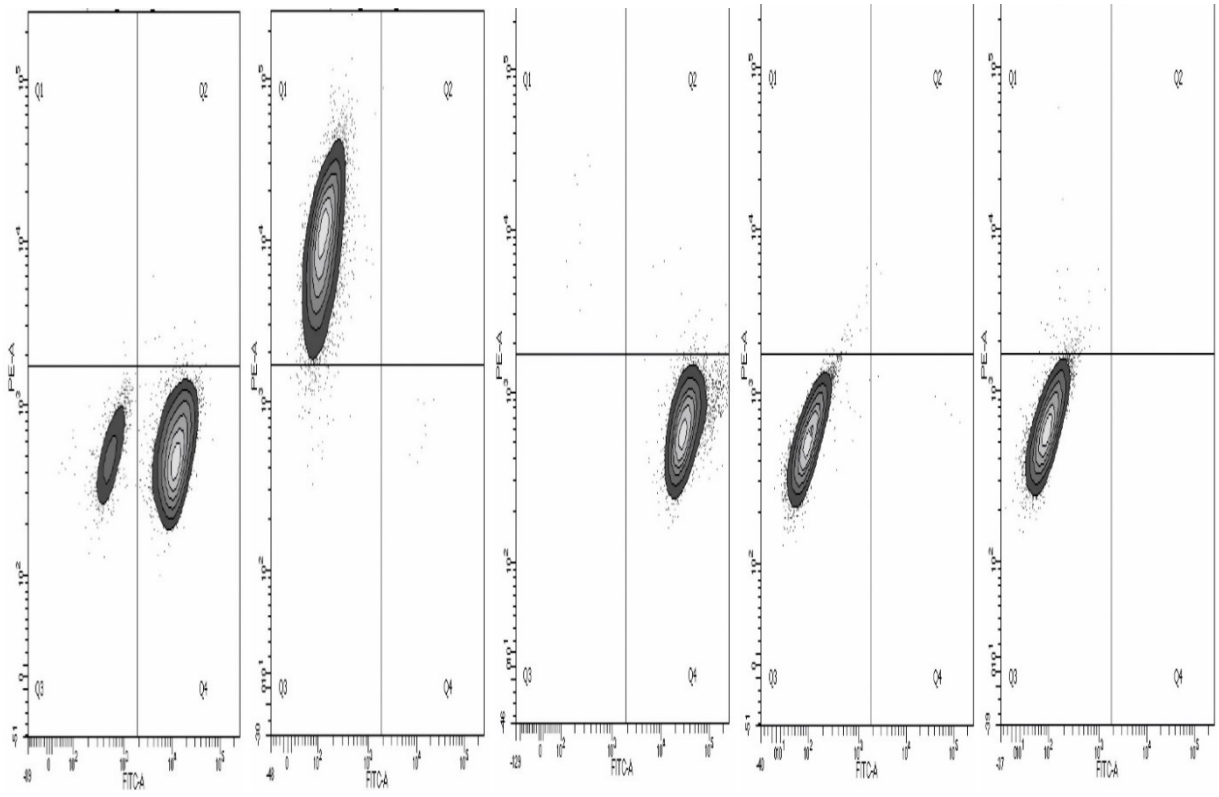



Figure 12: Characterization of WJ-MSCs by flow cytometry: as expected, WJ-MSC express CD29, CD90 and CD44 and lack CD45 and CD34, markers of hematopoietic lineage. Other markers, usually sought for WJ-MSC characterization are CD73, CD105, HLA-A.

**IPGTT**

In the contextual approach, the mice that received MSC transplantation ameliorated significantly their glycemia levels, as suggested by the overall areas under the curve. The smaller area drawn by the glycemia vs. time curve of transplanted mice indicates an improved glucose tolerance, especially in the descendent part, therefore the transplanted mice show an improved glucose disposal to the tissues. Likely, the better glucose tolerance might be due to an improved peripheral insulin signaling that allows an enhanced entry of glucose in peripheral tissues (Fig. 13).

A similar outcome is shown for the transplanted group in the delayed approach, even though the lower curve (and smaller AUC) does not reach significant values, perhaps due to the presence of high levels of FFA that still stimulate hepatic gluconeogenesis to an extent that the glucose disposal to the tissues does not compensate gluconeogenesis. It is evident that insulin signaling, although the tendency towards the improvement suggested by glycemia evaluation, has not been rescued in periphery (Fig. 14).

**Electrophysiology analyses**.

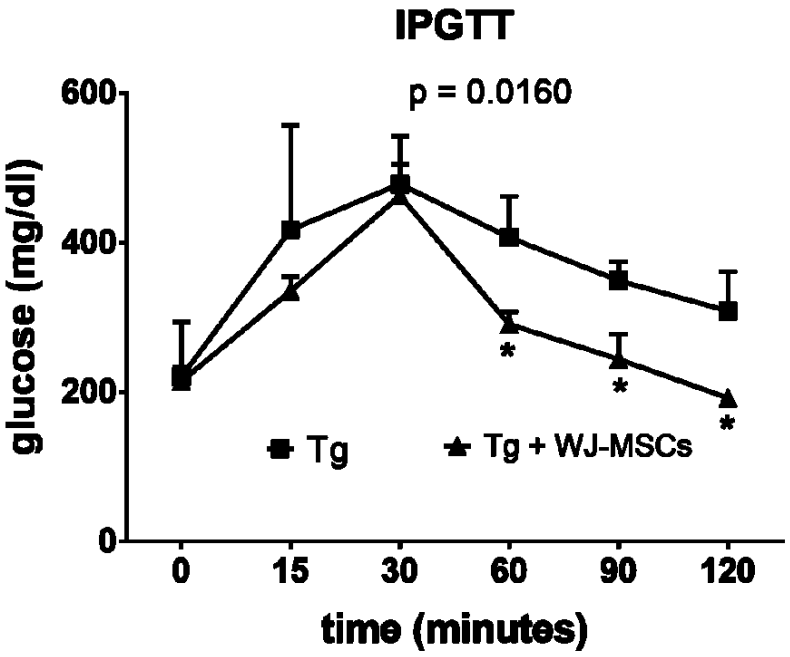
The analysis of fEPSP and consequent LTP revealed opposing results as well, between contextual and delayed approach. In fact, while in the first approach WJ-MSC treatment seemed to be beneficial for the rescue of LTP and overall excitatory transmission, in the delayed approach the outcomes are completely reverted, with transplanted mice that even result worse than non-recipient mice. It is of note that in both scenarios, though, fEPSP of non-transplanted reach the same height in the graph, thus suggesting that HFD halts LTP to a level that brings to memory and learning deficits in this transgenic model[180,181]. WJ-MSC seem to provide neuroprotective factors that reestablish synaptic transmission within the hippocampus, but also proposes that the brain-fat axis, impaired by systemic insulin resistance, has rebuilt the communication avenues as a consequence of the protection supplied by stem cells. Paradoxically, the same conclusions might be drawn for the transplanted group in the delayed approach even if there is no clue of amelioration. In fact, among the two approaches, different mechanisms can be involved to justify the different behavior of these animals. For example, while in the contextual approach the glutamatergic transmission seems to benefit from MSC transplantation bringing to LTP, in the delayed approach there is no

sign of glutamatergic potentiation, thus suggesting the improvement of an inhibitory transmission (GABAergic). However, the absence of LTD might be due to an incomplete rescue of glutamatergic transmission, whereas the GABAergic one results perhaps stabilized after MSC treatment.

Therefore, we can conclude that WJ-MSC transplantation is protective against impairment due to chronic high-caloric food intake and allows to rebuild a “clear” route of communication between AT and CNS, but is poorly effective if the metabolic and synaptic derangements have already taken place. Whether an adjustment of either dose, number of cells or number of injections is needed in order to improve glucose levels in a delayed approach will be further explored in future.

Figure 13: IntraPeritoneal Glucose Tolerance Test (IPGTT) in contextual approach, with relative area under the curves, AUC, shows lower plasma glucose levels after WJ-MSCs injection, suggesting amelioration of glucose uptake in the contextual approach.

Statistical analysis: Paired t-test with Mann-Whitney posthoc analysis for overall curves. Multiple t-test for each time point. Statistical significance with $p<0.05$.



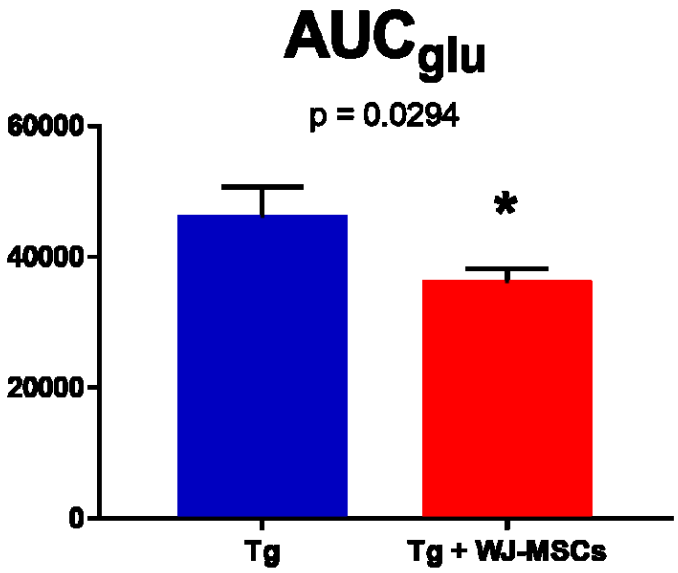
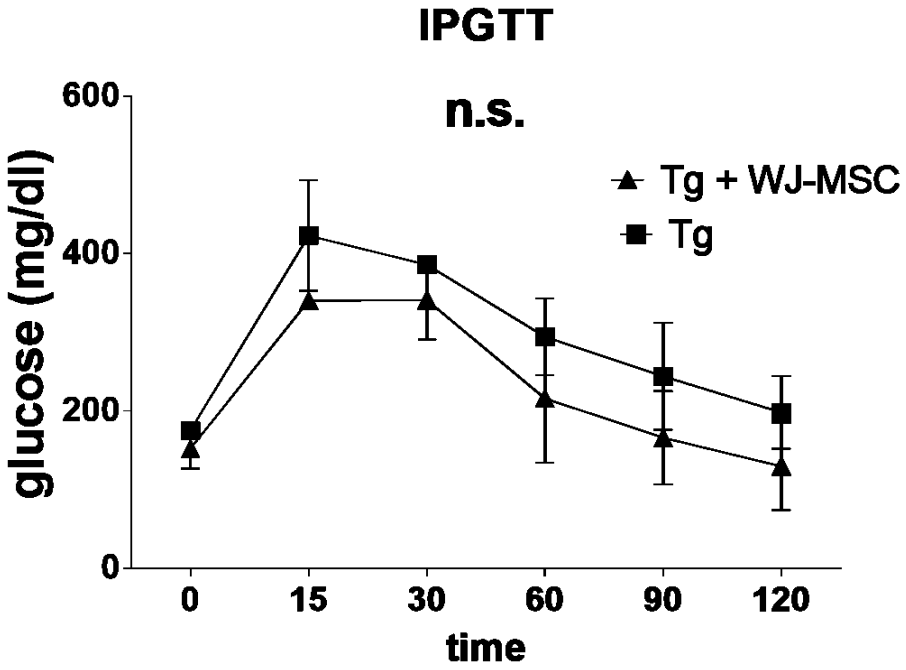



Figure 14: IntraPeritoneal Glucose Tolerance Test (IPGTT) in delayed approach, with relative area under the curves, AUC, shows lower plasma glucose levels after WJ-MSCs injection, but without significance, thus suggesting that the metabolic anomalies are not completely overcome by MSC treatment.

Statistical analysis: Paired t-test with Mann-Whitney posthoc analysis for overall curves. Multiple t-test for each time point. Statistical significance with $p<0.05$.



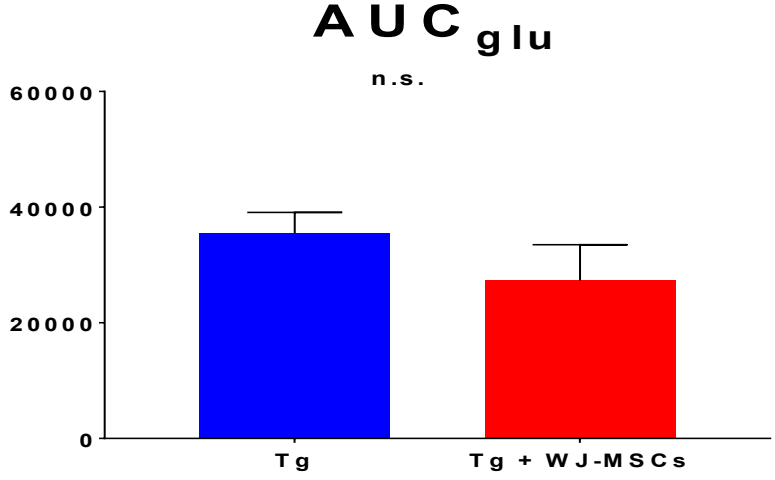



Figure 15: Electrophysiology analyses for mice included in contextual approach. The analyses show a clear improvement of synaptic transmission and LTP for transplanted mice. It is conceivable that MSC protected synapses from the alteration in lipids that characterizes AtENPP1Tg mice after HF-feeding.

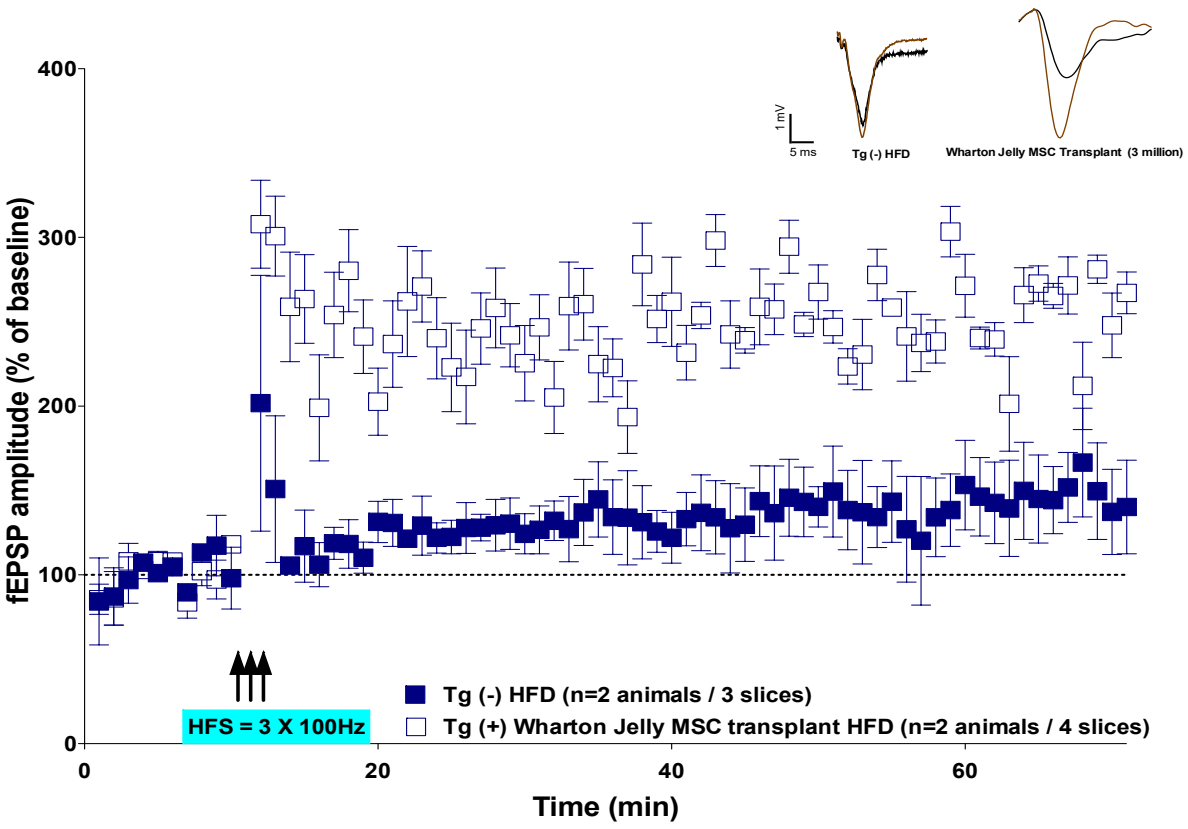


Figure 16: Summary of last 10 minutes of LTP for mice included in contextual approach. The improvement in LTP for recipient group is confirmed by statistical analyses (details of statistical analyses below graph).

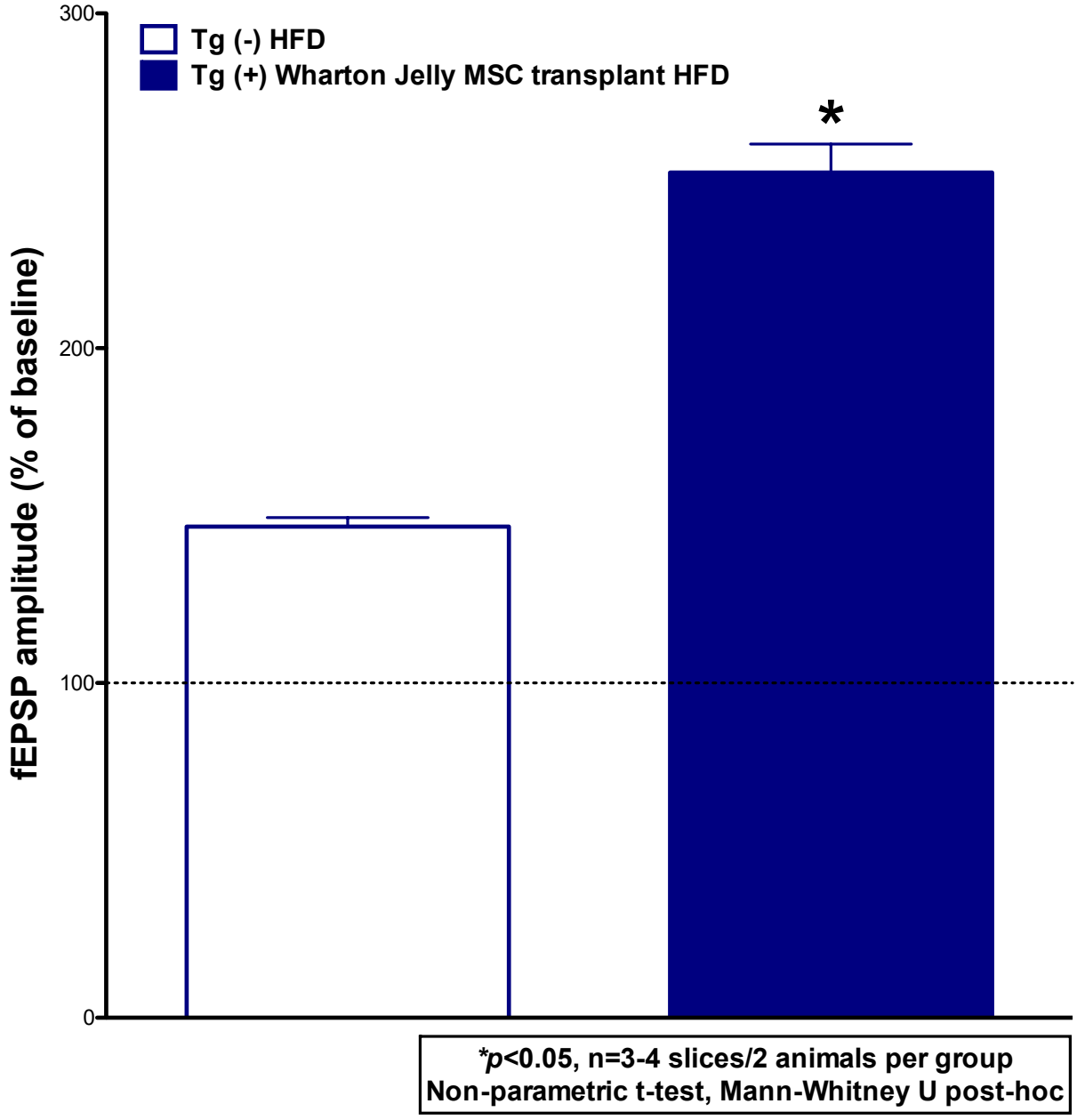
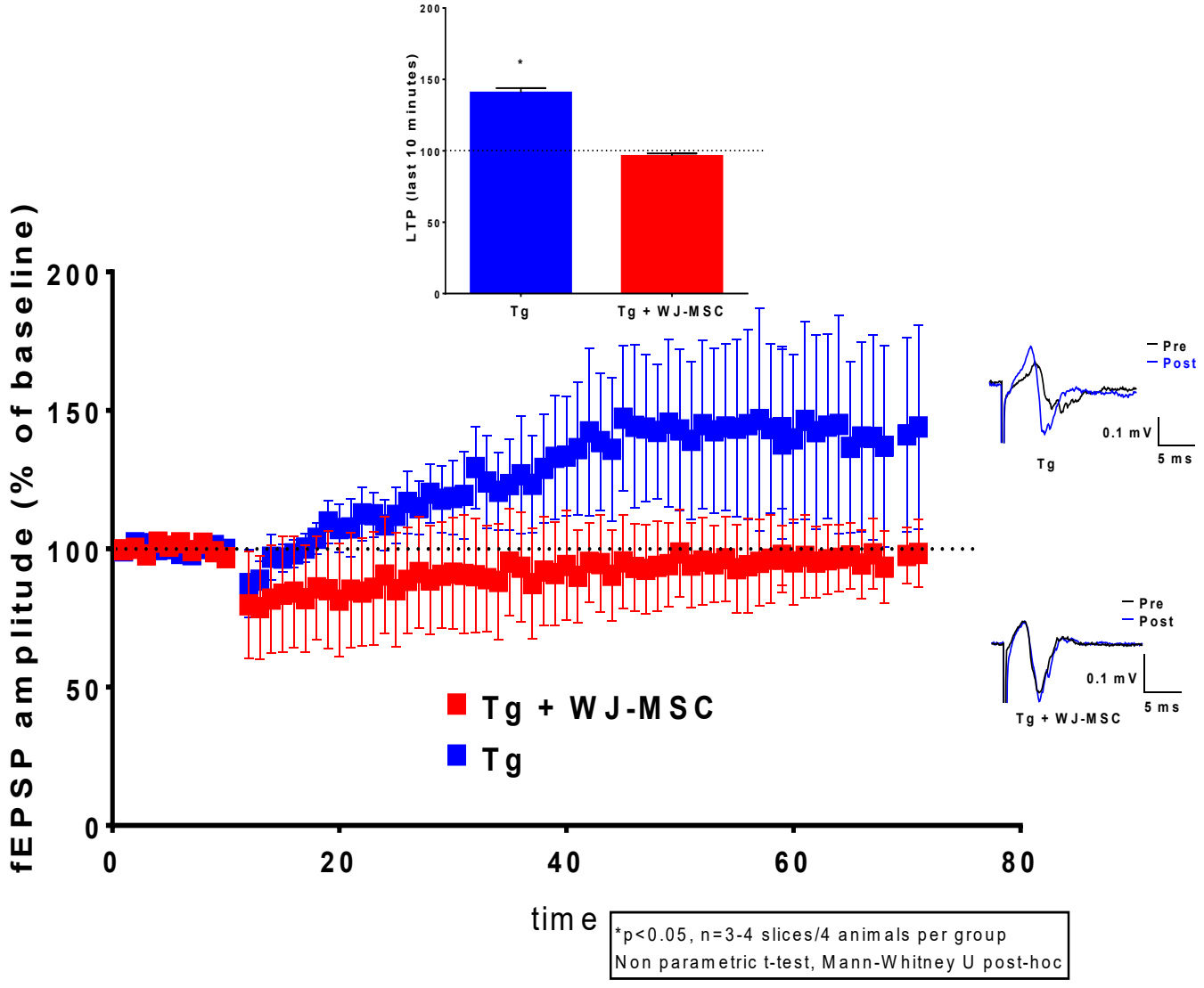


Figure 17: Electrophysiology analyses do not show any significant improvement in the delayed approach. WJ-MSC seem to worsen synaptic transmission even though not-transplanted group does not improve, as shown in contextual approach. Therefore, it is conceivable that glutamatergic transmission is still impaired when the HFD is began before MSC treatment. The little column graph on top of the main one represents last 10 minutes of LTP for both animal groups: the blunted LTP is confirmed for transplanted group as well as the slight LTP for not transplanted. Though WJ-MSC do not provoke LTD and not transplanted mice have the same outcomes in both approaches. (Details of statistical analyses below graph)



# DISCUSSION

Persuasive epidemiological evidence has established a link between Alzheimer's Disease (AD) and Type 2 Diabetes (T2D). T2D is characterized by insulin resistance, which has also been demonstrated as an early feature of AD, especially in CNS areas associated with cognitive performance, such as the hippocampus[137,147,255–261]. Insulin resistance implies a higher insulin secretion, due to reduced sensitivity to the hormone, however the resulting hyperinsulinemia, paradoxically, contributes to decrease insulin transport across the blood brain barrier (BBB), thus leading to brain InsRes[262,263]. In addition, postmortem analyses on human AD brains confirmed a decreased expression of insulin receptor (IRs) and reduced insulin signaling[93,264,265]. In the CNS, insulin has been shown to prevent Aβ deposition and promote learning and memory processes by modulating glutamatergic transmission via AMPA and/or NMDA receptors.

A major risk factor for T2D is AT dysfunction, which is usually accompanied by increased lipid metabolism and spillover of FFA, thus causing increased gluconeogenesis and systemic insulin resistance[42,43]. Besides, insulin regulates lipid metabolism by inhibiting lipolysis and increasing lipogenesis. If lipolysis inhibition lacks, there is a higher efflux of FFA, which are considered the mechanistic link between dysfunctional AT and insulin resistance. Elevated levels of FFA have been also reported in AD brains, and are correlated with impaired cognition in either high-fat-induced animal models of insulin resistance or human insulin resistant patients[56,57,89,151,266].

Therefore, I propose that a therapeutic approach aimed at improving insulin sensitivity might be preventive for brain insulin resistance while improves peripheral insulin resistance, especially in presence of an overload of nutrients. Testing one such approach is the overall goal of this project. Dr. Abate's group has developed an animal model of AT-targeted insulin resistance (AtENPP1-Tg mice), which is ideal to study mechanistic pathways underlying insulin resistance and its associated CNS deficits. When subject to a high-fat diet, AtENPP1-Tg mice show decreased adipocyte functionality (defective maturation, insulin signaling activation, fat storage ability and, adiponectin production), systemic insulin resistance, increased circulating FFA and ectopic fat deposition in the liver, thus recapitulating essential features of human insulin resistance states[173]. Moreover, Drs. Abate and Taglialatela groups found that AtENPP1-Tg mice display altered lipid composition and reduced expression of insulin and NMDA receptors in hippocampal synaptosomes, suggesting that CNS impairments occur in these mice as a result of peripheral

dyslipidemia[180]. Furthermore, recent studies from our group demonstrated impaired learning and memory processes for HFD-fed AtENPP1Tg mice compared to diet-matched wild type mice as well as to regular chow-fed transgenic mice[181].

Therefore, functional AT is crucial for the deposition of lipid and glucose after excessive caloric intake, hence adipocyte differentiation and maturation must be efficient to allow fat storage[267]. Indeed, the arrest of adipogenesis leads to immature and defective adipocytes, no longer able to store triglycerids, thereby a spillover of FFAs occurs with consequent insulin resistance which may contribute to development of associate CNS deficits[89,267,268]. Notably, T2D and hyperglycemia provoke a reduction in survival, regeneration and differentiation potential of mesenchymal stem cells (MSCs), known to participate to adipocytes turnover, thus leading to impaired ability to replace senescent cells. Since MSC exert their action by secreting numerous factors, it is plausible that a diminution of MSCs number reduces also information flow between AT and CNS, thus paving the way to brain insulin resistance, perhaps due to a warning message delivered to brain by peripheral organs[246,250,251,269,270].

Transplantation of different kinds of MSCs, included WJ-MSC, have been studied as treatment for T2D in both experimental animal models and diabetic humans. In these attempts, a significant improvement of glycemia and insulinemia was demonstrated, even though without addressing a clear mechanism of action[228,234–244]. More recently, studies have also reported beneficial effects of MSC-derived exosomes in improving cognitive function or reducing Aβ deposition[232,233], thus suggesting that transplantation of MSCs, directly into the AT, may correct T2D-associated metabolic deficits, while ameliorating associated CNS impairments. However, none but one of these studies mentioned any CNS deficit or considered any cross-talking between organs even though some of them employed animal models with AT dysfunction. This cross-talking gets impaired when insulin resistance occurs, hence we tested the hypothesis that a transplantation of **MSCs may ameliorate CNS deficits associated with insulin resistance** exploiting the availability of a model that holds metabolic issues along with CNS deficits.

In this project, I tested my hypothesis by employing human umbilical cord-derived Wharton's Jelly Mesenchymal Stem Cells (WJ-MSCs), a population of stem cells easy to obtain from ready available tissue and with relative high proliferation rates compared to other sources[193,194,196,216,220].

The previous above-mentioned studies were performed through a systemic administration of MSC, while in this study I considered an innovative approach aimed to deliver WJ-MSC with a local injection, directly in the AT of AtENPP1Tg mice in order to evaluate whether CNS deficits can be corrected by repairing peripheral abnormalities. The problem to choose a safe and efficacious route of administration is still under debate. Leibacher and Henschler reviewed some of the findings about biodistribution, homing and migration of MSC once they are injected systemically; researchers have found that they accumulate mostly in lungs when administered IV, even though they can sense the site of injury because attracted by chemokines and pro-inflammatory cytokines released by cells of the immune system which reach immediately the damaged sites. Moreover, many cells can clog the veins, thus potentially provoking disturbances in microcirculation. Besides, a comparison between intraarterial and intravenous injection demonstrated that the former one leads to better outcomes, less clogs and overall more homogenous distribution of cells after administration[271]. Another study that employed WJ-MSC in a model of T1D demonstrated that IV injection has to be preferred to intraperitoneal, since the cells transplanted through venous circulation did help the pancreas to recover its function, compared to the ones injected intraperitoneally. It is worthy of note, however, that the actual cells injected in this study were not undifferentiated WJ-MSC but insulin-producing cells derived from stem cells[225].

Therefore, in order to avoid any issue related to the route of administration, I preferred to use an innovative approach for the transplantation, aimed directly to the tissue from which all the metabolic anomalies due to reduced insulin responsiveness started.

Indeed, the retrieval of AT function seems to be crucial for systemic insulin sensitivity, included brain, as suggested at least by outcomes of contextual approach. It is established that nutrient excess is harmful for insulin sensitivity as well as for glucose disposal to an extent that involves also brain function. The improved glucose tolerance in transplanted group under the contextual approach shows that glucose disposal in periphery, governed by insulin signaling, has been improved, therefore the stem cell transplantation may help peripheral organ to face the dietary overload by improving their glucose utilization and overall metabolic capacity. In any case, further analyses on insulin blood levels after treatment are needed in order to establish if insulin tolerance has been restored as well.

However, when the mice undergo HFD before stem cell administration, most likely, all the metabolic derangements take place, thereby the injection of MSC seems ineffective, even though there is a tendency toward the amelioration of glucose tolerance. It is conceivable that a single injection of stem cells is not enough for a corrective purpose since the amelioration in blood glucose levels, although present, does not reach significance. Moreover, since the AT of transgenic mice shows immature adipocytes, it might be probable that the overnutrition burden already in place with the HFD is so big that our approach results unsuccessful.

An interesting observation comes from the evaluation of LTP registered in hippocampal slices isolated from both transplanted and not transplanted mice. Especially for the contextual approach, there is a significant improvement of LTP in recipient group, thus suggesting that the improvement of metabolic needs in periphery positively affects CNS to an extent that the hippocampal synaptic activity results improved. One hypothesis to support this observation is the improvement of brain insulin signaling that is known to be involved in synaptic plasticity and modulation of LTP. Despite the continuous supply of high caloric food, the stem cell transplantation improves the synaptic activity within the hippocampus, thus suggesting that stem cell treatment provides protective factors, which in both periphery and CNS allow a proper insulin signaling as well as a better nutrient utilization.

In the delayed approach, the stem cell treatment seems to worsen the LTP for recipient mice at a first impression; by the way, this data needs further interpretation. In fact, the apparently better LTP for non-recipient mice in the delayed approach reached in the graph fEPSP amplitude vs. time the same height seen for the contextual approach, while transplanted mice do not show neither LTP nor LTD in this approach, with the amplitude that stays at the baseline. If we assume that brain insulin signaling has been in somehow restored (as suggested by the peripheral ameliorated blood glucose levels, although not significant) and take into account that insulin signaling is able to modulate not only hippocampal glutamatergic transmission but also the GABAergic one, we can hypothesize a different mechanism. It might be conceivable that the metabolic overload that affects also brain is not completely overcome by MSC transplantation, in the delayed approach, consequently the GABAergic transmission might have been already reestablished, while the glutamatergic one results still impaired. GABA receptor can be phosphorylated by Akt, therefore it might be probable that the number and activity of GABA receptors have been rescued, compared

to AMPA glutamatergic receptors, likely still internalized, and unable to start the excitatory cascade that leads to LTP.

HFD may lead to post-translational modifications of these receptors may take place, such as lipid-acylation, in light of the fact that mice diet is rich in fat and previous studies from our group have demonstrated an elevated level of FFA receptors in brain as well as an increased content of triglycerides in hippocampal synaptosomes that leads to an altered lipid composition[180], similar to what happens in diabetic and AD humans[58,89,99,148,156]. In literature, growing evidence about the role of FFA and lipid-acylation (palmitoylation, miristylation, phosphatidilinositolylation…) in trafficking and recruiting of receptors have been published, therefore a similar mechanism could be taken into account in this study. It is established that palmitoylation of certain subunits of AMPA, NMDA and GABA receptors can either enhance or reduce their expression at synapses depending on which subunit and which aminoacid is modified. For example, AMPA receptor trafficking can be either promoted or inhibited (and AMPA-R accumulates in Golgi) on the basis of which palmitoylation prevails[209,272–280]. Hence, the high presence of palmitic acid in brain may either inhibit or not completely promote its recrutiment to the membrane thus impeding LTP activation, in the delayed approach. Indeed, the graph fEPSP vs. time shows for the transplanted group of animals a substantially steady amplitude, thus suggesting that the excitatory stimuli do not neither induce AMPA-R recruitment nor their endocytosis. Further support of this hypothesis comes from the observation that for non-transplanted mice LTP increases but reaches a plateau after some time, most likely because the increased content in CNS triglycerides impairs this process. Noteworthy, NMDA receptor-palmitoylation is beneficial for LTP since this modification keeps activated the receptor, thus maintaining LTP. On the other hand, GABA-receptor recruitment, their expression to the plasma membrane and their endocytosis seem to be palmitoylation-dependent. On top of these observations, it is worth to underline that insulin resistance might weaken receptor trafficking to the synapses, post-translational modification processes and overall synaptic plasticity[281].

Electrophysiological data from non-transplanted mice, regardless of the approach used, reveals that LTP occurs although at a small extent: this may be due to extremely high levels of triglycerides and FFA that perhaps accelerate trafficking of GABA receptors to/from the membrane, thus maintaining a certain degree of LTP, while simultaneously reduce AMPA trafficking, most likely

altered by improper insulin signaling. Therefore, while in contextual approach the excitatory post-synaptic events are restored after MSC transplantation, likely due to a higher presence of glutamatergic receptors (hypothetically due to a “good” palmitoylation) and to an amelioration of insulin signaling, in the delayed approach MSC treatment has perhaps modulated GABA-receptor trafficking (with a more stable expression at the plasma membrane), but is not sufficient to revert completely AMPA-receptor trafficking and NMDA activation. As a result, GABA transmission might be reestablished while glutamatergic receptors-trafficking is still unregulated. Of course, further experiments to understand whether or not dysregulated post-translational modification affect synaptic transmission in the hippocampus during insulin resistance are needed as well as the role of MSC in restoration of both insulin signaling and hippocampal synaptic transmission.

# CONCLUSIONS AND FUTURE DIRECTIONS

This study suggests that:

- a correction of CNS deficits through a peripheral approach is possible, thus suggesting the existence of a fat-brain axis that gets impaired during insulin resistant states
- cognitive deficits can be repaired by improving peripheral insulin responsiveness
- MSC delivery to AT reestablish proper insulin sensitivity with consequent positive effects on CNS, perhaps due to an ameliorated disposal of glucose which reestablishes a proper communication between periphery and CNS
- stem cell transplantation prevents HFD-induced-insulin synaptic deficiencies if stem cells are given contextually to HFD, but is ineffective after the initiation of HFD, likely due to incomplete restoration of both neurotransmitter transmission and receptor trafficking and recruitment.

As mentioned earlier, the beneficial effects for LTP seen in the contextual approach strongly suggest the existence of a brain-fat axis, impaired during insulin resistance-dependent AT dysfunctions. AT indeed secretes hormones (such as adiponectin and leptin), important for feeding regulation and control of body weight[48], or pro-inflammatory cytokines (TNFα, IL1β…), considered major contributors of dementia and neuroinflammation[172,282–284] even though our group did not show any significant increase in pro-inflammatory cytokine release from AT except for an increase of CD68, marker of macrophage recruitment[173]. Inflammation might be considered as "interfering factor" of brain-fat axis, given that high levels of pro-inflammatory cytokines characterize obesity, T2D and AD in humans. On the other hand, MSCs are acknowledged for their antinflammatory and immunomodulatory properties[204,285–290]. Therefore, a possible future path to pursue could be the assessment of inflammatory markers by use of specific arrays (such as, by means of the kits by Raybiotech®) which could allow to depict a frame of the entire panel of inflammatory molecules in brain. Also, it is legit to hypothesize that a single injection might not be sufficient to see positive effects after stem cell transplantation (especially for the delayed approach), hence a multiple injection protocol can be considered.

Furthermore, the improvement of AT and brain function could be due to an amelioration of AT secretion of adiponectin and leptin, which can help insulin action in brain and are significantly

changed in AD patients. Therefore, ELISA analyses aimed to detect these two hormones is a possible alternative path. Finally, limited to the brain, analyses of hypothalamic nuclei in which insulin activity is massive and where some researchers believe that insulin resistance starts, could be considered.

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