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Investigation of signaling pathways triggered by hyperglycemia and inflammation in obesity and type 2 diabetes

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ABSTRACT

Insulin resistance represents a central pathogenic feature of metabolic diseases and is tightly linked to chronic low-grade inflammation. While inflammatory cytokines are well established contributors to impaired insulin action, the molecular mechanisms through which inflammatory signalling integrates with metabolic dysfunction remain incompletely understood. In particular, the contribution of chemokine systems, traditionally studied in immune cell recruitment, to insulin resistance in metabolically active tissues is still poorly defined. This PhD thesis investigates the role of the chemokine receptors CXCR1 and CXCR2 in inflammation-driven insulin resistance, focusing on their functional involvement in the disruption of insulin signalling and cellular metabolism. Using two complementary in vitro models, differentiated 3T3-L1 adipocytes and FL83B hepatocyte-like cells, insulin resistance was induced by exposure to TNF-α, mimicking chronic inflammatory stress associated with metabolic disease. A combined transcriptional, biochemical and functional approach was employed to provide an integrated characterization of molecular signalling defects and metabolic alterations. Across both cellular models, TNF-α induced a profound impairment of the canonical insulin signalling cascade. Early and consistent reductions in IRS1 and IRS2 expression compromised signal transmission from the insulin receptor to downstream effectors, an effect further reinforced by the activation of stress-responsive kinases, particularly JNK. These proximal defects resulted in a marked attenuation of insulin-stimulated Akt phosphorylation, providing a mechanistic basis for impaired glucose handling. Functionally, these signalling alterations translated into reduced GLUT4 expression and translocation in adipocytes, decreased GLUT2 abundance in hepatocytes, and a significant reduction in insulin-stimulated glucose uptake.

Beyond glucose metabolism, inflammatory stress profoundly altered cellular energy homeostasis. Both adipocytes and hepatocytes exhibited mitochondrial dysfunction, characterized by reduced basal respiration, diminished ATP production and impaired maximal respiratory capacity. These bioenergetic defects were associated with metabolic inflexibility, altered glycogen storage and, in hepatocytes, increased intracellular lipid accumulation and lipotoxic features, highlighting the integration of mitochondrial dysfunction and lipid dysregulation in insulin-resistant states.

A central finding of this work is the identification of CXCR1 and CXCR2, together with their ligand CXCL1, as consistently upregulated components of the inflammatory response in insulin-resistant cells. The coordinated induction of receptors and ligand in both adipocytes and hepatocytes suggests the establishment of an autocrine/paracrine chemokine axis that actively sustains inflammatory and metabolic dysfunction. Importantly, pharmacological inhibition of CXCR1 and CXCR2 using Ladarixin partially restored insulin signalling, improved glucose uptake, enhanced mitochondrial bioenergetic performance and attenuated lipid dysregulation in both cellular models. Overall, this thesis demonstrates that inflammation-driven insulin resistance arises from the integration of signalling defects, stress pathway activation, mitochondrial dysfunction and altered substrate metabolism, and identifies the CXCR1/2 chemokine axis as a functional molecular link between inflammation and metabolic disease. By delineating the contribution of CXCR1 and CXCR2 to insulin resistance, this work provides a strong experimental foundation for considering chemokine receptor targeting as a potential therapeutic strategy to restore metabolic homeostasis in inflammatory metabolic disorders.

1 INTRODUCTION

1.1 Type 2 Diabetes Mellitus in Insulin Resistance

1.1.1 Epidemiology and clinical relevance

Type 2 diabetes mellitus (T2DM) is a chronic and multifactorial metabolic disorder characterized by impaired glucose regulation and reduced insulin sensitivity. Under normal physiological conditions, blood glucose levels are tightly controlled by the coordinated actions of insulin and glucagon, secreted by pancreatic β- and α-cells, respectively. Following a meal, the rise in plasma glucose triggers insulin release from β-cells, which promotes glucose uptake in peripheral tissues and glycogen storage in the liver, thus restoring euglycemia. Conversely, during fasting states, α-cells release glucagon to stimulate hepatic gluconeogenesis and maintain stable glucose availability. In T2DM, these regulatory mechanisms are progressively disrupted, leading to chronic hyperglycemia (Figure 1).

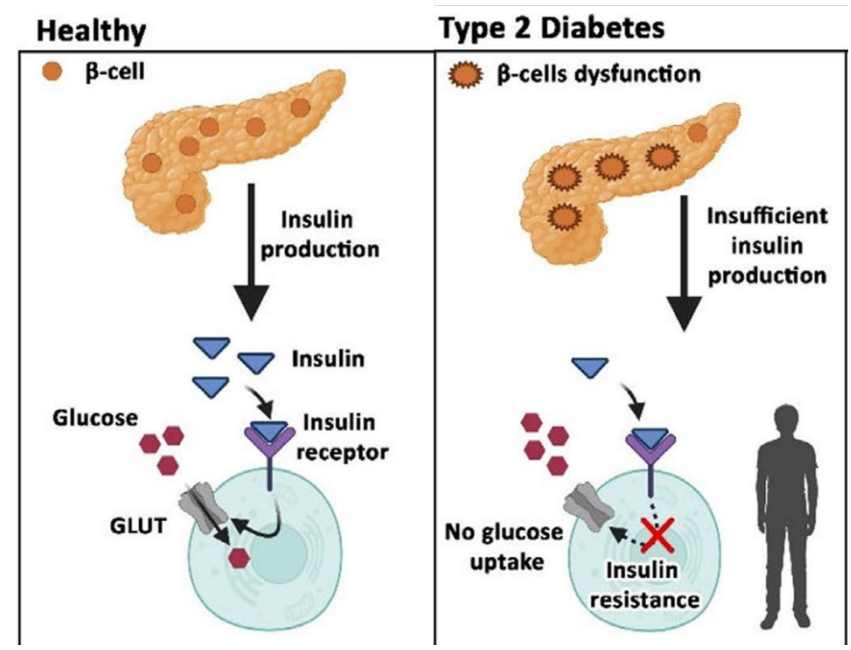

Figure 1. Schematic representation of T2DM

Persistent hyperglycemia drives the development of long-term complications, including microvascular damage (retinopathy, nephropathy, and neuropathy) and macrovascular outcomes such as atherosclerosis, myocardial infarction, stroke, and diabetic foot syndrome [1], [2].

The global burden of T2DM has risen dramatically over the last few decades, with the number of affected individuals increasing from 382 million in 2013 to an estimated 700 million projected by 2045 [3]. Although prevalence varies across countries, T2DM is recognized as a worldwide health challenge, with the steepest increases occurring in low- and middle-income nations where "Westernized" lifestyles, obesity, and physical inactivity are becoming more common. The highest risk is typically observed in adults aged 40-60 years in developed countries, whereas in developing countries onset often occurs later in life. However, pediatric T2DM is also emerging as a growing concern, with cases reported not only in the United States and Japan but increasingly worldwide. This global rise has major clinical and socioeconomic implications: diabetes already accounts for a substantial proportion of health care costs, and the burden is expected to increase further due to both population growth and rising incidence. Importantly, a considerable fraction of cases remain undiagnosed, particularly in developing regions, suggesting that the true prevalence is even higher than current estimates [4].

1.1.2 Risk factors

T2DM arises from the combined influence of genetic predisposition, metabolic disturbances, and a variety of environmental and behavioural exposures. Among modifiable determinants, overweight and obesity remain the most powerful predictors of disease onset. Excess adiposity, particularly the accumulation of visceral fat, provokes chronic low-grade inflammation, lipotoxicity, and oxidative stress, which together impair insulin signalling and damage pancreatic β-cells, ultimately precipitating the development of T2DM [5], [6], [7]. Sedentary behaviour and insufficient physical activity further intensify this metabolic vulnerability by reducing glucose uptake in skeletal muscle and diminishing overall insulin sensitivity. Dietary habits add another critical layer of risk: high consumption of saturated fats, refined carbohydrates, and processed meats has been consistently associated with greater incidence of T2DM, whereas diets rich in fibre, whole grains, and unsaturated fats appear to confer protection and delay disease progression [5], [6].

Beyond lifestyle-related exposures, several non-modifiable factors also shape individual susceptibility. A strong family history of diabetes, particularly in first-degree relatives, represents one of the most robust predictors of future T2DM and reflects both inherited susceptibility and shared environmental conditions [7]. Advancing age further amplifies vulnerability, in part due to cumulative exposure to metabolic stressors, declining β-cell function, and age-related changes in body composition, such as the redistribution of fat toward more visceral depots [7]. Although T2DM has traditionally been considered an adult-onset disease, the dramatic rise in paediatric and adolescent obesity has been paralleled by an increasing number of T2DM diagnoses in younger populations. Cases once considered rare are now reported globally, indicating that the boundaries between “adult” and “youth” diabetes are becoming increasingly blurred [5], [6].

A growing body of evidence also implicates other, often overlooked determinants in the pathogenesis of T2DM. Chronic psychosocial stress, smoking, and sleep disturbances such as short or fragmented sleep have each been linked to metabolic dysregulation, heightened inflammation, and impaired glucose homeostasis [5]. Socioeconomic disadvantage and adverse environmental conditions (including urbanisation, air pollution, limited access to healthy foods, and reduced opportunities for physical activity) further exacerbate the risk landscape, particularly in low and middle-income countries where the steepest increases in prevalence are being observed [5]. Collectively, these findings illustrate that T2DM risk does not derive from a single cause but from a complex, synergistic interaction between genetic and non-genetic factors. They also underscore the importance of comprehensive prevention strategies that target both individual behaviours and the broader social and environmental context in order to curb the growing global burden of the disease.

1.1.3 Pathophysiological hallmarks

T2DM is a heterogeneous disorder whose clinical picture reflects a constellation of interrelated pathophysiological disturbances rather than a single defect. At its core, the disease is defined by a progressive mismatch between insulin demand and insulin action, resulting from the combination of insulin resistance in peripheral tissues and β-cell dysfunction. Skeletal muscle, liver, and adipose tissue (the main insulin-responsive organs) gradually lose their sensitivity to insulin, leading to impaired glucose uptake, excessive hepatic glucose output, and dysregulated lipid metabolism [8], [9], [10]. As this resistance develops, pancreatic β-cells initially respond by increasing insulin secretion, but chronic metabolic stress eventually exhausts this compensatory

mechanism, causing loss of first-phase insulin release, impaired proinsulin processing, and eventual β-cell failure (Fig. 2) [8], [9].

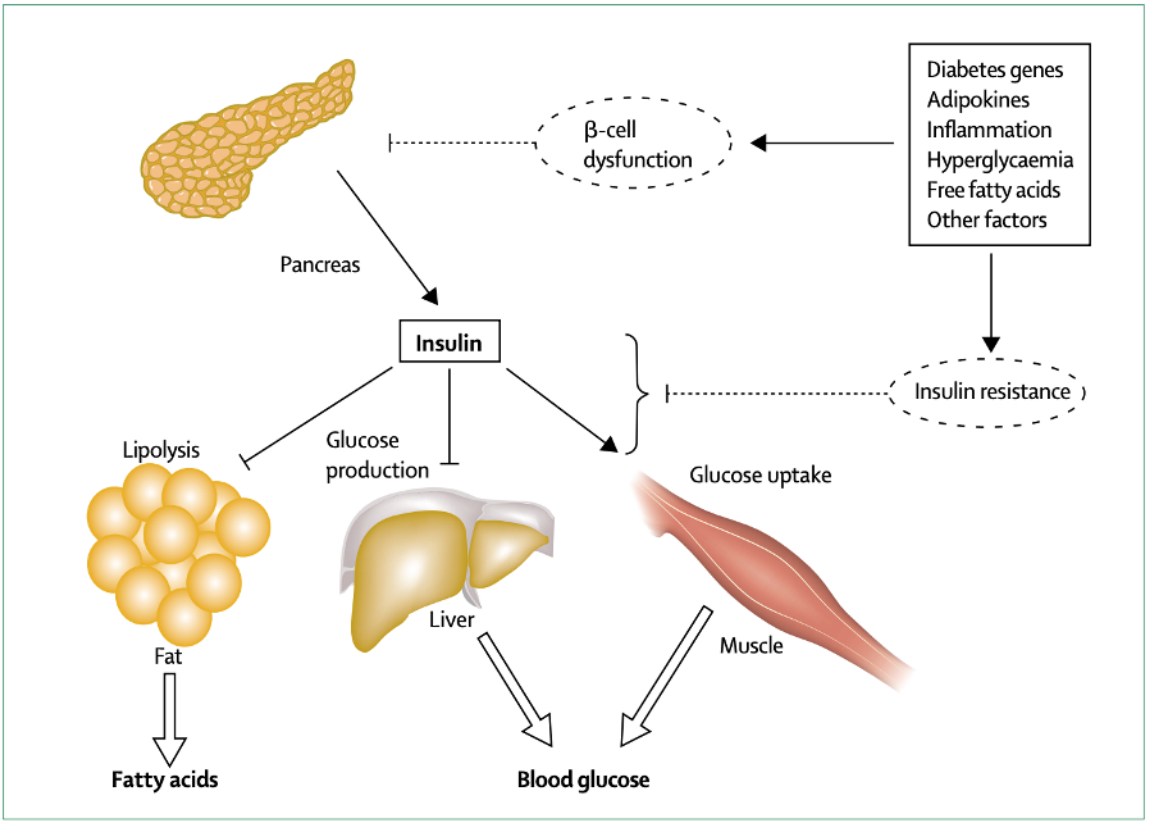

Figure 2.Pathophysiology of T2DM.

Visceral adiposity is a key driver of this metabolic environment. Excess central fat releases free fatty acids and pro-inflammatory adipokines while reducing protective hormones such as adiponectin. This "lipotoxic" environment disrupts insulin signalling pathways (particularly at the level of insulin receptor substrate proteins) and fuels low-grade, chronic inflammation that further aggravates insulin resistance [8], [9], [10]. In parallel, mitochondrial dysfunction and oxidative stress emerge as central features of T2DM: chronic hyperglycaemia and elevated lipid flux increase the production of reactive oxygen species (ROS), which damage mitochondrial DNA, impair ATP production, and activate stress kinases such as c-Jun N-terminal kinases (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

These processes compromise insulin signalling in muscle and liver and also induce endoplasmic reticulum (ER) stress, unfolded protein response (UPR), and apoptosis in β-cells, thereby diminishing their secretory capacity [9].

Beyond this classical "triumvirate" of muscle, liver, and pancreas, T2DM involves a wider network of organs and regulatory pathways. The concept of the "ominous octet" recognises that defects in α-cell function (hyperglucagonaemia), altered incretin secretion in the gut, enhanced renal glucose reabsorption, and central nervous system insulin resistance all contribute to chronic hyperglycaemia [8], [9]. More recent work has highlighted additional signalling pathways. For example, studies in obese and insulin-resistant livers report upregulated platelet-derived growth factor AA (PDGF-AA) signalling, which downregulates insulin receptor (IR) and Insulin receptor substrate 1 (IRS1) expression; pharmacological inhibition of this pathway restored insulin responsiveness *in vitro* [10]. Such findings illustrate how inflammatory and growth factor networks, beyond traditional metabolic pathways, can actively shape the course of T2DM.

Altogether, these mechanisms create a self-reinforcing cycle: insulin resistance and β-cell stress lead to worsening hyperglycaemia, which in turn amplifies oxidative and ER stress, accelerates β-cell dysfunction, and sustains the inflammatory milieu. This multifaceted pathophysiology explains why T2DM is closely linked to dyslipidaemia, hypertension, and a pro-atherogenic state, and why interventions targeting single pathways often provide only partial benefit. Understanding these hallmarks provides the biological rationale for new therapeutic strategies, including those that target chemokine or growth factor signalling in metabolic tissues [8], [9], [10].

1.2 Molecular Mechanisms Underlying Insulin Resistance

1.2.1 Canonical insulin signaling pathway

The canonical insulin signaling pathway is initiated by insulin binding to its membrane receptor (IR), a tyrosine kinase expressed in all insulin-responsive tissues, including liver, adipose tissue, and skeletal muscle. Ligand engagement induces activation of the receptor's intracellular kinase domain, which undergoes autophosphorylation on specific tyrosine residues, creating docking surfaces for downstream adaptor proteins [11]. This first step enables the recruitment of major signaling intermediates and establishes the foundation for insulin-dependent metabolic regulation.

One of the principal substrates of the activated receptor is the family of insulin receptor substrates (IRS), predominantly IRS-1 and IRS-2. Upon insulin stimulation, IRS proteins become phosphorylated on multiple tyrosine motifs that serve as binding sites for SH2-domain-containing effector molecules [12]. Their phosphorylation is required for the assembly of signaling complexes that relay insulin's metabolic actions. In parallel, the receptor can recruit additional adaptor proteins, including Shc, which participates in alternative signaling routes and contributes to the diversification of cellular responses to insulin [13].

A central event downstream of IRS activation is the recruitment of phosphoinositide 3-kinase (PI3K), which docks to phosphorylated IRS via its regulatory subunit. Activated PI3K catalyzes the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the plasma membrane, enabling the localization of phosphoinositide-dependent kinase-1 (PDK1) and Akt, also known as protein kinase B [14]. PDK1 phosphorylates Akt on its activation loop, while full activation requires an additional phosphorylation mediated by Mammalian target of rapamycin 2 (mTORC2). Once activated, Akt orchestrates a broad metabolic program that includes stimulation of glycogen synthesis through inhibition of glycogen synthase kinase-3 (GSK3), suppression of hepatic gluconeogenesis via phosphorylation-dependent

inactivation of FOXO transcription factors, and modulation of lipogenic and oxidative pathways in hepatocytes (Fig. 2) [11], [14].

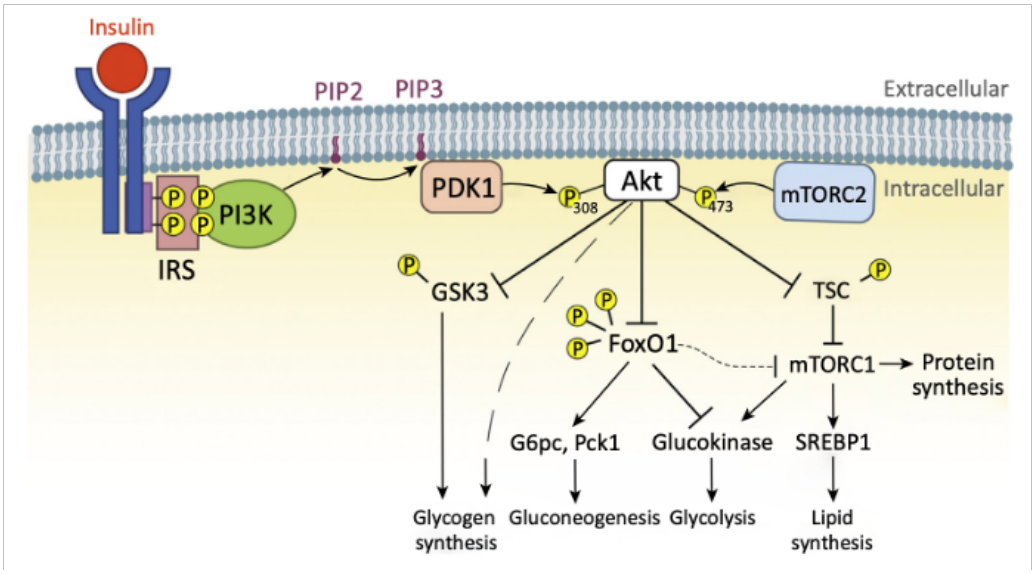

Figure 3. Insulin canonical signaling

A major metabolic consequence of Akt signaling is the regulation of glucose transport. In insulin-responsive tissues, insulin promotes the redistribution of glucose transporters from intracellular compartments to the cell surface. Studies in adipose tissue and skeletal muscle demonstrate that insulin signaling through PI3K and Akt facilitates the mobilization of Glucose transporter type 4 (GLUT4)-containing vesicles toward the plasma membrane, a process essential for the rapid increase in glucose uptake after hormonal stimulation [11]. Work in transgenic models has shown that defects in this pathway markedly impair insulin-stimulated glucose transport, confirming the centrality of the PI3K/Akt cascade in glucose homeostasis [15], [11].

IRS-dependent signaling can also intersect with regulatory scaffold proteins. Among them, Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1) has been identified as a modulator of insulin action in metabolic tissues. APPL1 interacts with components of the insulin signaling pathway and enhances the efficiency of Akt activation under insulin-stimulated conditions, contributing to the fidelity and amplification of downstream responses. Through these interactions, APPL1 supports optimal signaling flux and participates in maintaining insulin sensitivity in adipocytes and hepatocytes [16].

In addition to PI3K/Akt activation, insulin can engage the Shc-dependent branch of signaling, which contributes to broader cellular responses. Shc phosphorylation upon insulin stimulation enables its association with Growth factor receptor-bound protein 2 (Grb2) and the recruitment of additional intermediates that shape downstream signal propagation. Although less directly involved in acute metabolic regulation, this pathway integrates with the overall network of insulin-responsive signals and contributes to cellular adaptation [16].

1.2.2 Alterations in insulin signaling during IR

IR represents a pathological state characterized by the compromised ability of insulin-sensitive tissues (primarily skeletal muscle, adipose tissue, and the liver) to respond appropriately to normal circulating levels of insulin [17], [18]. The molecular basis of this defect lies in significant perturbations within the canonical insulin signal transduction cascade, a pathway crucial for maintaining systemic glucose homeostasis (Fig. 3) [17], [19].

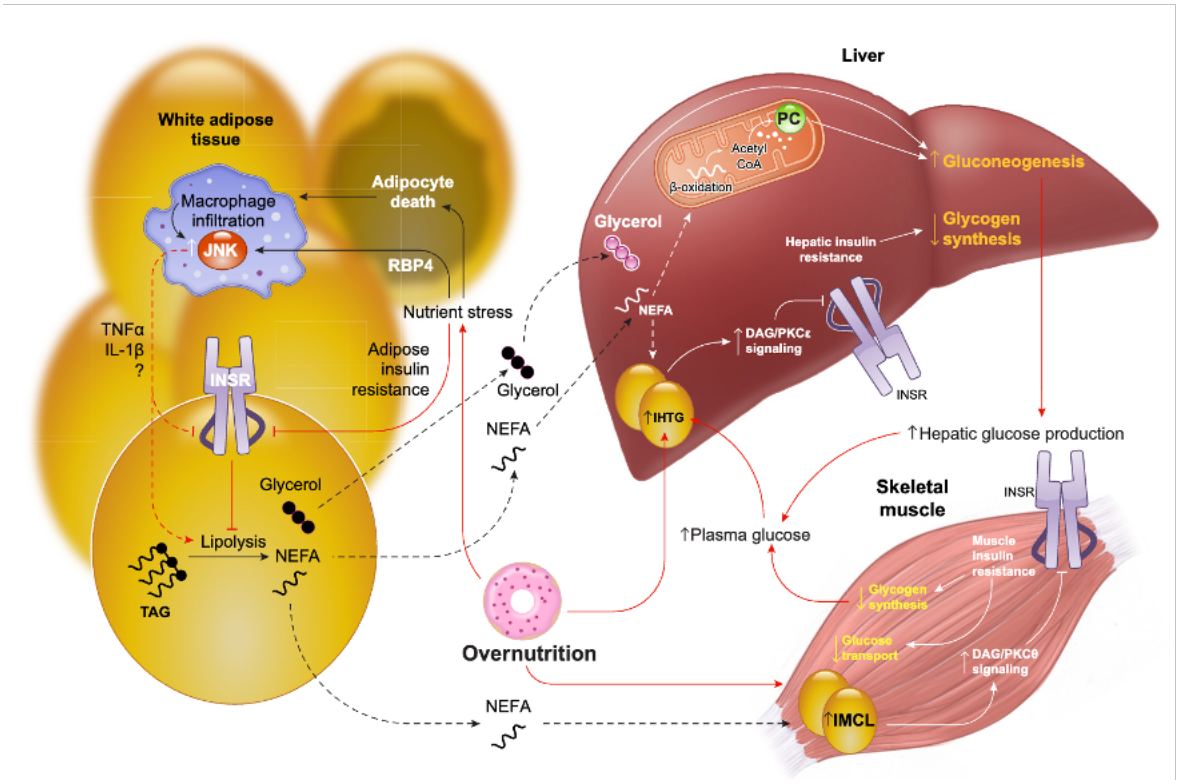

Figure 4.IR mechanism in different tissues

The process is initiated by the binding of insulin to the Insulin Receptor (IR), a heterotetrameric transmembrane glycoprotein. This binding activates the intrinsic tyrosine kinase activity of the IR β-subunits, leading to autophosphorylation of the receptor and subsequent phosphorylation of key cytoplasmic adaptor proteins, notably the Insulin Receptor Substrates (IRS), predominantly IRS-1 and IRS-2 [18]. In the insulin-sensitive state, tyrosine phosphorylation of IRS proteins provides crucial docking sites for regulatory molecules, particularly Phosphatidylinositol 3-Kinase (PI3K) [17].

Activated PI3K then catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 accumulation is critical for recruiting and activating the serine/threonine protein kinase Akt (also known as Protein Kinase B) [17]. Active Akt is the central effector of insulin's metabolic actions, regulating diverse processes including:

1. Glucose Transport: Akt promotes the translocation of the glucose transporter type 4 (GLUT4) from intracellular vesicles to the plasma membrane of muscle and adipose cells, thereby facilitating cellular glucose uptake [17], [19].
2. Glucose Storage: Akt stimulates glycogen synthesis by inactivating Glycogen Synthase Kinase-3 (GSK-3) [17].
3. Hepatic Glucose Production: Akt inhibits gluconeogenesis in the liver [17], [20].

Conversely, in states of chronic IR, the efficiency of this cascade is severely impaired, primarily at the level of the IRS proteins [18]. This molecular failure is strongly correlated with chronic, low-grade inflammation, which activates several intracellular stress pathways [18], [19]. A pivotal mechanism underlying the progression of IR is the inappropriate phosphorylation of IRS proteins on serine residues rather than the activating tyrosine residues [18], [19]. This inhibitory serine phosphorylation is mediated by a variety of stress-activated protein kinases (SAPKs) that are upregulated in the inflammatory milieu, most notably the c-Jun N-terminal kinase (JNK) and the inhibitor of nuclear factor κB kinase (IKKβ) [18], [19]. Serine phosphorylation of IRS-1 has two primary detrimental consequences: it physically inhibits the binding of IRS-1 to the Insulin Receptor (IR), thereby preventing its essential tyrosine phosphorylation, and it can tag IRS-1 for subsequent degradation [17], [18]. The resulting lack of IRS-1 tyrosine phosphorylation significantly compromises the activation of PI3K and the subsequent generation of PIP_3. Consequently, Akt activity is diminished [17], [18], [19]. The functional outcome of this signal attenuation is the loss of insulin's metabolic control, leading to:

1. Reduced Glucose Uptake: the failure to adequately activate Akt impairs GLUT4 translocation in peripheral tissues, resulting in decreased glucose disposal and contributing to hyperglycemia [17], [19].

2. Persistent Hepatic Gluconeogenesis: Reduced Akt activity fails to suppress the hepatic production of glucose, contributing substantially to fasting and postprandial hyperglycemia [17], [20].

Furthermore, inflammatory signaling pathways, such as those involving Toll-like Receptor 4 (TLR4) and the subsequent activation of the Nuclear Factor κB (NF-κB) pathway, directly contribute to this disruption [19]. TLR4 is a pattern recognition receptor whose activation, often by saturated fatty acids or lipopolysaccharide (LPS), triggers signaling that culminates in NF-κB activation [19]. The resulting NF-κB cascade drives the expression of pro-inflammatory cytokines, which in turn activate the stress kinases JNK and IKKβ, thereby reinforcing the inhibitory serine phosphorylation loop on IRS proteins [18], [19]. This detrimental cross-talk between inflammatory kinases and the insulin signaling machinery establishes a vicious cycle that is central to the development and perpetuation of chronic systemic IR, resulting in the hallmark features of hyperinsulinemia and hyperglycemia [18].

1.3 Role of lipid metabolism and lipotoxicity

1.3.1 Diacylglycerols, ceramides, and other lipotoxins

Lipotoxicity is the phenomenon of cellular dysfunction, damage, and ultimately organ failure caused by the ectopic accumulation of lipid metabolites in non-adipose tissues such as skeletal muscle, liver, pancreas, and heart [21], [22], [23]. This condition is often initiated when the storage capacity of enlarged fat cells in obese adipose tissue is overwhelmed, leading to a diminished ability to store fat and a resistance to insulin anti-lipolytic effects [23]. This impaired function causes an uncontrolled release of free fatty acids (FFAs) into the circulation (fatty acid spillover), which subsequently reach toxic levels within ectopic tissues [21], [21]. Within these tissues, FFAs and their metabolic products act as lipotoxic intermediates that directly antagonize insulin signaling

[22], [23]. Among the numerous bioactive lipid species associated with the pathogenesis of insulin resistance (IR), diacylglycerols (DAGs) and ceramides are considered the primary lipotoxins responsible for disrupting glucose metabolism [21], [22], [23]. DAGs are neutral lipids that accumulate within cells alongside triglycerides (TGs) and are strongly linked to the development of IR in the liver and skeletal muscle [22], [23]. Their mechanistic link to IR centers on their ability to activate the conventional and novel isoforms of Protein Kinase C (PKC) [22], [23]. Once activated, PKC initiates a signaling cascade that culminates in the inhibitory serine phosphorylation of IRS-1, effectively blocking the downstream propagation of the insulin signal through the PI3K/Akt pathway and impairing glucose uptake [22].Ceramides, which are intermediates in the biosynthetic pathway that produces complex sphingolipids, are perhaps the most detrimental lipotoxic players, particularly within the skeletal muscle and liver [21], [22], [23]. Ceramides are proposed as a key intermediate that links both the oversupply of nutrients (specifically long-chain saturated fatty acids) and inflammatory cytokines (TNFα) to the induction of IR [21], [23]. The mechanisms by which ceramides impair insulin signaling are multifaceted:

- Inhibition of the PI3K/Akt Pathway: Ceramides are potent inhibitors of Akt (PKB) activation. By blocking Akt, ceramides interfere with the signaling required for GLUT4 translocation to the plasma membrane, thereby suppressing glucose entry into muscle and fat tissue [21], [22], [23].
- Mitochondrial Dysfunction and Apoptosis: Ceramides disrupt mitochondrial metabolism, leading to impaired lipid oxidation, increased production of Reactive Oxygen Species (ROS), and, ultimately, the induction of apoptosis (lipopapoptosis) in highly sensitive cell types, such as pancreatic β-cells and cardiomyocytes, which contributes to the complications associated with metabolic diseases [22], [23].

- ER Stress and Inflammation: Ceramide accumulation is also linked to the induction of endoplasmic reticulum (ER) stress, and inflammatory agents selectively upregulate the sphingolipid pathway, which is essential for their induction of IR [22], [23].

The chronic accumulation of these bioactive lipids (DAGs, ceramides, and FFAs) in non-adipose tissues is thus instrumental in establishing the link between obesity, inflammation, and metabolic disorders, resulting in a vicious cycle of hyperglycemia and hyperinsulinemia [21], [23]. Remarkably, studies in rodents have demonstrated that inhibiting ceramide biosynthesis or catalyzing its degradation can improve many metabolic disorders, including IR, diabetes, and steatohepatitis [22].

1.3.2 PKC activation and interference with insulin signaling

Protein kinase C (PKC) plays a central role in linking lipid excess to impaired insulin signaling, particularly under conditions of elevated free fatty acids (FFAs), diacylglycerol (DAG) accumulation, and metabolic stress characteristic of insulin-resistant states. The studies provided consistently show that DAG overload functions as a primary activator of several PKC isoforms, including conventional and novel PKCs, which subsequently interfere with key nodes of the insulin signaling cascade [24], [25], [26].

Elevated intracellular DAG levels promote membrane translocation and activation of PKC isoforms, an event widely reported in insulin-resistant tissues such as liver, skeletal muscle, and adipose tissue. Activation of PKC leads to inhibitory serine/threonine phosphorylation of insulin receptor substrates (IRS), particularly IRS-1, which reduces their ability to undergo insulin-stimulated tyrosine phosphorylation and to recruit downstream effectors such as PI3K [24], [25]. This mechanism results in impaired activation of Akt and a

downstream reduction in the metabolic actions of insulin, including glucose uptake, glycogen synthesis, and suppression of hepatic gluconeogenesis.

Evidence from human and animal studies indicates that PKC activation is tightly associated with impaired insulin signaling in metabolic tissues. In liver, DAG-dependent activation of PKC isoforms correlates with decreased insulin-stimulated IRS-1 function and reduced suppression of hepatic glucose production, contributing to the development of systemic hyperglycemia [25]. In skeletal muscle, similar PKC-driven IRS-1 serine phosphorylation disrupts PI3K/Akt activation and thereby impairs GLUT4 translocation, diminishing insulin-dependent glucose transport [24], [26]. These findings position PKC as a critical mediator of lipid-induced insulin resistance in both hepatic and peripheral tissues.

In addition to IRS-1 inhibition, PKC activation is also reported to interfere more proximally by attenuating insulin receptor (IR) signaling. Increased PKC activity contributes to reduced IR kinase function, further diminishing the ability of insulin to propagate its signal along the PI3K/Akt axis [25]. Combined, these effects show that PKC alters the insulin pathway at multiple levels, amplifying the degree of metabolic impairment seen under lipotoxic conditions. The studies further highlight that chronic PKC activation triggers downstream inflammatory responses and oxidative stress, both of which exacerbate insulin resistance and reinforce the cycle of serine phosphorylation–mediated IRS inhibition [24], [26]. This integrated process explains how lipid overload can quickly translate into persistent defects in insulin responsiveness, contributing to the pathophysiology of type 2 diabetes mellitus.

Overall, the provided evidence supports a model in which DAG-driven PKC activation disrupts insulin signaling at the receptor and IRS levels, impairing PI3K/Akt signaling and reducing cellular glucose handling. PKC therefore acts as a molecular bridge between lipid excess and insulin resistance, positioning

it as a potential therapeutic target in metabolic diseases characterized by altered lipid homeostasis and chronic insulin dysfunction.

1.3.3 Crosstalk between adipose tissue and liver in lipid overflow

During conditions of chronic caloric excess, white adipose tissue (WAT) progressively loses its ability to store lipids efficiently, leading to adipocyte hypertrophy, extracellular matrix remodeling, and a low-grade inflammatory state [27], [28]. As WAT becomes dysfunctional, the antilipolytic action of insulin is impaired, resulting in increased release of free fatty acids (FFAs) and glycerol into the circulation [28]. This excess lipid flux is directly delivered to the liver, where it contributes to triglyceride accumulation and hepatic steatosis, a key metabolic consequence of adipose tissue overload [27], [29].

The liver responds to elevated FFA influx by enhancing triglyceride synthesis; however, persistent lipid overload also leads to the generation of lipotoxic lipid species that disrupt hepatocellular function and insulin sensitivity [29]. According to recent findings, the progression from simple steatosis to metabolic dysfunction–associated steatotic liver disease (MASLD) is strongly influenced by adipose-derived lipid overflow, positioning WAT dysfunction as a major determinant of hepatic metabolic impairment [27].

Beyond lipid flux, adipose tissue dysfunction alters its secretory profile. Inflammatory cytokines and other adipokines released from enlarged or stressed adipocytes exert direct effects on hepatic metabolism, promoting inflammation and impairing insulin-mediated suppression of gluconeogenesis [28], [29]. Reduced production of protective adipokines, such as adiponectin, further amplifies hepatic metabolic dysregulation, since adiponectin normally supports fatty acid oxidation and improves insulin responsiveness in the liver [28].

The interplay is bidirectional: hepatic steatosis and inflammation may feedback on adipose tissue by modulating whole-body lipid handling, thereby perpetuating a cycle of lipid overflow and systemic insulin resistance [29]. Collectively, the evidence provided by these studies highlights a coordinated and interdependent relationship between dysfunctional WAT and the liver, where excess lipid mobilization from adipose depots constitutes a central mechanism driving hepatic steatosis and insulin resistance in metabolic diseases [27], [28], [29].

1.4 Mitochondrial dysfunction and oxidative stress

1.4.1 Altered mitochondrial respiration and dynamics

Mitochondrial dysfunction in metabolic tissues is a recurring feature of insulin resistance and type 2 diabetes, and it encompasses both impaired respiratory capacity and altered organelle dynamics. Mitochondrial oxidative phosphorylation (OXPHOS) efficiency is commonly reduced in insulin-resistant states: electron transport chain (ETC) activity, ADP-stimulated respiration and ATP production are diminished in skeletal muscle and liver of insulin-resistant subjects and animal models, yielding lower cellular energetic output and reduced metabolic flexibility [30].

Loss of respiratory capacity can arise from decreased mitochondrial content and biogenesis (for example via reduced PGC-1α-driven transcriptional programs), from defects in individual ETC complexes, or from impaired substrate oxidation that reduces NADH/FADH2 supply to the ETC. These alterations lead to decreased ATP generation and to an increased propensity for electron leak from the ETC, which enhances formation of reactive oxygen species (ROS) under nutrient-excess conditions [31], [32].

Mitochondrial dynamics - the balance between fusion and fission - and the quality-control processes that govern mitophagy are also disrupted in metabolic

disease. Changes in fusion/fission proteins, reductions in mitochondrial mass and altered morphology (fragmentation or enlargement depending on context) have been reported in insulin-resistant muscle and adipose tissue and are associated with decreased oxidative capacity and higher ROS production. These structural and turnover defects compromise organelle function and the cell's ability to adapt to fluctuating energy demands, thereby contributing to systemic metabolic inflexibility [30], [31].

There is a two-way relationship between mitochondrial dysfunction and insulin signaling. On one hand, impaired insulin action blunts insulin's stimulatory effects on mitochondrial biogenesis and function (for example, insulin-dependent increases in mitochondrial ATP production and expression of oxidative enzymes are diminished in insulin-resistant subjects), which further reduces mitochondrial respiratory capacity [30].

On the other hand, primary defects in mitochondrial fatty acid oxidation can promote accumulation of lipid intermediates (e.g., acylcarnitines, diacylglycerols) that interfere with insulin signaling, linking mitochondrial impairment causally to insulin resistance in muscle and liver [30], [31].

Because mitochondria are a major source of intracellular ROS, respiratory impairment and altered substrate handling increase mitochondrial ROS generation. When ROS production overwhelms antioxidant defenses, oxidative damage to proteins, lipids and mitochondrial DNA follows, which in turn exacerbates dysfunction of the ETC and depolarizes the inner mitochondrial membrane, a feed-forward loop that sustains oxidative stress and promotes insulin resistance at the cellular level [32].

The degree and consequences of mitochondrial alterations vary by tissue and experimental model. Human studies frequently show reduced ADP-stimulated respiration and diminished insulin-stimulated mitochondrial responses in skeletal muscle of people with T2DM or with a family history of T2DM,

whereas some animal models with severe mitochondrial defects paradoxically show preserved or even improved insulin sensitivity depending on the nature and severity of the mitochondrial perturbation. These nuances indicate that mitochondrial dysfunction can be both a cause and a consequence of impaired insulin action, and that the relationship depends on the specific molecular context and compensatory responses [30], [31].

Interventions that restore mitochondrial biogenesis, improve fatty acid oxidation, or reduce mitochondrial ROS show promise in ameliorating insulin resistance in preclinical models; likewise, lifestyle measures such as exercise increase mitochondrial content and function and reduce oxidative stress, which is consistent with improvements in insulin sensitivity observed clinically [31].

1.4.2 ROS production and activation of stress pathways

Mitochondrial dysfunction in insulin resistant cells is closely associated with an imbalance between reactive oxygen species generation and the antioxidant capacity of the cell. Under physiological conditions, mitochondria produce low amounts of superoxide as a byproduct of oxidative phosphorylation, which is rapidly converted to hydrogen peroxide and detoxified through enzymatic systems such as superoxide dismutases, catalase and glutathione peroxidases [33]. In the context of nutrient excess, chronic lipid exposure and impaired electron transport chain activity, this balance is disrupted and the rate of mitochondrial ROS production increases significantly. Accumulation of diacylglycerols, ceramides and other lipid intermediates exacerbates this process by altering mitochondrial membrane integrity and favouring electron leakage [34], [35].

Elevated ROS levels initiate a cascade of stress responses that profoundly interfere with insulin signalling. Superoxide and hydrogen peroxide promote

the activation of redox sensitive kinases including JNK and I kappa B kinase (IKK). These kinases exert inhibitory effects on key nodes of the insulin signalling cascade, mainly through serine phosphorylation of IRS1, which reduces the ability of IRS1 to generate downstream PI3K and Akt activation. In parallel, ROS trigger the nuclear translocation of NF kappa B by promoting the degradation of IKK, thereby initiating the transcription of a broad array of pro inflammatory mediators. This creates a feed forward loop where oxidative stress and inflammation reinforce each other [36], [37].

Beyond direct effects on signalling molecules, ROS also influence mitochondrial biogenesis and dynamics. Altered expression of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), reduced mitochondrial turnover and impaired fusion and fission contribute to an overall decline in metabolic flexibility. As a consequence, cells become less capable of modulating substrate preference according to insulin availability, further undermining glucose uptake and lipid handling. Together, these events position ROS as central mediators linking mitochondrial dysfunction to defective insulin action and chronic metabolic inflammation [38], [39].

1.4.3 Consequences on metabolic flexibility and insulin action

Metabolic flexibility refers to the ability of a cell to adapt fuel utilization according to nutrient availability and hormonal signals. In healthy adipocytes and hepatocytes, insulin plays a pivotal regulatory role by promoting glucose uptake, enhancing glycolysis, stimulating lipogenesis when appropriate and suppressing lipolysis. Mitochondria support these transitions by modulating substrate oxidation rates, balancing NADH and FADH2 production and maintaining adequate ATP generation. When mitochondrial function becomes compromised, as in insulin resistant states, this adaptive capacity progressively deteriorates [40], [41].

Impaired oxidative phosphorylation reduces the efficiency of glucose oxidation, forcing cells to rely disproportionately on fatty acid oxidation or, conversely, to accumulate incompletely oxidized lipid intermediates depending on the metabolic context. In adipocytes, this results in a diminished ability to incorporate glucose into triglycerides and an inappropriate activation of lipolysis, which increases free fatty acid release into the circulation. These circulating lipids further exacerbate hepatic and peripheral insulin resistance through lipid induced activation of serine kinases and by contributing to ectopic lipid deposition [42], [43].

In hepatocytes, reduced metabolic flexibility alters the balance between gluconeogenesis and glycogen synthesis. Under physiological insulin stimulation, hepatic glucose production is suppressed and glycogen storage is favoured. In insulin resistant hepatocytes, defective Akt activation and impaired mitochondrial function converge to sustain gluconeogenic flux even in the presence of high insulin levels. This inappropriate endogenous glucose output is a major contributor to fasting hyperglycaemia in type 2 diabetes [17], [44].

At the signalling level, the loss of metabolic flexibility heightens cellular susceptibility to inflammatory and oxidative cues. Reduced mitochondrial capacity increases ROS production, which amplifies the activation of stress kinases such as JNK and IKK. These pathways further impair IRS1 function, creating a self-sustaining cycle where defective substrate oxidation, oxidative stress and inflammatory activation perpetuate insulin resistance. Therefore, the disruption of metabolic flexibility is not merely a consequence of insulin resistance but a central mechanistic link connecting mitochondrial dysfunction,

lipid oversupply and impaired insulin signalling [45], [46].

1.5 Pro-inflammatory signaling pathways

1.5.1 NFκB pathway and cytokine production

The NFκB pathway represents one of the principal inflammatory cascades activated during insulin resistance and plays a decisive role in sustaining chronic low grade inflammation in metabolic tissues. Under basal conditions, the transcription factor NFκB is retained in the cytoplasm through its association with inhibitory proteins of the IκB family. This prevents its nuclear translocation and maintains inflammatory gene expression under tight control. Metabolic stressors such as elevated free fatty acids, ceramides, hyperglycaemia and excessive mitochondrial ROS disrupt this equilibrium by activating upstream kinases, most notably IKK. When IKK phosphorylates IκB, the inhibitor becomes ubiquitinated and degraded, liberating NFκB to enter the nucleus [47], [48].

Once translocated, NFκB induces a transcriptional program that encompasses a wide range of pro inflammatory cytokines, chemokines, adhesion molecules and stress related mediators. Among the genes most robustly upregulated are tumour necrosis factor alpha (TNF-α), interleukin six (IL-6), monocyte chemoattractant protein one (MCP-1) and chemokines of the CXC family. These mediators amplify inflammation both locally, within adipose tissue and the liver, and systemically through endocrine like effects. Persistent NFκB activation also enhances the recruitment of immune cells into metabolic tissues, contributing to the shift from homeostatic resident immune populations to pro inflammatory phenotypes [49], [50].

Importantly, NFκB driven inflammation exerts direct inhibitory actions on insulin signalling. Cytokines such as TNF-α and IL-6 trigger serine phosphorylation of IRS1 through multiple kinase cascades, diminish Akt activation and reduce the transcription of metabolic genes essential for glucose disposal. In adipocytes, this translates into decreased GLUT4 expression and impaired lipid storage capacity, favouring lipolysis and the release of free fatty acids. In hepatocytes, NFκB activation supports gluconeogenic gene transcription and contributes to inappropriate hepatic glucose output. Through these mechanisms, NFκB acts not only as a sensor of metabolic imbalance but also as a central driver of the inflammatory milieu that perpetuates insulin resistance [51], [52].

1.5.2 JNK pathway and inhibitory phosphorylation of IRS1

The c-Jun N terminal kinase pathway is another major stress responsive signaling cascade implicated in the development of insulin resistance. JNK belongs to the mitogen activated protein kinase family and is activated by a wide array of metabolic stressors, including elevated free fatty acids, ceramides, endoplasmic reticulum stress and increased mitochondrial ROS. These stimuli converge on upstream MAP kinase kinases such as MKK4 and MKK7, which phosphorylate and activate JNK. Once activated, JNK translocates to the nucleus or acts in the cytoplasm to modify key metabolic proteins [53], [54].

A defining feature of JNK activation in insulin resistant states is its direct impact on IRS1. JNK phosphorylates IRS1 on specific serine residues, which reduces the ability of IRS1 to undergo proper tyrosine phosphorylation in

response to insulin. This modification destabilizes the formation of the IRS1 PI3K complex, limiting the propagation of downstream signaling and strongly impairing Akt activation. As a result, canonical metabolic responses to insulin, including glucose uptake, suppression of lipolysis and hepatic inhibition of gluconeogenesis, become markedly blunted [55], [56].

Beyond its actions on IRS1, JNK contributes more broadly to inflammatory amplification. Activated JNK enhances the transcription of pro inflammatory genes through c Jun dependent mechanisms and promotes apoptotic and pro fibrotic pathways in metabolic tissues. In adipose tissue, sustained JNK activation alters adipokine secretion by decreasing adiponectin and increasing the release of pro inflammatory mediators, which further aggravate systemic insulin resistance. In the liver, JNK activity reinforces lipid accumulation, contributes to hepatocyte stress responses and potentiates inflammatory signalling through cross activation of other kinases [57], [58].

Importantly, JNK and oxidative stress form a reciprocal loop. ROS activate JNK, and the activity of this protein promotes additional ROS generation by impairing mitochondrial function and encouraging lipid derived stress. This establishes a chronic inflammatory environment in both adipocytes and hepatocytes, stabilising the insulin resistant phenotype and rendering cells less responsive to metabolic cues. Through these combined actions, the JNK pathway represents a pivotal molecular axis linking nutrient excess, oxidative stress and defective insulin signalling [59], [60].

1.5.3 Crosstalk between NF-κB, JNK and lipid-induced stress

The development of insulin resistance is not driven by the isolated activation of individual inflammatory pathways, but rather by the convergence of multiple stress signals that reinforce one another. Among these, the interplay between NFκB, JNK and lipid induced stress represents a central integrative hub linking nutrient excess to chronic metabolic dysfunction. Elevated circulating free fatty acids, accumulation of intracellular diacylglycerols and the formation of bioactive lipids such as ceramides provide potent stimuli that activate both NFκB and JNK. These lipids interact with pattern recognition receptors, including Toll like receptor four, and alter intracellular membrane microdomains, thereby initiating kinase cascades that converge on inflammatory transcriptional programs [61], [62].

NFκB and JNK are highly interconnected at multiple regulatory levels. ROS generated during lipid overload activate both pathways simultaneously, creating a shared redox sensitive environment that fosters persistent inflammatory signalling. In addition, NFκB driven cytokines such as TNF-α can activate JNK, while JNK activity enhances the transcription of genes that indirectly potentiate NFκB signalling. This reciprocal activation ensures that once triggered, the inflammatory response becomes self-sustaining even when the initial metabolic insult fluctuates [48], [63].

Lipid induced stress further amplifies this network by directly impairing metabolic organelles. Ceramides inhibit Akt, reduce mitochondrial efficiency and promote additional ROS production, all of which enhance the activation of NFκB and JNK. Diacylglycerols activate novel protein kinase C isoforms that interfere with insulin receptor signalling and contribute to the phosphorylation

of IRS1 on serine residues. These events not only propagate inflammation but also reinforce the inhibition of insulin signalling at multiple levels [64], [65].

The combined actions of NFκB, JNK and lipid derived mediators create a metabolic environment in which inflammatory activation and defective insulin signalling mutually reinforce each other. This crosstalk contributes to the transition from acute, adaptive stress responses to chronic, maladaptive inflammation characteristic of adipose and hepatic insulin resistance. By integrating oxidative stress, lipid overload and pro inflammatory cytokine production, these interconnected pathways establish a stable molecular framework that maintains and aggravates the insulin resistant state [62], [66].

1.6 TNF-α as a Central Mediator in Insulin Resistance

1.6.1 TNF-α production in adipose tissue and liver

TNF-α is one of the most extensively studied inflammatory mediators involved in the pathogenesis of insulin resistance, and its production increases markedly in both adipose tissue and the liver during metabolic dysfunction. In adipose tissue, hypertrophic expansion of adipocytes is accompanied by local hypoxia, mechanical stress, altered extracellular matrix remodelling and excessive lipid turnover, conditions that strongly favour inflammatory activation. As adipocytes enlarge and become metabolically stressed, they begin secreting higher levels of pro inflammatory cytokines, including TNF-α. However, the majority of TNF-α in obese adipose tissue originates from infiltrating immune cells, particularly classically activated macrophages that accumulate around necrotic or dysfunctional adipocytes. This shift from resident anti-inflammatory macrophages to a pro inflammatory macrophage phenotype

represents a crucial turning point in the inflammatory amplification that characterises adipose insulin resistance [67], [68], [69].

In the liver, TNF-α production arises from multiple cellular sources. Kupffer cells, the tissue resident macrophages of the liver, are highly responsive to metabolic cues such as endotoxin leakage from the gut, elevated free fatty acids and oxidative stress. Upon activation, they release substantial amounts of tumour necrosis factor alpha, which acts locally on hepatocytes as well as systemically. Hepatocytes themselves can produce TNF-α under lipotoxic conditions, especially when exposed to high concentrations of saturated fatty acids that trigger endoplasmic reticulum stress and mitochondrial dysfunction. This autocrine and paracrine cytokine production contributes to the inflammatory microenvironment characteristic of non-alcoholic fatty liver disease and hepatic insulin resistance [70], [71].

The increased release of TNF-α from adipose tissue and liver has profound systemic consequences. Circulating TNF-α contributes to chronic low grade inflammation by acting on distant tissues, including skeletal muscle and the vascular endothelium, altering insulin responsiveness and promoting further cytokine production. In addition, TNF-α stimulates the expression of chemokines such as CXCL1, which enhance immune cell recruitment and establish positive feedback loops that intensify tissue inflammation. Through these mechanisms, elevated TNF-α production in adipose tissue and liver serves as a central driver of the inflammatory milieu that underlies metabolic dysfunction in obesity and type 2 diabetes [72], [73].

1.6.2 Mechanisms of action on IRS1, Akt, GLUT4/GLUT2

TNF-α interferes with insulin signalling through several coordinated mechanisms that act at multiple molecular levels, ultimately impairing glucose transport and metabolic regulation in both adipocytes and hepatocytes. One of the most well characterised actions of TNF-α is its capacity to promote inhibitory serine phosphorylation of IRS1. This occurs through the activation of a network of kinases, including JNK, I kappa B kinase and certain isoforms of protein kinase C, all of which are stimulated by tumour necrosis factor alpha driven inflammatory signalling. Serine phosphorylated IRS1 exhibits reduced affinity for the insulin receptor and diminished ability to undergo tyrosine phosphorylation following insulin stimulation. As a consequence, the recruitment of PI3K is compromised, limiting the propagation of downstream insulin signals [63], [74].

Further downstream, TNF-α inhibits Akt activation through both direct and indirect mechanisms. In adipocytes, defective IRS1 PI3K engagement reduces PIP3 generation at the plasma membrane, which is essential for Akt phosphorylation and activation. In addition, TNF-α driven ceramide accumulation can directly interfere with Akt by stimulating protein phosphatase 2A, a phosphatase that dephosphorylates and inactivates Akt. Reduced Akt activity has profound metabolic consequences because Akt is required for the suppression of lipolysis, the stimulation of glycogen synthesis and, crucially, the promotion of glucose transporter translocation [65], [75].

GLUT4 translocation to the plasma membrane in adipocytes is particularly sensitive to tumour necrosis factor alpha. By limiting Akt activity, TNF-α diminishes the activity of AS160, a GTPase activating protein that regulates Rab dependent trafficking of GLUT4 storage vesicles. As a result, GLUT4

containing vesicles remain sequestered in the cytoplasm, preventing efficient glucose uptake despite the presence of insulin. Additionally, chronic TNF-α exposure suppresses GLUT4 gene expression by altering the activity of transcription factors that control adipocyte differentiation and metabolic gene programs, further contributing to impaired glucose disposal [76], [77].

In hepatocytes, TNF-α disrupts insulin mediated regulation of glucose production by targeting the GLUT2 mediated arm of hepatic glucose handling. Although GLUT2 is not insulin responsive in the same manner as GLUT4, its function is strongly influenced by the integrity of the IRS1/Akt pathway. Reduced Akt activity leads to inadequate suppression of gluconeogenic enzymes and diminished glycogen synthesis, thereby maintaining elevated hepatic glucose output. Moreover, tumour necrosis factor alpha enhances the expression of gluconeogenic drivers such as PGC1 alpha, creating a metabolic environment in which insulin is unable to effectively restrain glucose production [78], [79].

Through these combined actions on IRS1, Akt and the glucose transport machinery, TNF-α exerts a powerful inhibitory influence on insulin signalling in metabolic tissues. This multi-level disruption not only impairs acute insulin responses but also contributes to the long term establishment of a chronically insulin resistant phenotype [80].

1.6.3 Autocrine and paracrine effects in metabolic tissues

TNF-α exerts its biological effects in metabolic tissues through a combination of autocrine and paracrine mechanisms that amplify local inflammation and progressively deteriorate insulin sensitivity. In adipose tissue, adipocytes exposed to metabolic stress begin secreting TNF-α, which in turn acts back on the same adipocytes (autocrine signalling) to further impair insulin

responsiveness. This self-reinforcing loop promotes inhibitory phosphorylation of IRS1, reduces Akt activation and limits GLUT4 translocation, thereby compromising glucose uptake. In parallel, TNF-α depresses the expression of key adipogenic and insulin responsive genes, contributing to a dedifferentiated, dysfunctional adipocyte phenotype [77], [81].

Paracrine signalling is even more influential. TNF-α released by infiltrating macrophages diffuses within adipose tissue and potently activates neighbouring adipocytes, endothelial cells and additional immune cells. This creates a microenvironment enriched in pro inflammatory mediators, chemokines and lipid derived stress signals. One of the major consequences of this paracrine activity is the recruitment and activation of further macrophages, often forming crown like structures around stressed or dying adipocytes. These newly recruited immune cells become an additional source of TNF-α, reinforcing the inflammatory loop and sustaining chronic metabolic dysfunction [67], [82].

A similar dual mechanism operates in the liver. Kupffer cells produce substantial quantities of TNF-α in response to free fatty acids, gut derived endotoxins and oxidative stress. This TNF-α acts paracrinally on hepatocytes to inhibit IRS1 activation, reduce Akt mediated suppression of gluconeogenesis and impair normal glucose handling. Hepatocytes themselves can also release tumour necrosis factor alpha under conditions of lipotoxicity or endoplasmic reticulum stress, creating an autocrine loop that further compromises mitochondrial function and enhances oxidative stress. Over time, these interactions contribute to steatosis, inflammatory infiltration and the establishment of a metabolically inflamed hepatic niche [71], [83].

The systemic metabolic consequences of these autocrine and paracrine processes are substantial. TNF-α released from adipose tissue and the liver enters the circulation and acts on skeletal muscle, pancreatic islets and vascular endothelium, promoting widespread insulin resistance and contributing to the progression of type 2 diabetes. Through this integrated network, tumour necrosis factor alpha serves both as a local amplifier of inflammation and as a systemic mediator linking adipose and hepatic dysfunction to whole body metabolic impairment [72], [84].

1.7 Chemokines and CXCR1/2 Signaling in Metabolic Regulation

1.7.1 Chemokines (CXCL8/IL-8, CXCL1) and their role in inflammation

Chemokines are central coordinators of immune cell trafficking and play a crucial role in shaping the inflammatory responses associated with obesity and insulin resistance. Among the CXC chemokines, CXCL8 (also known as interleukin 8) and CXCL1 are particularly important because they mediate the recruitment and activation of neutrophils, monocytes and other inflammatory cells within metabolic tissues. Although originally characterized for their role in acute inflammation, these chemokines acquire distinct pathogenic functions under chronic metabolic stress, where their sustained production contributes to the low grade inflammatory state typical of adipose and hepatic insulin resistance [85], [86].

CXCL8 and CXCL1 are produced by multiple cell types in response to metabolic and inflammatory stimuli. In adipose tissue, hypertrophic adipocytes exposed to elevated free fatty acids, ceramides and oxidative stress upregulate chemokine synthesis through NFκB and AP1 dependent transcriptional programs. Immune cells that infiltrate expanding adipose depots, such as macrophages and neutrophils, further contribute to chemokine release, amplifying local inflammation. Endothelial cells and fibroblasts in the stromal vascular fraction also participate in chemokine production, particularly when exposed to cytokines such as TNF-α or interleukin one beta (IL-1β) [85], [87].

These chemokines exert potent chemotactic activity by binding to receptors of the CXC family, primarily CXCR1 and CXCR2. Their engagement promotes the migration of immune cells toward regions of metabolic stress, enhancing local inflammation. Importantly, the accumulation of immune cells is not simply a consequence of chemokine release but a key driver of insulin resistance. Recruited immune cells secrete additional cytokines, chemokines and reactive oxygen species, establishing a self-reinforcing inflammatory circuit that affects adipocyte and hepatocyte insulin signalling [86], [88].

Beyond immune cell recruitment, CXCL8 and CXCL1 can exert direct effects on metabolic cells. Emerging evidence indicates that activation of CXCR1 and CXCR2 on adipocytes and hepatocytes promotes intracellular signalling cascades involving ERK1/2, p38 MAPK and NFκB, which can interfere with insulin responsiveness. In adipose tissue, chemokines contribute to altered adipokine secretion and impaired lipid storage capacity, while in the liver they promote inflammatory stress that exacerbates steatosis and disrupts insulin mediated metabolic control. Thus, CXCL8 and CXCL1 act not only as inflammatory messengers but also as modulators of cellular function within metabolic organs [81], [88].

Through these combined mechanisms, CXCL8 and CXCL1 play a central role in bridging metabolic stress and immune activation. Their persistent production under conditions of nutrient excess and inflammation contributes to the chronic, maladaptive immune responses that drive insulin resistance in obesity and type 2 diabetes [86].

1.7.2 Expression of CXCR1/2 in adipocytes and hepatocytes

CXCR1 and CXCR2, the principal receptors for CXCL8 and CXCL1, were historically studied within the context of immune cell biology, particularly neutrophil recruitment and activation. However, growing evidence demonstrates that these receptors are also expressed in non-immune metabolic tissues, including adipose tissue and the liver, where they participate directly in the regulation of inflammatory signalling and metabolic homeostasis. Their presence in these organs provides a mechanistic link through which chemokine driven inflammation can exert cell autonomous effects on insulin signalling [89], [90].

In adipose tissue, both adipocytes and cells of the stromal vascular fraction express CXCR1 and CXCR2 at variable levels. Mature adipocytes show upregulation of CXCR1 and CXCR2 under conditions of metabolic stress, such as exposure to free fatty acids, oxidative stress or pro inflammatory cytokines including TNF-α. This inducible expression pattern suggests that adipocytes become increasingly sensitive to chemokine signalling as inflammation progresses. Stromal vascular cells, including macrophages, preadipocytes, endothelial cells and fibroblasts, also express these receptors, thereby

contributing to a complex chemokine responsive microenvironment within adipose depots [85], [91].

In the liver, CXCR1 and CXCR2 are expressed on hepatocytes as well as on non-parenchymal cells such as Kupffer cells, hepatic stellate cells and sinusoidal endothelial cells. Hepatocytes upregulate CXCR2 expression in response to lipotoxic stress, mitochondrial dysfunction and pro inflammatory cytokines, allowing them to respond directly to chemokine gradients generated within the hepatic microenvironment. Kupffer cells, which are major sources of chemokines in the liver, also express these receptors, creating autocrine and paracrine loops that reinforce inflammatory activation and immune cell recruitment [92], [93].

The functional consequences of CXCR1 and CXCR2 expression in metabolic tissues are increasingly recognised. Activation of these receptors in adipocytes can stimulate intracellular pathways including ERK1/2, JNK, p38 MAPK and NFκB, promoting cytokine production, oxidative stress and impaired insulin signalling. In hepatocytes, CXCR1 and CXCR2 activation amplifies inflammatory responses, supports lipid accumulation and interferes with insulin mediated suppression of gluconeogenesis. These effects position CXCR1 and CXCR2 as active participants in the metabolic deterioration characteristic of obesity and type 2 diabetes [90], [94].

Overall, the expression of CXCR1 and CXCR2 in adipocytes and hepatocytes highlights the capacity of chemokines to influence metabolic cell behaviour independently of immune cell recruitment. By enabling direct crosstalk between chemokine signalling and insulin responsive pathways, these receptors extend the impact of chronic inflammation deep into the metabolic architecture of adipose tissue and the liver [81].

1.7.3 Evidence linking CXCR1/2 signaling in obesity, IR and T2DM

A growing body of experimental, clinical and translational evidence implicates CXCR1 and CXCR2 signalling in the development and progression of obesity associated inflammation, insulin resistance and type 2 diabetes. Initial clues emerged from studies showing that circulating levels of CXCL8, CXCL1 and related CXC chemokines are markedly elevated in individuals with obesity, metabolic syndrome and T2DM, with plasma concentrations correlating positively with indices of insulin resistance, hepatic steatosis and systemic inflammation. These findings suggest that aberrant chemokine production reflects not only immune activation but also metabolic dysfunction at the tissue level [85], [86].

Preclinical studies have provided mechanistic insight into these associations. In mouse models of diet induced obesity, genetic or pharmacological inhibition of CXCR2 reduces adipose tissue inflammation, limits macrophage and neutrophil infiltration and improves glucose tolerance and insulin sensitivity. Mice lacking CXCR2 exhibit reduced expression of pro inflammatory cytokines in adipose tissue and display lower hepatic triglyceride accumulation, highlighting a multi organ improvement in metabolic function when chemokine signalling is suppressed. Conversely, overactivation of the CXCR2 axis aggravates inflammatory recruitment and accelerates the decline of insulin responsiveness, emphasizing the causal contribution of chemokine receptor signalling to metabolic deterioration [90], [95].

In adipocytes, activation of CXCR1 and CXCR2 interferes directly with insulin signalling by promoting the activation of stress kinases such as ERK1/2, JNK

and NFκB. These pathways converge on serine phosphorylation of IRS1, reduced Akt activation and impaired GLUT4 translocation, ultimately diminishing glucose uptake. Similar effects have been observed in hepatocytes, where CXCR2 activation exacerbates lipotoxicity, enhances gluconeogenic gene expression and interferes with insulin mediated metabolic regulation. These cell autonomous responses provide a mechanistic basis for the observed systemic associations between chemokine levels and insulin resistance [53], [96].

Clinical studies further support the involvement of CXCR1 and CXCR2 in human metabolic disease. Elevated expression of CXCL8 and CXCL1 has been documented in adipose tissue biopsies from individuals with obesity and type 2 diabetes, particularly in depots enriched in inflammatory macrophages. Increased hepatic expression of CXCR2 has been reported in patients with non-alcoholic fatty liver disease, where it correlates with inflammation, fibrosis and metabolic impairment. Importantly, the chemokine profile of both plasma and tissue samples is strongly modulated by weight loss interventions and insulin sensitizing therapies, suggesting that chemokine signalling is dynamically linked to metabolic status and not merely a passive byproduct of obesity [97], [98].

Together, these findings establish CXCR1 and CXCR2 as key molecular nodes integrating inflammation and metabolic dysfunction. By promoting immune cell recruitment, amplifying inflammatory circuits and directly impairing insulin signalling in adipocytes and hepatocytes, CXCR1/2 signalling contributes substantially to the pathogenesis of insulin resistance and type 2 diabetes. This mechanistic and translational evidence provides a strong rationale for the exploration of CXCR1/2 antagonists as potential therapeutic strategies for metabolic disease [81], [95].

1.8 PPARα and Nuclear Receptors in Metabolic Regulation

1.8.1 PPARα as a regulator of lipid metabolism and anti-inflammatory responses

Peroxisome proliferator activated receptor alpha (PPARα) is a nuclear receptor that plays a central regulatory role in lipid metabolism, energy homeostasis and the resolution of inflammation. PPARα is highly expressed in metabolically active tissues with substantial oxidative capacity, including the liver, brown adipose tissue, skeletal muscle and the heart. In these organs, PPARα functions as a lipid sensor that responds to fluctuations in fatty acid availability by modulating gene expression programs that promote fatty acid oxidation and maintain metabolic balance [99], [100].

Activation of PPARα by endogenous ligands such as long chain fatty acids and their derivatives induces the expression of a broad network of genes involved in mitochondrial and peroxisomal beta oxidation, fatty acid transport, ketogenesis and lipid handling. Among the target genes upregulated by PPARα are Carnitine palmitoyltransferase I (CPT1A), Peroxisomal acyl-coenzyme A oxidase 1 (ACOX1), Medium-chain specific acyl-CoA dehydrogenase (MCAD) and Fatty Acid-Binding Protein 1 (FABP1), which collectively enhance cellular capacity to oxidize fatty acids and prevent the intracellular accumulation of lipotoxic lipid intermediates. By channelling fatty acids into oxidative pathways, PPARα alleviates metabolic stress and reduces the formation of diacylglycerols and ceramides that otherwise impair insulin signalling [99], [101].

Beyond its metabolic functions, PPARα exerts strong anti-inflammatory actions that contribute to its protective effects in metabolic disease. Activation of PPARα interferes with NFκB and AP1 signalling through multiple mechanisms, including trans-repression of pro inflammatory transcriptional complexes and inhibition of co activator recruitment. As a result, PPARα reduces the expression of cytokines, chemokines and adhesion molecules that drive chronic low grade inflammation in adipose tissue and the liver. It also promotes the expression of genes involved in oxidative stress defence, thereby limiting ROS production and attenuating the activation of stress kinases such as JNK [102], [103].

These combined metabolic and anti-inflammatory activities position PPARα as a key regulator of insulin sensitivity. In the liver, PPARα activation enhances fatty acid oxidation, reduces hepatic steatosis and lowers gluconeogenic drive, thereby improving insulin mediated control of glucose production. In adipose tissue, PPARα helps maintain lipid homeostasis and counterbalances inflammatory activation, reducing the spillover of free fatty acids and systemic inflammatory mediators that contribute to insulin resistance [99], [100].

Overall, PPARα acts at the intersection of lipid metabolism and inflammation, orchestrating adaptive responses that protect metabolic tissues from nutrient excess and inflammatory stress. Its role as a stabilizing factor in metabolic homeostasis provides an important conceptual framework for understanding how perturbations in nuclear receptor signalling can exacerbate insulin resistance and how pharmacological modulation of this pathway may confer therapeutic benefit [104], [105].

1.8.2 Interaction between PPARα and CXCR1/2 in adipose tissue and liver

The interplay between PPARα and the CXCR1/2 chemokine axis represents an emerging area of interest in the understanding of how metabolic and inflammatory signals converge to shape insulin sensitivity in adipose tissue and the liver. Although these pathways have traditionally been studied separately, accumulating evidence suggests that alterations in PPARα activity can influence chemokine signalling and, conversely, that CXCR1/2 driven inflammation can disrupt PPARα mediated metabolic regulation. This bidirectional relationship provides a mechanistic framework through which chronic inflammation and impaired lipid handling jointly contribute to insulin resistance [106], [107].

PPARα activation exerts a restraining influence on the chemokine system by suppressing the expression of CXCL8, CXCL1 and related inflammatory mediators. Through its well characterised ability to antagonise NFκB and AP1 activities, PPARα reduces the transcription of pro inflammatory chemokines in both adipocytes and hepatocytes. This dampening effect limits the recruitment of immune cells to metabolic tissues and decreases the autocrine and paracrine inflammatory loops that otherwise amplify CXCR1/2 signalling. By promoting fatty acid oxidation and reducing the intracellular accumulation of diacylglycerols and ceramides, PPARα also lowers the lipotoxic burden that stimulates chemokine production, reinforcing a state less permissive to chronic inflammation [108], [109].

Conversely, activation of CXCR1 and CXCR2 can impair PPARα function. Chemokine driven activation of ERK1/2, JNK and NFκB interferes with nuclear receptor signalling by promoting inhibitory phosphorylation events,

reducing co activator availability and altering chromatin accessibility at PPARα responsive gene promoters. In this context, hepatocytes and adipocytes exposed to sustained CXCR1/2 activation exhibit reduced expression of PPARα target genes involved in fatty acid oxidation, which contributes to lipid accumulation, mitochondrial dysfunction and the exacerbation of insulin resistance. Such reciprocal inhibition highlights a competitive dynamic between pro inflammatory chemokine signalling and lipid oxidative pathways [108], [110].

In adipose tissue, this antagonism results in impaired lipid buffering capacity, increased free fatty acid release and enhanced recruitment of inflammatory cells. In the liver, diminished PPARα activity under heightened CXCR1/2 signalling promotes steatosis, increases oxidative stress and amplifies inflammatory circuits, further disrupting insulin mediated control of glucose production. Thus, the cross regulation between PPARα and CXCR1/2 creates a self-reinforcing cycle in which inflammatory and metabolic dysfunctions perpetuate each other [111], [112].

This reciprocal relationship underscores the importance of integrating metabolic and inflammatory pathways when studying insulin resistance. The close interaction between PPARα and the CXCR1/2 axis provides a compelling biological rationale for exploring chemokine receptor antagonists as modulators of metabolic homeostasis, forming the conceptual basis that leads directly into the experimental aims of your study.

2 AIM OF THE STUDY

Type 2 diabetes and obesity are characterized by a chronic, low-grade inflammatory condition that profoundly affects insulin sensitivity. Among the mediators linking inflammation to metabolic dysfunction, TNF-α plays a central role in impairing insulin signalling through mechanisms that involve defects in IRS1 and IRS2 phosphorylation, inhibition of the PI3K/Akt pathway and dysregulation of glucose transporters. Increasing evidence suggests that the chemokine network activated downstream of TNF alpha, in particular the

CXCR1 and CXCR2 receptors and their ligands, may represent an additional regulatory system capable of sustaining and amplifying insulin resistance. However, the specific contribution of these receptors to metabolic dysfunction in insulin responsive cells is still not completely clarified.

The overall objective of this study is to elucidate the role of CXCR1 and CXCR2 in TNF alpha induced insulin resistance in *in vitro* models, through integrated molecular, biochemical and functional approaches. In particular, the study pursued the following objectives:

1. Characterize the expression and modulation of CXCR1 and CXCR2 in insulin responsive murine cell models under basal conditions and after TNF alpha treatment.
2. Determine whether the inhibition or silencing of CXCR1 and CXCR2 can restore insulin signalling pathways, with specific attention to the IRS to PI3K to Akt cascade, and recover the expression and membrane translocation of glucose transporters (GLUT4 in adipocytes and GLUT2 in hepatocyte like cells).
3. Evaluate the functional relevance of blocking CXCR1 and CXCR2 by measuring glucose uptake and metabolic competence in TNF alpha treated insulin resistant cells.
4. Investigate how the inhibition of CXCR1 and CXCR2 influences stress related pathways, including JNK activation and inflammatory gene expression, in order to better understand the molecular mechanisms behind the observed metabolic improvements.
5. Assess the potential of Ladarixin, a dual inhibitor of CXCR1 and CXCR2, as a molecule capable of counteracting inflammation associated insulin resistance.

By addressing these objectives, the study aims to define the CXCR1 and CXCR2 axis as a mechanistic link between inflammation and impaired insulin action, and to explore its potential as a therapeutic target in metabolic diseases. This framework provides a comprehensive basis for understanding how chemokine signalling intersects with insulin pathways and supports the development of anti-inflammatory strategies designed to improve metabolic homeostasis.

3 MATERIAL AND METHODS

3.1 Insulin-resistant adipocytes

3T3-L1 murine preadipocytes cell line was bought from ATCC (American Type Culture Collection, Manassas, VA, USA), and cultured in DMEM with 10% newborn calf serum (NCS) at 37 °C in a 5% CO2 atmosphere until confluence. At 2 days after full confluence, cells were differentiated using DMEM (10% FBS) supplemented with 0.5 mM isobutylmethylxanthine (IBMX), 1 µM dexamethasone, 1 µg/ml insulin for 48 h, and then for 2 days in DMEM (10% FBS) containing 1 µg/ml insulin alone following manufacturer's protocols (Sigma-Aldrich, St. Louis, MO, USA) [18]. Cells were maintained and refed every 2 days with DMEM containing 10% FBS. After 22 days, more than 80% of the cells exhibited the adipocyte phenotype with large lipid droplets in the cytoplasm.

The IR condition was obtained by incubating the differentiated cells with TNF-a 50 ng/mL for 24 h. Induction of IR was proven by the ability of insulin to stimulate glucose uptake. Once the model was established, adipocytes were

treated for 24 h with Ladarixin at 10 μM, a concentration that has already shown to be safe and effective in previous *in vitro* study [18], then cellular biochemical parameters were analyzed in the presence or absence of stimulation with insulin (1 μM) for 30 min (short-term insulin). To activate the insulin pathway, insulin was added at the end of 24h at 1 μM concentration for 20 min. Different treatments were used to study the effects on TNF-induced IR adipocytes.Adipocytes cells were divided into 6 groups:

1. Control cells (CTR)

2. Control cells challenged with a short-term exposure to insulin (CTR + INS)

3. TNF-a treated cells (TNF)

4. TNF-a treated cells subsequently challenged with a short-term exposure to insulin (TNF-a+INS)

5. TNF-a treated cells upon Ladarixin treatment (TNF-a+LAD)

6. TNF-a treated cells upon Ladarixin treatment subsequently challenged with a short-term exposure to insulin (TNF-a+LAD + INS)

3.2 Insulin-resistant hepatocytes

Mouse liver cell line FL83B CRL-2390™ was purchased from ATCC and cultured following the manufacturer's protocols. Specifically, cells were incubated in F12-K Medium containing 10% FBS (all products from ATCC) in Petri dishes at 37 °C and 5% CO2. After 2 days from seeding, the IR condition was obtained by incubating the cells with TNF-α 20 ng/mL for 24 h with and

without 10 μM of Ladarixin. To activate the insulin pathway, insulin was added at the end of 24h at 1 μM concentration for 30 min. Different treatments were used to study the effects on TNF-induced IR hepatocytes.

Hepatocytes cells were divided into 6 groups:

1. Control cells (CTR)

2. Control cells challenged with a short-term exposure to insulin (CTR + INS)

3. TNF-a treated cells (TNF)

4. TNF-a treated cells subsequently challenged with a short-term exposure to insulin (TNF-a+INS)

5. TNF-a treated cells upon Ladarixin treatment (TNF-a+LAD)

6. TNF-a treated cells upon Ladarixin treatment subsequently challenged with a short-term exposure to insulin (TNF-a+LAD + INS)

3.3 CXCR1/2 silencing

siRNA knockdown was performed using Lipofectamine™ RNAiMAX Transfection Reagent (ThermoFisher Scientific, Waltham, MA, USA) and CXCR1 (ID s105602) and/or CXCR2 (ID s64085) specific siRNA duplexes (ThermoFisher Scientific) according to the manufacturer's instructions. FL38B hepatocyte and differentiated 3T3-L1-adipocytes were seeded to 1 × 104 cells/cm2 in a 96 well plate overnight and trasfected with 1 pmol of CXCR1

and/or CXCR2 siRNA duplexes, or 1 pmol of negative control siRNA duplexes. Knockdown of CXCR1/CXCR2 expression was confirmed after 72 h by evaluating CXCR1 and CXCR2 gene expression via qRT-PCR.

3.4 Neutralization with antibodies

Mature adipocytes and hepatocytes were treated with neutralizing antibodies against CXCR1 and CXCR2. Cells were incubated with anti-CXCR1 (CD Creative Diagnostics, Shirley, NY, USA) and anti-CXCR2 (GeneTex, Irvine, CA, USA) antibodies (according to the manufacturer's protocols) for 24 h at 37 °C in a humidified atmosphere containing 5% CO2. Control cells were treated with an equal concentration of isotype-matched control antibodies.

3.5 Glucose uptake

To monitor the uptake of glucose, mature adipocytes and hepatocytes upon different treatments were incubated with the fluorescent tracer 2-NBDG (2-Deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose; Sigma-Aldrich) as previously described [18]. Data are reported as 2-NBDG fluorescence intensity.

3.6 Nile Red Staining

Nile Red is a commonly used neutral lipid stain to detect the adipogenesis of different types of cultured cells. Briefly, after culturing and treating as previously described, cells were washed with PBS and incubated with Nile Red (final concentration 50 ng/mL) for 30 min, then washed and observed at fluorescence microscopy (Olympus, Leica, Germany).

3.7 Western blotting

Control and treated hepatocytes and adipocytes were collected and lysed in ice-cold RIPA buffer with freshly added protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific). 30 μg of proteins were loaded on Bolt Bis-Tris Glycine gradient precast gel (Invitrogen, Carlsbad, CA, USA) and transferred onto a PVDF membrane using a power blotter-semi-dry transfer system (Thermo Fisher Scientific). Non-specific binding sites were blocked by Blocking Buffer (Invitrogen) for 10 min at RT. Membranes were then incubated overnight at 4 °C with the following primary antibodies, diluted in blocking buffer: anti-Akt 1:1000 (4691 s Cell Signaling Technology, Danvers, MA, USA), anti-pAkt 1:1000 (4060 s Cell Signaling Technology, Danvers, MA, USA), anti-GLUT4 1:1000 (2213 s Cell Signaling Technology, Danvers, MA, USA), anti-JNK 1:200 (9252 s Cell Signaling Technology, Danvers, MA, USA), anti-PPARα 1:250, anti-pJNK 1:200 (9255 s Cell Signaling Technology, Danvers, MA, USA), and conjugated Actin 1:20000 (5125S, Cell Signaling Technology, Danvers, MA, USA). As secondary antibodies, 1:20,000 peroxidase-conjugated anti-rabbit or anti-mouse IgG were used. Immunoreactive bands were visualized by luminol (Thermo Fisher Scientific), according to the manufacturer's protocols, and visualized at iBright digital system (Thermo Fisher Scientific). The relative densities of the immunoreactive bands were determined and normalized to tubulin or actin or histone H3 or their respective total forms using Fiji software. Values were reported as relative units.

3.8 Subcellular protein fractionation assay

For the subcellular protein fractionation, cells were cultured as described above, collected and incubated with provided buffer to extract the

cytoplasmatic, membrane nuclear components as previously published [18] and then performed the Western blotting analyses as described above.

3.9 Real-Time PCR

Total RNA was extracted by Miniprep column Qiagen according to the manufacturer's instructions. To improve the extraction procedure of adipocytes, detergents (Deoxycholate-Nonidet P40) were added. The total RNA concentration was determined in RNAse-free water using Nanodrop, while the concentration was determined using Qubit Fluorometer 3.0 (Thermo Fisher Scientific). 1 µg of total RNA was reverse transcribed using a 5X All-In-One RT MasterMix (Applied Biological Materials, Richmond, BC, Canada) into cDNA using Thermo-block (Eppendorf AG, Hamburg, Germany). Finally, the real-time PCR was carried out on ABI 7300HT sequence detection system (Applied Biosystems, Waltham, MA, USA), containing 2X TaqMan Gene Expression Master Mix (Invitrogen), DEPC water and 5 µL of cDNA and 1 µL of the following primers. Prime Time qPCR Assays: mouse CXCL1, CXCR1, CXCR2, IRS1, IRS2, GLUT2, IGF, GLUT4, Adiponectin were purchased from ThermoScientific. Triplicate samples were run for each gene. The reference gene GAPDH was used as an internal control to normalize the expression of target genes. Relative expression levels were analyzed for each sample after normalization against the reference gene, using the ΔΔCt method for comparing relative fold expression differences.

3.10SeaHorse analysis

Seahorse XF96e Extracellular Flux Analyzer (Agilent Technologies, CA, USA) to investigate the bioenergetic profiles of our IR *in vitro* models upon different conditions was used. Live-cell evaluations of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were detected by the Mito Stress assay (Agilent). Cells were seeded at a density of 5 × 104 cells/well (optimized

to guarantee a proportional response of FCCP) on a Seahorse XF96 plates then treated as described above. Mito Stress assay was performed following manufacturer's protocols, and the concentrations of the drugs were: 1 µM oligomycin, 1 µM FCCP, 0.5 µM rotenone/antimycin. For the normalization in port D, Hoechst 33,342 solution was added. Data were obtained using Wave software.

3.11 Adiponectin mouse ELISA assay

To assess the released adiponectin in cell culture media from mature 3T3-L1 at the different conditions tested, an adiponectin mouse ELISA assay was used as previously described [18]. The results were read immediately at 450 nm in a plate reader. Data were expressed as ng/ml.

3.12 GLUT4 mouse ELISA assay

The amount of glucose transporter type 4 (GLUT4) in adipocyte plasma membranes was evaluated using the ELISA KIT by MyBioSource, Inc (San Diego, CA, USA) (#MBS2022770) following the manufacturer's instructions and as previously published [18]. Briefly, the cells were counted and lysed as suggested, then the Standard curve as well as the reagents were prepared and used following the assay procedure. The microplate provided in this kit has been pre-coated with an antibody specific to GLUT4. Standards or samples are then added to the appropriate microplate wells with a biotin-conjugated antibody specific to GLUT4. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After the TMB substrate solution is added, only those wells that contain GLUT4, biotin-conjugated antibody, and enzyme-conjugated Avidin will exhibit a color change. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured

spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of GLUT4 in the samples is then determined by comparing the O.D. of the samples to the standard curve. Data were presented as ng/ml.

3.13 CXCL1/KC (mouse IL-8) ELISA assay

To evaluate the CXCL1/KC released from mature adipocytes in different conditions CXCL1/KC ELISA assay was performed following the manufacturer's protocol (R&D System, Minneapolis, USA). Briefly, the plate was prepared the day before using Capture Antibody. Then, we followed the assay procedure preparing the standard curve and the samples, then the substrate solution was incubated for 20 min at room temperature, followed by the addition of stop solution. The optical density of each well was quickly determined using a microplate reader set to 450 nm. Data were expressed as pg/ml.

3.14 Adipocyte lipolysis colorimetric assay

To evaluate the lipolysis, cells were grown and differentiated as previously described in a 48 wells plate. After 22 days, cells were washed two times with wash buffer and the wash buffer was replaced with lipolysis assay buffer. Then, isoproterenol (100 nM) was added to stimulate lipolysis 2 h and the medium was collected. 25 µl of media were added into 96well plate and the volume was adjusted with lipolysis Assay buffer to 50 µl. Standard curve was prepared following manufacturer's protocol as well as reaction mix. The plate was incubated at room temperature for 30 min protected from light. The results were read at OD 570 nm in a plate reader. Data were expressed as glycerol content (nmol/well).

3.15 High Sensitivity Lipolysis Assay for hepatocytes

To evaluate the lipolysis in hepatocytes, High Sensitivity Lipolysis assay was performed following manufacturer's instruction (Sigma-Aldrich, #MAK215). Cells were grown in a black 96-well plate and treated as described above. Cells were washed two times with wash buffer, and then with lipolysis assay buffer. To stimulate lipolysis, isoproterenol (100 nM) was added and incubated for 8 h, and the medium was collected. 25 µL of media were added into 96 well-plates, and the volume was adjusted with lipolysis Assay buffer to 50 µL. The standard curve of glycerol and reaction mix were prepared. The plate was incubated at room temperature for 1 h protected from light. Lipolysis was determined by measuring a fluorescent product (λex = 535/ λem = 587 nm) proportional to the amount of glycerol present using the microplate reader Spark, Tecan and analyzed following instruction provided.

3.16 Glycolysis assay

To evaluate the glycolysis, hepatocytes were grown in a black 96-well plate and treated as described above and glycolysis assay was performed following manufacturer's instruction (Abcam, Waltham, USA #ab197244). While preparing all the required reagents, tested compound and Oxamic acid (negative control, decreases ECA) cells, a CO2 purge by incubating cells was performed in a CO2-free incubator at 37 °C with 95% humidity, approx. 3 h prior to performing the Glycolysis assay measurement.

We then washed cells with Respiration Buffer twice. Add 150 µL of Respiration Buffer to all wells containing cells. To the negative control 10 µL of oxamic acid solution (750 mM in ddH2O) to wells containing cells were added. 10 µL reconstituted Glycolysis Assay Reagent to each sample well were added and 10 µL of test compound (vehicle control and/or stock) were added to the wells.

Plate was read at λex = 380/ λem = 615 nm using the microplate reader Spark, Tecan and analyzed following instruction provided.

3.17 Glycogen assay

To evaluate the glycogen, hepatocytes were grown and treated as described above and glycogen assay was performed following manufacturer's instruction (Abcam, #ab169558). For the sample preparation cells were rapidly homogenized on ice and then boiled for 10 mins to inactivate enzymes and then centrifuged. Supernatants were collected to perform the assay using 50 µg of proteins each well. In parallel, standards were prepared as indicated and both samples and standards were added to each well at the final volume of 50 µl. Hydrolysis enzyme was then added and incubated for 30 min at room temperature. Finally, reaction mix was added and incubated at room temperature for 30 mins. Plates were read at OD450 nm using a microplate reader Spark, Tecan and analyzed following instruction provided.

3.18 Statistical analyses

All data were presented as mean ± SE. Data analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). For multiple comparisons one-way analysis of variance (ANOVA) followed by Tukey post-hoc tests was used. The level of significance was set at $p < 0.05$.

4 RESULTS

4.1 Evaluation of the involvement of CXCR1/CXCR2 in IR-adipocytes and hepatocytes

To investigate the contribution of chemokine receptors CXCR1 and CXCR2 to inflammation associated insulin resistance, we first assessed their expression and regulation in two complementary cellular systems: differentiated 3T3-L1 adipocytes and FL83B hepatocyte like cells. These models were selected because they capture key aspects of insulin action in peripheral tissues and allow a controlled evaluation of the molecular consequences of exposure to pro inflammatory stimuli.

The induction of insulin resistance was achieved by treating both cell types with TNF-α, a cytokine known to interfere with insulin signaling and to promote a transcriptional program consistent with chronic low grade inflammation. As expected, TNF-α produced a reproducible impairment of insulin responsiveness, which provided a suitable background for examining how CXCR1 and CXCR2 participate in this process.

Analysis of gene expression revealed that TNF-α significantly increased the transcription of both CXCR1 and CXCR2 in 3T3-L1 adipocytes. The elevation of these receptors was accompanied by an increase in the expression of their cognate ligand CXCL1, indicating the activation of an autocrine or paracrine chemokine loop potentially capable of sustaining a pro inflammatory state. A similar trend was observed in FL83B cells, where TNF-α promoted the upregulation of CXCR1, CXCR2 and CXCL1. These findings suggest that the TNF-α induced inflammatory environment enhances the chemokine signalling machinery in both adipocytes and hepatocyte like cells.

The increase in CXCR1 and CXCR2 expression in insulin resistant conditions supports the hypothesis that these receptors may act as amplifiers of TNF-α mediated dysfunction. Their upregulation is in line with the known ability of inflammatory cytokines to activate transcription factors such as NFκB, which can in turn regulate chemokine receptors and their ligands. The parallel rise of CXCL1 suggests that TNF-α does not simply induce the receptors in isolation, but rather promotes a coordinated chemokine axis that may contribute to the maintenance and propagation of insulin resistance (Figure 5).

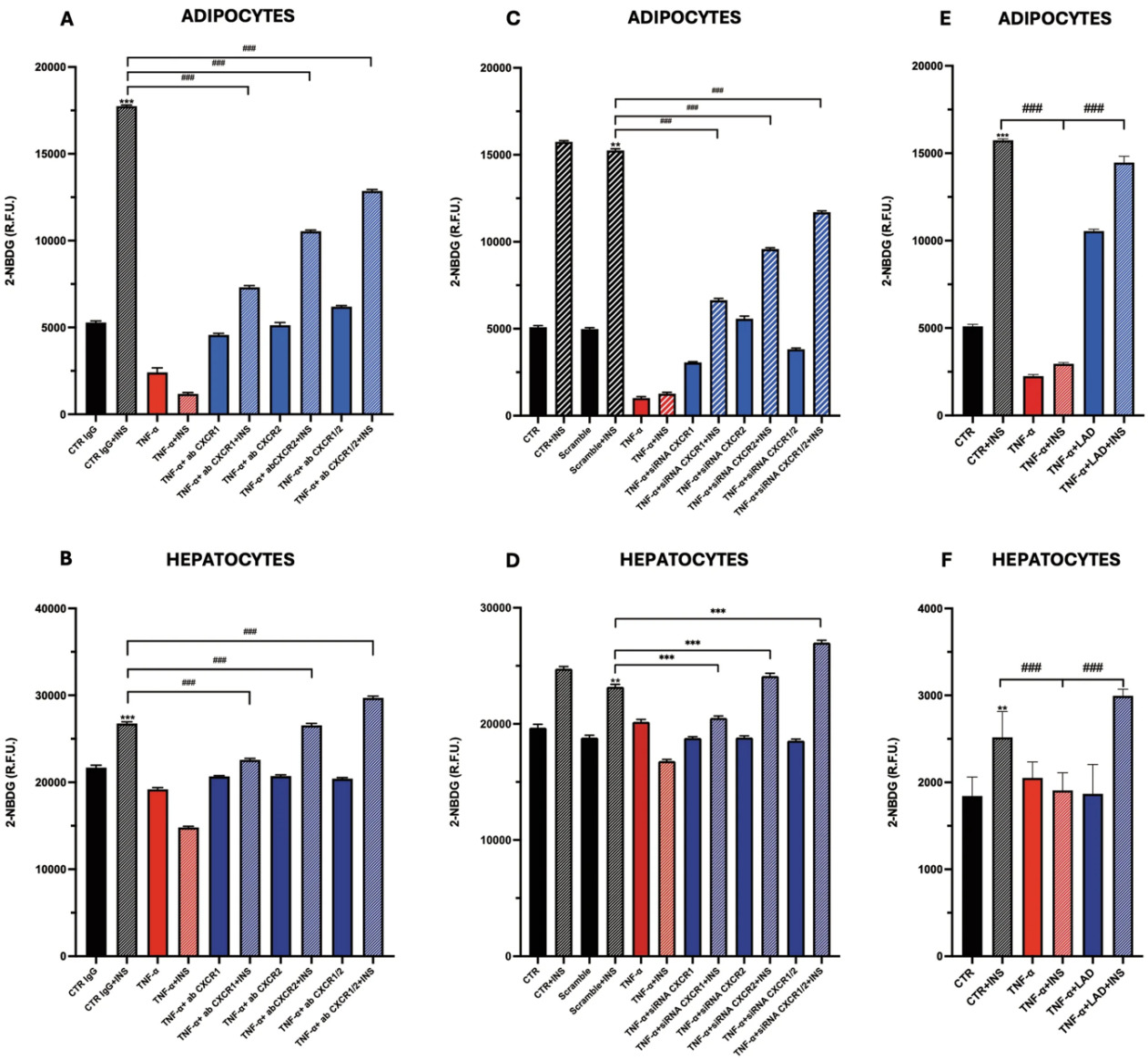

Figure 5. Glucose Uptake of silenced adipocytes (**A**), of silenced hepatocytes (**B**), of neutralized adipocytes (**C**) and neutralized hepatocytes (**D**) are shown. Glucose Uptake of treated adipocytes (**E**) and hepatocytes (**F**) with Ladarixin (LAD) are shown. Data are mean ± SE of 3 different experiments; One way ANOVA; $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, $^{\#\#\#}p < 0.0005$ vs the silencing/inhibition/treated condition ($N = 3$), $p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ vs. their respective condition without INS stimulation ($N = 3$).

Taken together, these observations indicate that CXCR1 and CXCR2 are responsive to inflammatory cues and are consistently upregulated in insulin resistant adipocytes and hepatocyte like cells. These changes highlight a

potential mechanistic link between inflammation and altered insulin signaling. Establishing this regulatory pattern was a necessary first step for the subsequent analyses aimed at determining whether targeting CXCR1 and CXCR2 could ameliorate the molecular and functional defects associated with insulin resistance in these cellular systems.

Importantly, the functional relevance of CXCR1 and CXCR2 upregulation was further supported by glucose uptake experiments performed under conditions of receptor inhibition or silencing (Figure 5). In both adipocytes and hepatocyte like cells, blockade of CXCR1 and CXCR2 using neutralizing antibodies or receptor specific siRNA partially restored insulin stimulated glucose uptake in TNFα treated cells, indicating that activation of this chemokine axis contributes directly to the insulin resistant phenotype. Notably, pharmacological inhibition of CXCR1 and CXCR2 with Ladarixin produced a more pronounced and consistent recovery of glucose uptake compared with genetic or antibody mediated approaches, approaching levels observed in insulin responsive control cells. This superior efficacy suggests that simultaneous and sustained inhibition of CXCR1 and CXCR2 signalling is required to effectively counteract inflammation induced metabolic dysfunction. Based on these observations, Ladarixin was selected for subsequent experiments as the most suitable tool to interrogate the functional contribution of CXCR1 and CXCR2 to insulin resistance.

4.2 Adipokines secretion and metabolism in adipocytes

To understand how inflammatory stress alters the secretory and metabolic behaviour of adipocytes, we examined the production of adipokines and related metabolic markers in 3T3-L1 cells rendered insulin resistant by TNF alpha

treatment (Figure 6). Adipocytes are highly active endocrine cells, and their ability to coordinate metabolic homeostasis depends on a tightly regulated network of cytokines, chemokines and lipid mediators. Disruption of this network is widely recognized as a central event in the establishment of systemic insulin resistance.

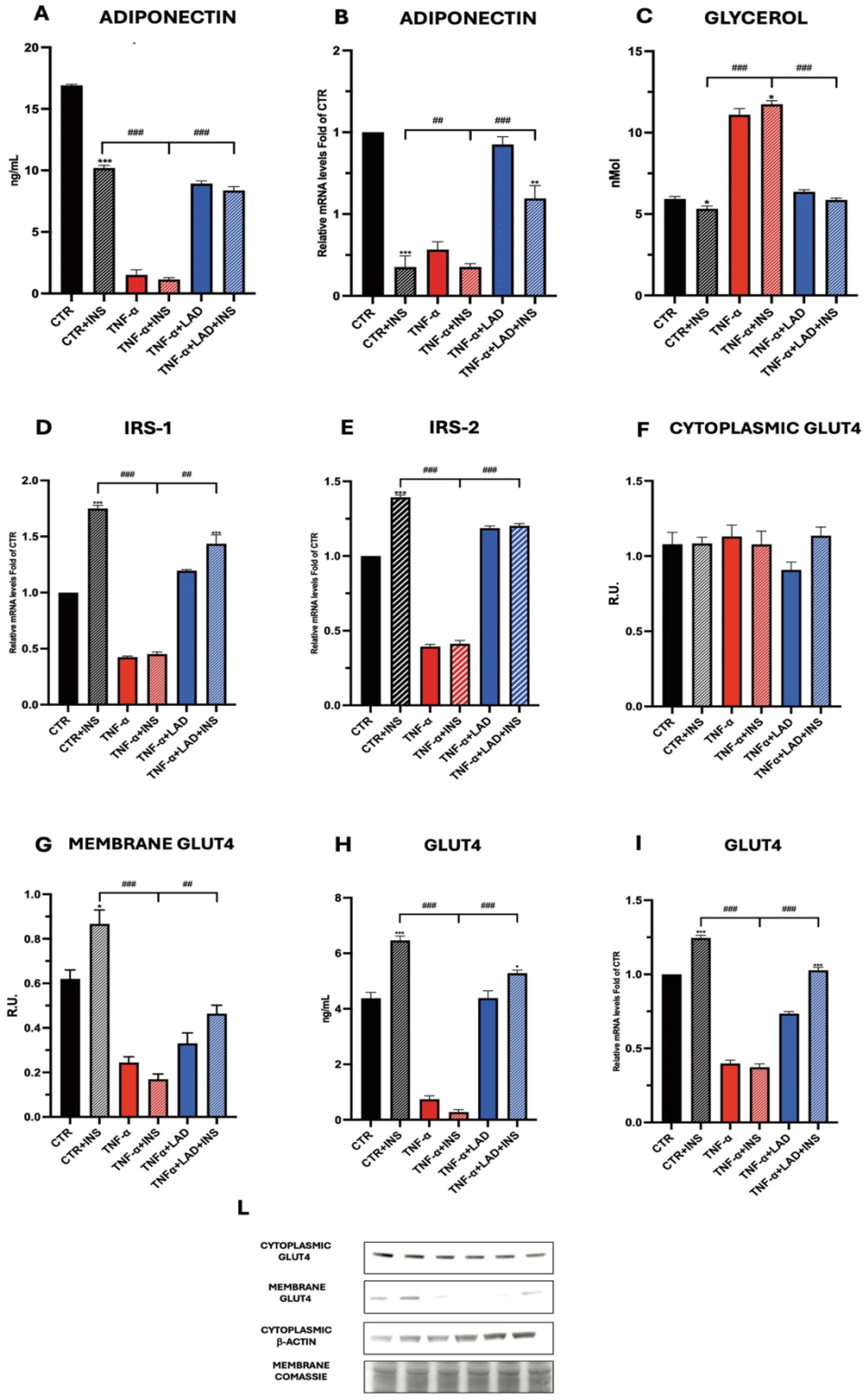

*Figure 6. **A** Adiponectin ELISA assay, **B** Adiponectin Real-Time PCR, **C** Glycerol, **D** IRS1 and **E** IRS2 expressions by Real-Time PCR in adipocytes upon different conditions. **F**, **G**, **L** Western blotting of cytoplasmic fraction and of the membrane and GLUT-4 and relative densitometric analyses. A representative figure is shown. **H** ELISA assay for the quantification of GLUT-4 protein level, **I** GLUT-4 expression by Real-Time PCR in adipocytes are reported. Data are mean ± SE of 3 different experiments; One way ANOVA. $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, $^{\#\#\#}p < 0.0005$ vs. the other conditions with the same INS stimulation (N = 3); $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ vs. their respective condition without INS stimulation (N = 3).*

Exposure of differentiated 3T3-L1 adipocytes to TNF-α led to a marked alteration of their secretory phenotype. One of the most prominent changes was the significant increase in CXCL1 secretion, consistent with the transcriptional upregulation of the CXCL1 gene observed in the same experimental conditions. This result indicates that adipocytes exposed to inflammatory stress actively contribute to the amplification of the chemokine environment. Elevated CXCL1 levels are particularly relevant because this ligand can activate CXCR1 and CXCR2, which were themselves upregulated in insulin resistant adipocytes. The coordinated induction of receptors and ligand suggests the establishment of a chemokine axis capable of reinforcing the inflammatory and metabolic imbalance.

Alongside chemokine production, TNF-α also modulated the release of key adipokines involved in glucose and lipid metabolism. The cytokine caused a reduction in adiponectin expression, a hallmark of dysfunctional adipocytes. Since adiponectin promotes insulin sensitivity through pathways that enhance fatty acid oxidation and improve the insulin signalling cascade, its downregulation is consistent with the impaired metabolic phenotype induced by TNF-α. This shift in adipokine balance further destabilizes the intracellular environment and contributes to defective insulin responsiveness.

The metabolic consequences of these secretory modifications were confirmed by assessing additional markers of adipocyte function. TNF-α exposure resulted in the reduction of GLUT4 expression and impaired GLUT4 recruitment to the plasma membrane, indicating defective insulin mediated glucose transport. Moreover, TNF-α influenced lipid metabolism, leading to alterations in lipolytic activity detectable through the lipolysis assay. These changes reflect a broader reprogramming of adipocyte metabolic behaviour, in

which inflammation drives both reduced insulin sensitivity and increased catabolic responses.

Together, these findings demonstrate that TNF-α not only impairs the intracellular insulin signalling machinery but also reshapes the endocrine output of adipocytes. By increasing CXCL1 secretion and reducing insulin sensitizing adipokines such as adiponectin, TNF-α drives the adipocyte toward a pro inflammatory and metabolically compromised state. This altered secretory profile is likely to act in concert with intracellular signalling defects to sustain the insulin resistant condition. Establishing these changes in adipokine patterns forms an essential basis for evaluating whether CXCR1 and CXCR2 inhibition can restore a more physiological metabolic and secretory profile in inflamed adipocytes.

Consistent with the involvement of the CXCL1–CXCR1/2 axis in adipocyte dysfunction, pharmacological blockade of these receptors with Ladarixin markedly attenuated the deleterious effects of TNF-α not on both the secretory and metabolic profile of adipocytes (Figure 6). Ladarixin treatment partially restored adiponectin expression and secretion, reduced CXCL1 levels, and normalized the expression of key insulin signalling components, including IRS1, IRS2 and GLUT4. At the functional level, Ladarixin improved GLUT4 recruitment to the plasma membrane and dampened the inflammatory induced increase in lipolytic activity, indicating a recovery of insulin mediated metabolic control. Overall, Ladarixin shifted adipocytes from a pro inflammatory, insulin resistant state toward a phenotype more closely resembling insulin sensitive cells. These data support the use of Ladarixin as a robust pharmacological tool to interrogate the contribution of CXCR1 and CXCR2 signalling to inflammation driven insulin resistance in subsequent experiments.

4.3 Adipocytes: Analysis of the pathways involved in IR

To gain insight into the intracellular mechanisms responsible for the impaired metabolic state observed in TNF-α treated adipocytes, we examined the key signaling pathways that regulate insulin action and inflammatory responses in differentiated 3T3-L1 cells. Insulin resistance in adipose tissue emerges from the convergence of multiple defects, including alterations in receptor proximal signaling, stress activated kinases, and transcriptional regulators of metabolic genes. Our analysis focused on the integrity of the IRS to PI3K to Akt axis, the activation of stress pathways such as JNK, and the expression of downstream effectors involved in glucose transport (Figure 7).

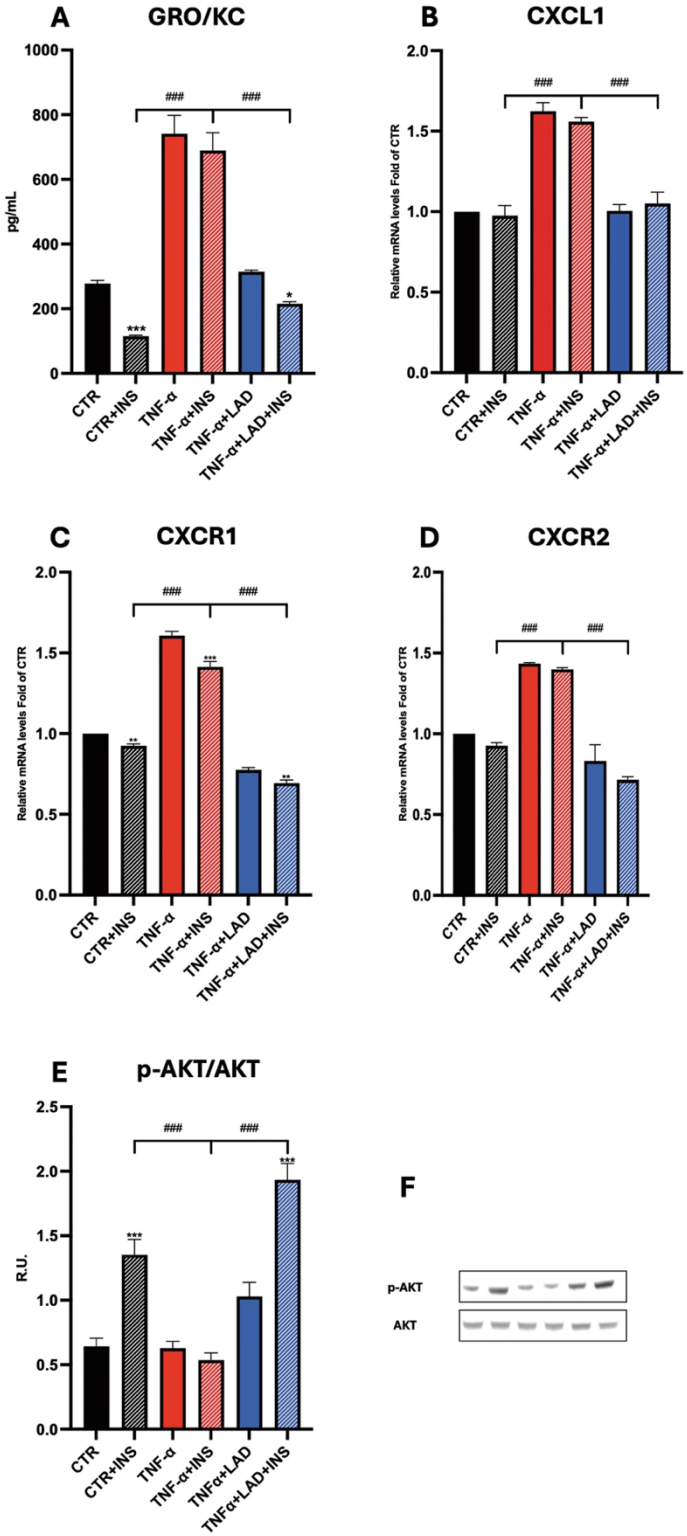

*Figure 7. **A** ELISA assay for the quantification of IL-8, **B** IL-8, **C** CXCR1, **D** CXCR2 expressions by Real-Time PCR, **E** Western Blotting for p-AKT and AKT protein levels and relative densitometric analysis in adipocytes upon different conditions. A representative figure is shown. Data are mean ± SE of 3 different experiments; One way ANOVA. $^{\#}p < 0.05$, $^{\#\#}p < 0.005$, $^{\#\#\#}p < 0.0005$ vs. the other conditions with the same INS stimulation (N = 3); $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ vs. their respective condition without INS stimulation (N = 3).*

As expected, exposure of adipocytes to TNF-α produced a profound disruption of insulin signalling. One of the earliest alterations was the reduction in the expression of IRS1 and IRS2, two essential adaptors required for transmitting the insulin signal from the receptor to the PI3K complex. The decrease in IRS proteins is consistent with the known ability of inflammatory cytokines to accelerate proteasomal degradation or inhibit transcription of these molecules. The loss of IRS1 and IRS2 significantly reduces the capacity of adipocytes to activate downstream signalling in response to insulin.

This defect was reflected in the behaviour of Akt, a central kinase responsible for mediating the metabolic effects of insulin. In control adipocytes, insulin stimulation produced a robust increase in Akt phosphorylation, confirming the integrity of the signalling cascade. In contrast, TNF-α treated adipocytes displayed a marked reduction in insulin induced Akt activation. Since Akt phosphorylation is required for GLUT4 translocation and glucose uptake, this impairment provides a direct mechanistic explanation for the functional alterations observed in insulin resistant cells.

In parallel with the weakening of the canonical insulin signalling cascade, TNF-α activated stress related pathways, particularly the JNK pathway. JNK is known to inhibit insulin signalling by promoting serine phosphorylation of IRS1, which interferes with its ability to propagate the insulin signal. Consistent with this mechanism, adipocytes exposed to TNF-α showed increased levels of phosphorylated JNK, indicating its active engagement in the inflammatory response. The co-occurrence of reduced IRS expression and enhanced JNK activation suggests a dual inhibitory pressure on the insulin

signaling system, both at the transcriptional level and through post translational modifications.

The combined influence of these pathways culminated in a significant alteration of glucose transporter dynamics. GLUT4 expression was reduced, and its recruitment to the plasma membrane was compromised, demonstrating that both the abundance and the mobilization of the transporter were impaired. Since GLUT4 translocation is a direct downstream event of Akt activation, these results further reinforce the link between defective insulin signaling and the metabolic phenotype of TNF-α treated adipocytes.

Overall, the analysis of intracellular pathways demonstrates that TNF-α induces insulin resistance in adipocytes through a multifaceted mechanism involving downregulation of IRS proteins, suppression of Akt activation, induction of JNK mediated stress responses and impairment of GLUT4 trafficking. These coordinated alterations establish a robust cellular framework of insulin resistance that provides the foundation for evaluating the functional relevance of CXCR1 and CXCR2 modulation in subsequent sections.

In line with the activation of the CXCL1–CXCR1/2 axis under inflammatory conditions, inhibition of CXCR1 and CXCR2 signaling significantly modulated the intracellular pathways altered by TNF-α (Figure 7). Pharmacological treatment with Ladarixin reduced the TNF-α induced upregulation of CXCL1, CXCR1 and CXCR2 and was associated with a marked restoration of insulin signalling, as evidenced by increased Akt phosphorylation in response to insulin stimulation. This recovery occurred in parallel with attenuation of stress related signalling and improvement of downstream insulin responsive processes. Compared with TNF-α treated

adipocytes, cells exposed to Ladarixin displayed a signalling profile more closely resembling insulin sensitive conditions, indicating that CXCR1 and CXCR2 activation contributes directly to the disruption of the IRS–PI3K–Akt pathway. These findings support the concept that targeting CXCR1 and CXCR2 effectively counteracts inflammation driven impairment of insulin signalling and further justify the use of Ladarixin as a key experimental tool in subsequent mechanistic and functional analyses.

4.4 Metabolic profile of IR adipocytes

To characterize the functional metabolic consequences of the signalling alterations induced by TNF-α, we examined the metabolic profile of differentiated 3T3 L1 adipocytes rendered insulin resistant, with a particular focus on glucose handling and mitochondrial bioenergetics (Figure 8). Since adipocytes integrate hormonal signals with nutrient availability and play a central role in whole body glucose and lipid homeostasis, the assessment of their metabolic competence provides critical insight into the downstream effects of inflammation driven insulin resistance.

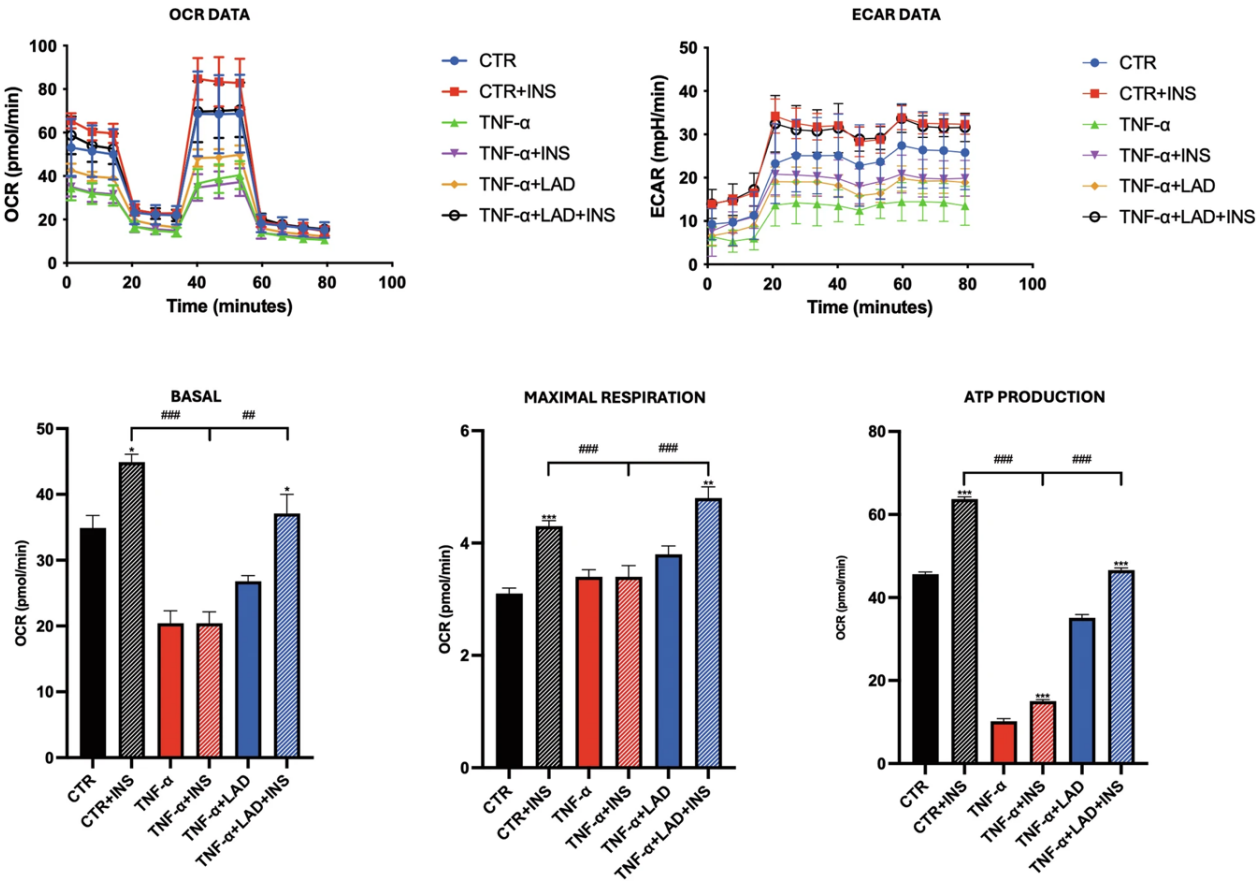

*Figure 8. Seahorse Mitostress assay of IR adipocytes upon different conditions are shown. Data are mean ± SE of 3 different experiments; One way ANOVA. #p < 0.05, ##p < 0.005, ###p < 0.0005 vs. the other conditions with the same INS stimulation (N = 3); *p < 0.05, **p < 0.005, ***p < 0.0005 vs. their respective condition without INS stimulation (N = 3).*

A first indication of metabolic dysfunction emerged from the analysis of insulin stimulated glucose uptake. In control adipocytes, insulin treatment resulted in a robust increase in glucose internalization, confirming the presence of an intact GLUT4 dependent transport system. In contrast, TNF-α treated adipocytes exhibited a marked reduction in insulin stimulated glucose uptake, consistent with impaired GLUT4 expression and defective translocation previously described. This functional defect represents a key hallmark of insulin resistant adipocytes and reflects the compromised integrity of the IRS to PI3K to Akt signalling axis.

To obtain a comprehensive view of cellular energy metabolism, mitochondrial bioenergetics were assessed using Seahorse extracellular flux analysis (Figure 8). TNF-α exposure caused a pronounced reduction in oxygen consumption rate (OCR) across multiple parameters of oxidative metabolism. Basal respiration was significantly decreased in TNF-α treated cells, indicating reduced mitochondrial activity under resting conditions. Similarly, ATP linked respiration was markedly impaired, demonstrating a diminished capacity to generate ATP through oxidative phosphorylation. Maximal respiratory capacity, assessed following mitochondrial uncoupling, was also reduced, revealing a loss of mitochondrial reserve capacity and an inability to meet increased energetic demand. Together, these alterations indicate a profound impairment of mitochondrial oxidative function in insulin resistant adipocytes. In parallel, extracellular acidification rate (ECAR) measurements revealed alterations in glycolytic activity. While control adipocytes displayed an increase in ECAR in response to insulin stimulation, TNF-α treated cells showed a blunted glycolytic response, suggesting that inflammatory stress compromises not only oxidative metabolism but also the flexibility to shift toward glycolysis when required. This combined reduction in OCR and ECAR reflects a state of metabolic inflexibility in which adipocytes fail to appropriately adapt their energy producing pathways.

In addition to glucose and mitochondrial metabolism, lipid handling was also affected. TNF-α treatment increased lipolytic activity, as detected by the lipolysis assay, indicating a shift toward a more catabolic phenotype characterized by enhanced release of free fatty acids. Since insulin normally suppresses lipolysis, this increase highlights a loss of insulin mediated control over lipid turnover. Elevated lipolysis further contributes to metabolic stress by increasing fatty acid availability, which can exacerbate mitochondrial dysfunction and insulin resistance in both adipose tissue and peripheral organs.

Collectively, these data demonstrate that TNF-α induced insulin resistance profoundly disrupts adipocyte metabolism at multiple levels, including glucose uptake, mitochondrial oxidative capacity and lipid homeostasis. The coordinated reduction in OCR parameters, the altered ECAR profile and the increase in lipolytic activity reveal a global breakdown of metabolic flexibility. This dysfunctional metabolic state, driven by inflammatory signaling, provides a strong rationale for investigating whether modulation of CXCR1 and CXCR2 can restore mitochondrial function and re-establish a more physiological metabolic profile in inflamed adipocytes.

Importantly, pharmacological inhibition of CXCR1 and CXCR2 with Ladarixin consistently ameliorated the metabolic defects induced by TNF-α (Figure 8). Ladarixin treatment partially restored mitochondrial oxidative function, as evidenced by increased OCR parameters, including basal respiration, ATP linked respiration and maximal respiratory capacity, compared with TNF-α treated adipocytes. In addition, insulin stimulated responses were improved in the presence of Ladarixin, indicating a recovery of mitochondrial metabolic flexibility. ECAR analysis further supported this effect, showing a partial normalization of glycolytic activity and an improved ability to adapt energy production pathways.

Beyond mitochondrial metabolism, Ladarixin also attenuated the catabolic shift induced by TNF-α, reducing excessive lipolytic activity and suggesting a partial re-establishment of insulin mediated control over lipid turnover. Collectively, these findings indicate that inhibition of CXCR1 and CXCR2 mitigates the inflammatory metabolic reprogramming of adipocytes, leading to an overall improvement in glucose handling, mitochondrial bioenergetics and lipid homeostasis. This rescue effect supports a functional role for CXCR1 and CXCR2 signaling in sustaining metabolic dysfunction under inflammatory

conditions and provides a strong experimental rationale for targeting this axis to restore adipocyte metabolic competence.

4.5 Hepatocytes: analysis of the pathways involved in IR

To complement the observations obtained in adipocytes and to determine whether similar molecular mechanisms operate in other insulin responsive tissues, we analyzed the intracellular pathways affected by TNF-α in FL83B hepatocyte like cells (Figures 9 and 10). Hepatocytes play a pivotal role in systemic glucose homeostasis by integrating insulin signaling with glucose uptake, storage and production, and even modest defects in hepatic insulin responsiveness can have profound metabolic consequences. The FL83B model therefore provides a suitable experimental system to dissect inflammation induced insulin resistance at the hepatic level. Consistent with the adipocyte model, exposure of FL83B cells to TNF-α resulted in a pronounced impairment of insulin signaling. TNF-α treatment significantly reduced the expression of IRS1 and IRS2, two essential adaptor proteins required for effective signal propagation from the insulin receptor to downstream pathways (Figure 9). The loss of IRS proteins compromises proximal insulin signaling and is consistent with the ability of inflammatory cytokines to repress transcription and promote protein degradation in insulin resistant states.

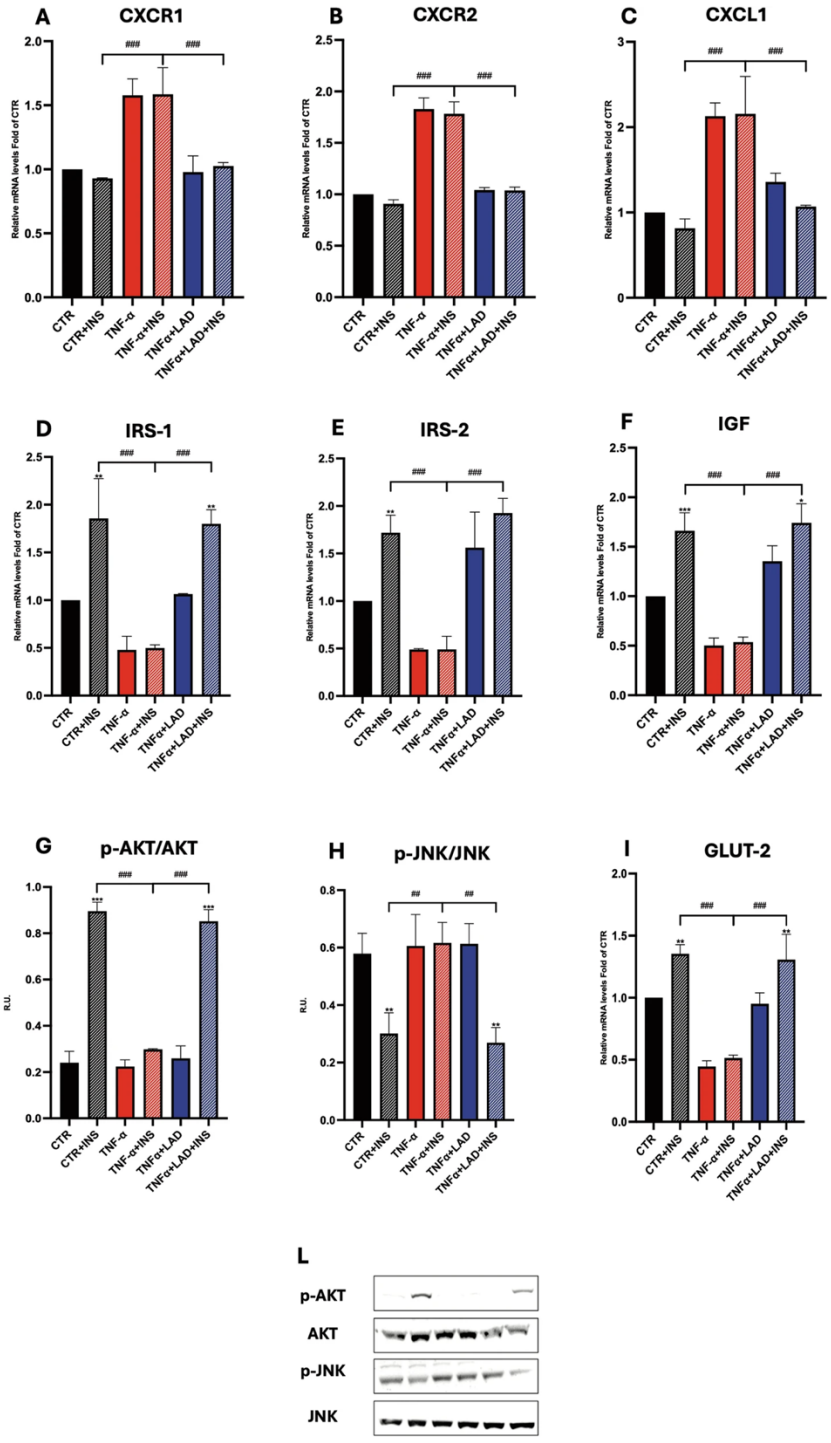

*Figure 9. A CXCR1, B CXCR2 and C CXCL1 expressions by Real-Time PCR in hepatocytes upon different conditions. D IRS-1 and E IRS-2 expressions by Real-Time PCR in hepatocytes upon different conditions. Western Blotting for (G) p-AKT and AKT and (H) p-JNK/JNK protein levels and relative densitometric analysis in hepatocytes upon different conditions. A representative figure is shown. F IGF and I GLUT-2 expressions by Real-Time PCR in hepatocytes upon different conditions. L Representative WB figures. Data are mean ± SE of 3 different experiments; One way ANOVA. #p < 0.05, ##p < 0.005, ###p < 0.0005 vs. the other conditions with the same INS stimulation (N = 3); *p < 0.05, **p < 0.005, ***p < 0.0005 vs. their respective condition without INS stimulation (N = 3).*

Defects in receptor proximal signalling were further confirmed by analysis of the PI3K–Akt axis. In control hepatocytes, insulin stimulation induced a robust increase in Akt phosphorylation, indicating intact insulin responsiveness. In contrast, TNF-α treated cells displayed a marked reduction in p-Akt levels, reflecting impaired activation of this central metabolic kinase (Figure 9). Since Akt signaling is required to suppress hepatic glucose production and promote glycogen synthesis, attenuation of Akt activation provides a mechanistic explanation for inflammation driven hepatic insulin resistance.

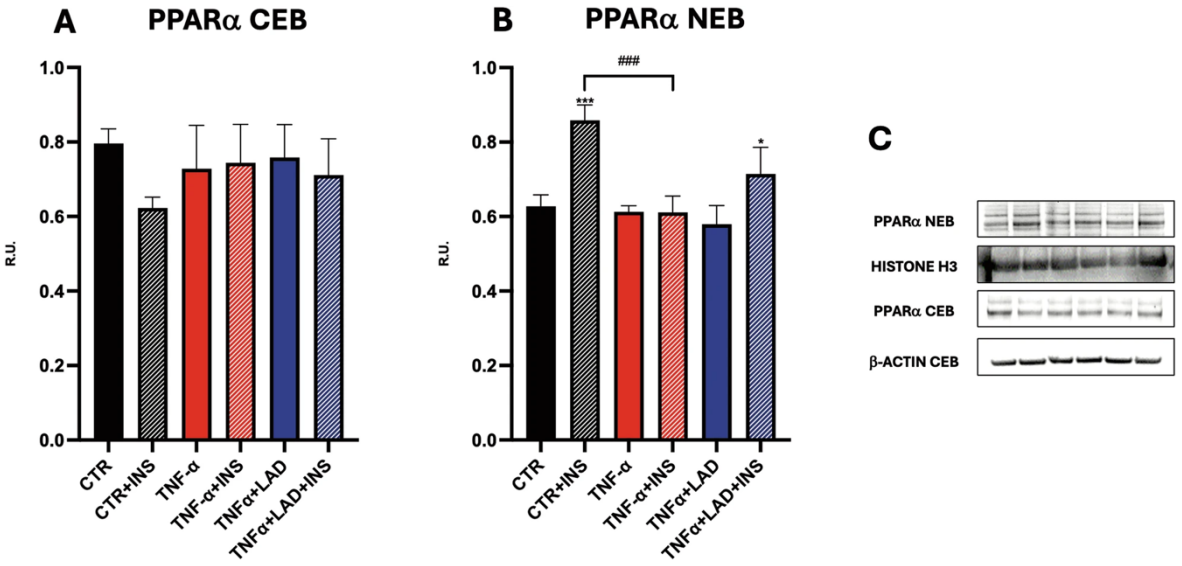

*Figure 10. A, B Western Blot analysis for nuclear and cytoplasmic PPARα in hepatocytes. C Representative WB figures are shown. Data are mean ± SE of 3 different experiments; One way ANOVA. #p < 0.05, ##p < 0.005, ###p < 0.0005 vs. the other conditions with the same INS stimulation (N = 3); *p < 0.05, **p < 0.005, ***p < 0.0005 vs. their respective condition without INS stimulation (N = 3).*

In parallel, TNF-α activated stress responsive pathways in hepatocytes, as evidenced by increased phosphorylation of JNK (Figure 9). JNK activation is particularly relevant in the liver, where it promotes inhibitory serine phosphorylation of IRS1, thereby amplifying defects in insulin signaling. The concomitant reduction of IRS proteins and activation of JNK establishes a dual inhibitory mechanism that profoundly disrupts insulin signal transduction in FL83B cells.

Alterations in intracellular signalling were accompanied by functional changes in hepatic glucose handling. TNF-α significantly reduced the expression of GLUT2, the principal glucose transporter in hepatocytes, which is essential for bidirectional glucose flux across the plasma membrane (Figure 9). Reduced GLUT2 abundance suggests that inflammatory stress affects not only signalling intermediates but also the metabolic machinery responsible for glucose sensing and transport, further contributing to hepatic insulin resistance.

In addition to classical insulin signalling components, TNF-α also altered pathways involved in metabolic regulation and nuclear receptor signalling. In particular, TNF-α reduced nuclear PPARα levels, while cytoplasmic PPARα content was less affected (Figure 10). This redistribution suggests impaired nuclear activity of PPARα, a key regulator of fatty acid oxidation and anti-inflammatory responses in hepatocytes. Reduced PPARα nuclear localization is consistent with a shift toward lipid accumulation, mitochondrial dysfunction and heightened inflammatory signalling under insulin resistant conditions.

Importantly, pharmacological inhibition of CXCR1 and CXCR2 with Ladarixin consistently mitigated the TNF-α induced alterations observed in hepatocytes. Ladarixin treatment restored IRS1 and IRS2 expression, enhanced insulin

stimulated Akt phosphorylation and reduced JNK activation (Figures 9 and 10). In parallel, Ladarixin partially normalized GLUT2 expression and promoted recovery of nuclear PPARα levels, indicating an improvement in both insulin signalling and metabolic regulatory pathways. Compared with TNF-α treated cells, hepatocytes exposed to Ladarixin displayed a molecular profile more closely resembling insulin sensitive conditions.

Collectively, these results demonstrate that TNF-α induces a robust insulin resistant phenotype in hepatocyte like cells through coordinated disruption of insulin signalling, activation of stress pathways, impairment of glucose transport and inhibition of nuclear PPARα activity. The ability of Ladarixin to reverse these defects supports a functional role for CXCR1 and CXCR2 signalling in sustaining hepatic insulin resistance and identifies Ladarixin as an effective pharmacological tool for restoring insulin sensitivity in inflammatory conditions.

4.6 Metabolic profile analysis in IR hepatocytes

To complement the metabolic characterization performed in adipocytes and to delineate the impact of inflammatory stress on hepatic metabolic function, we evaluated the metabolic profile of FL83B hepatocyte like cells exposed to TNF-α (Figures 11 and 12). The liver is a central metabolic organ responsible for coordinating glucose uptake, glycogen storage and energy distribution, and even modest impairments in hepatic insulin sensitivity can significantly influence systemic metabolic homeostasis.

A first indication of metabolic dysfunction was obtained from the analysis of glucose uptake. Under physiological conditions, FL83B cells respond to insulin by increasing glucose internalization through GLUT2 mediated transport. In

contrast, TNF-α treated hepatocytes displayed a marked reduction in insulin stimulated glucose uptake, confirming that inflammatory stress compromises hepatic glucose handling (Figure 11). This functional defect is consistent with the reduction in GLUT2 expression and the impaired activation of the IRS/PI3K/Akt pathway described in the previous section, and highlights the tight coupling between insulin signalling and glucose transport in hepatocytes. To further characterize cellular energy metabolism, mitochondrial bioenergetics were assessed using Seahorse extracellular flux analysis (Figure 11).

TNF-α exposure resulted in a significant reduction in oxygen consumption rate across multiple parameters of oxidative metabolism. Basal respiration, ATP linked respiration and maximal respiratory capacity were all markedly decreased, indicating a broad impairment of oxidative phosphorylation. In parallel, extracellular acidification rate analysis revealed a blunted glycolytic response, suggesting that hepatocytes exposed to inflammatory stress are unable to compensate for reduced mitochondrial function by appropriately engaging glycolysis. Together, these alterations reflect a state of pronounced metabolic inflexibility.

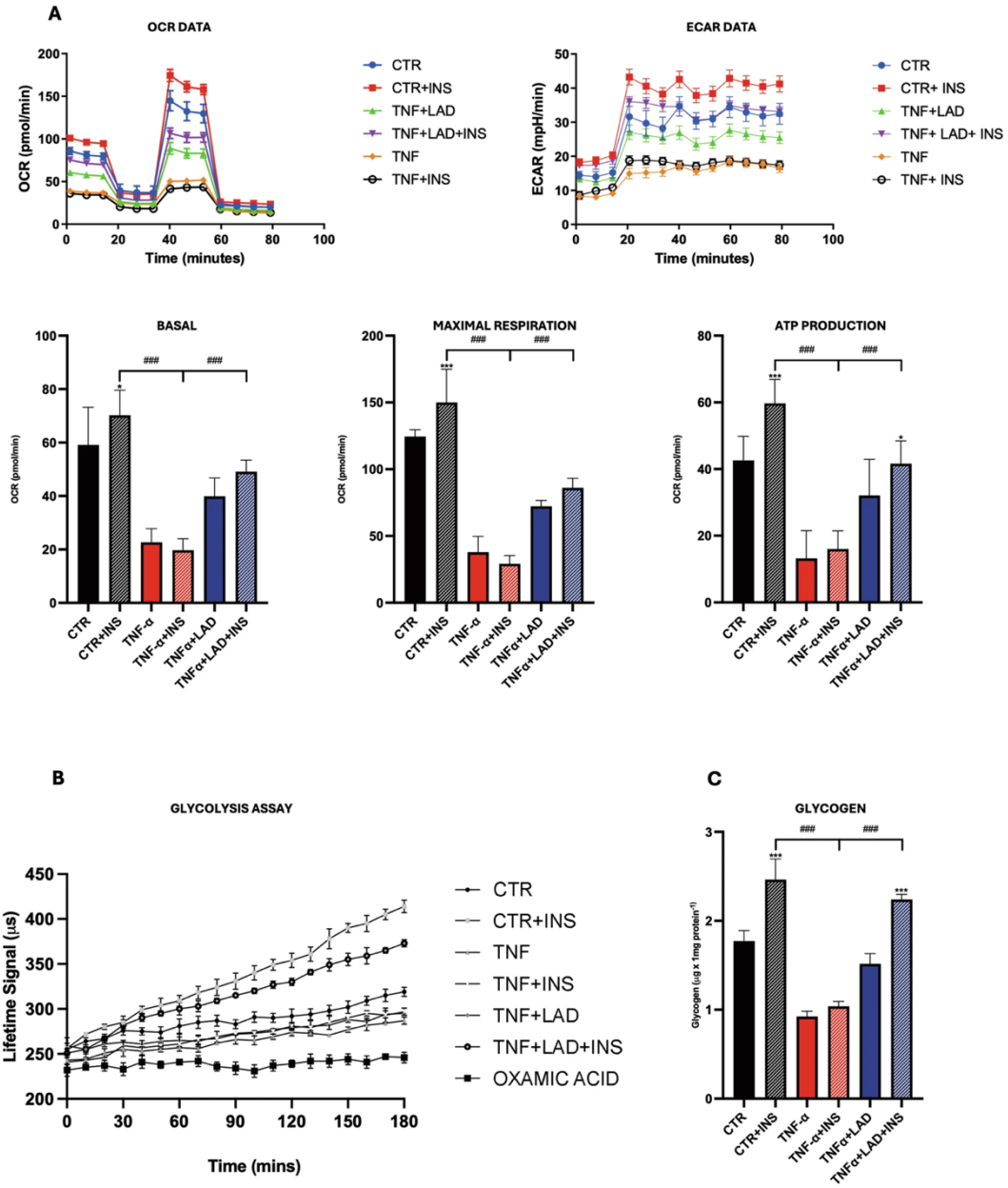

*Figure 11. A Seahorse Mitostress assay and B Glycolysis assay in hepatocytes upon different conditions, data were expressed as Lifetime Signal (µs) (Significativity in a Suppl table), C Glycogen concentration in hepatocytes upon different conditions. Dataare mean ± SE of 3 different experiments; One way ANOVA. #p < 0.05, ##p < 0.005, ###p < 0.0005 vs. the other conditions with the same INS stimulation (N = 3); *p < 0.05, **p < 0.005, ***p < 0.0005 vs. their respective condition without INS stimulation (N = 3).*

Consistent with this interpretation, TNF-α treated hepatocytes exhibited a reduced spare respiratory capacity, indicating a diminished ability to respond to increased energetic demand. Operating close to their bioenergetic limit

renders hepatocytes particularly vulnerable to additional metabolic stress, a condition that aligns with the chronic low grade inflammatory environment associated with insulin resistance and metabolic disease.

In addition to alterations in glucose metabolism and mitochondrial function, TNF-α profoundly affected hepatic lipid handling. Confocal microscopy analysis revealed a significant accumulation of intracellular lipid droplets in TNF-α treated cells, indicative of altered lipid storage and ectopic lipid deposition (Figure 12). This morphological evidence was supported by biochemical measurements showing increased glycerol release, reflecting dysregulated lipid turnover and a shift toward a lipotoxic phenotype. Such lipid accumulation is known to further impair insulin signaling and mitochondrial function, thereby reinforcing hepatic insulin resistance.

Notably, pharmacological inhibition of CXCR1 and CXCR2 with Ladarixin consistently ameliorated the metabolic defects induced by TNF-α. Ladarixin treatment partially restored mitochondrial oxidative capacity, as evidenced by increased OCR parameters, improved ATP production and recovery of spare respiratory capacity (Figure 11). In parallel, Ladarixin enhanced insulin stimulated glycolytic responses and promoted glycogen accumulation, indicating a re-establishment of coordinated glucose utilization and storage. Moreover, Ladarixin markedly reduced intracellular lipid droplet accumulation and attenuated glycerol release, demonstrating an improvement in hepatic lipid homeostasis (Figure 12). Collectively, these findings demonstrate that TNF-α induces a profound metabolic reprogramming in hepatocyte like cells, characterized by impaired glucose uptake, mitochondrial dysfunction, metabolic inflexibility and lipid accumulation. The ability of Ladarixin to reverse these alterations supports a functional role for CXCR1 and CXCR2 signaling in sustaining inflammation driven hepatic insulin resistance. These

results further strengthen the rationale for targeting the CXCR1/2 axis as a strategy to restore metabolic competence in insulin resistant hepatocytes and reinforce the systemic relevance of chemokine mediated metabolic dysregulation.

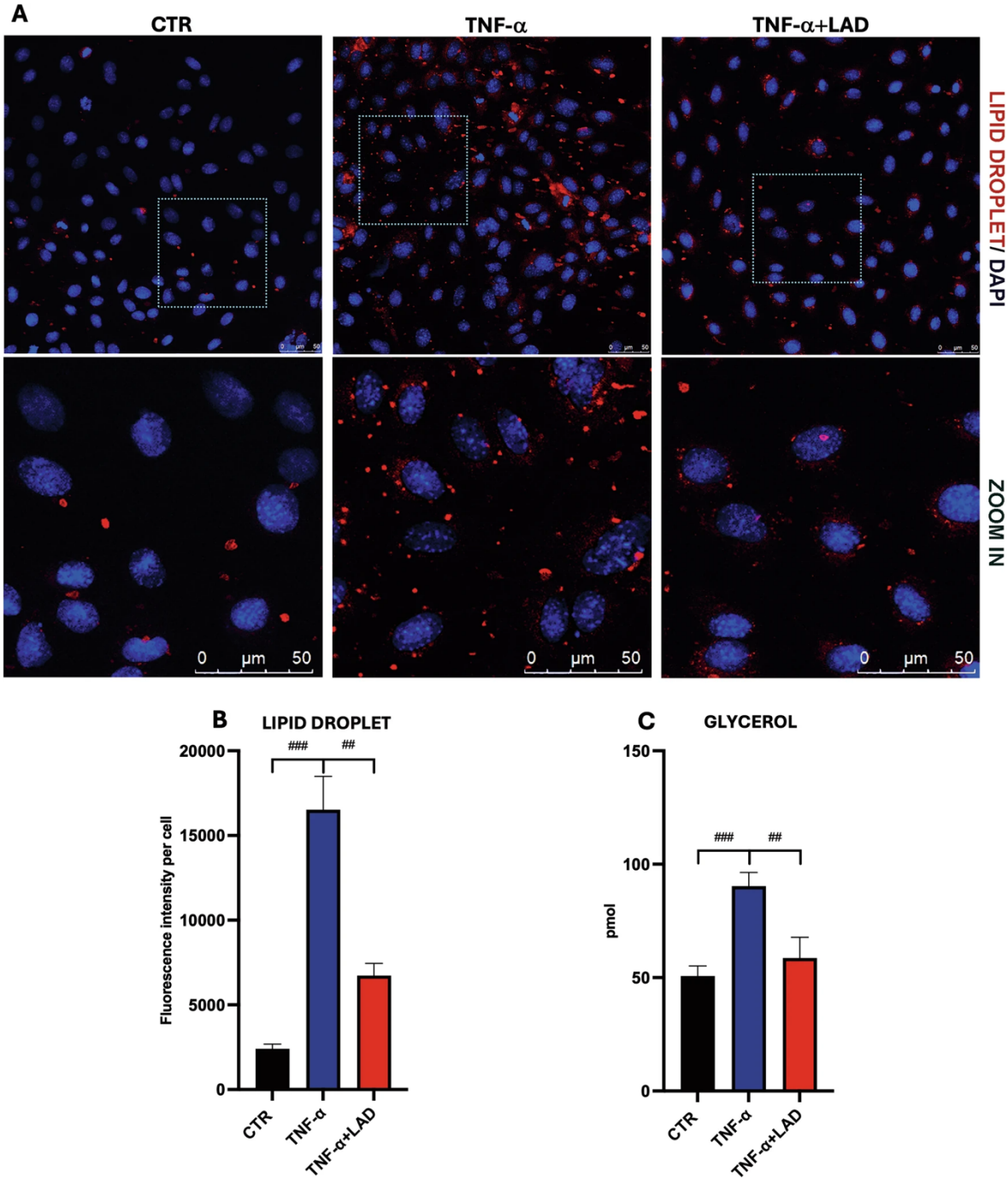

*Figure 12. A Nile Red staining marking LD in hepatocytes untreated (CTR), treated with TNF-a and TNF-a+LAD. Bar=50 µm, B LD intensity per cell count, C Lipolysis assay in IR-hepatocytes. Strippled blue square: Zoom in. Data are mean ± SE of 3 different experiments; One way ANOVA. #p < 0.05, ##p < 0.005, ###p < 0.0005 vs. the other conditions with the same INS stimulation (N = 3); *p < 0.05, **p < 0.005, ***p < 0.0005 vs. their respective condition without INS stimulation (N = 3).*

5 DISCUSSION

The present study examined the role of the chemokine receptors CXCR1 and CXCR2 in inflammation associated insulin resistance using two complementary *in vitro* models, differentiated 3T3-L1 adipocytes and FL83B hepatocyte like cells. By combining molecular, biochemical and functional approaches, we provide a comprehensive characterization of how TNF alpha driven inflammatory stress disrupts insulin signaling and metabolic homeostasis, and we identify the CXCR1/2 chemokine axis as a consistent and functionally relevant component of this process.

Across both cellular systems, TNF-α induced a robust impairment of the canonical insulin signalling cascade. A central and early event was the reduction in IRS1 and IRS2 expression, which compromises signal transmission from the insulin receptor to downstream effectors. This defect was accompanied by a marked attenuation of insulin stimulated Akt phosphorylation, confirming that inflammatory stress disrupts the IRS/PI3K/Akt axis at multiple levels. These findings are consistent with previous reports describing the susceptibility of IRS proteins to inflammatory regulation through transcriptional repression, enhanced degradation and inhibitory post translational modifications, and they reinforce the concept that IRS dysfunction represents a core molecular lesion in insulin resistance.

In parallel with the suppression of insulin signalling, TNF-α alpha activated stress responsive pathways, particularly the JNK pathway, in both adipocytes and hepatocytes. JNK activation is known to interfere with insulin action by promoting inhibitory serine phosphorylation of IRS proteins, thereby further weakening insulin signal propagation. The coexistence of reduced IRS abundance and enhanced JNK activity establishes a dual inhibitory mechanism

that acts both upstream and downstream of the insulin receptor. This convergence of transcriptional and post translational inhibition provides a mechanistic explanation for the persistence and severity of the insulin resistant phenotype observed in our models.

The molecular defects induced by TNF-α translated into clear functional consequences. In adipocytes, insulin resistance was associated with reduced GLUT4 expression and impaired translocation to the plasma membrane, while in hepatocytes TNF-α markedly decreased GLUT2 abundance. Since these transporters are essential for maintaining glucose flux and metabolic flexibility, their downregulation resulted in a pronounced reduction in insulin stimulated glucose uptake. These observations highlight how alterations in signalling pathways are directly coupled to defects in cellular glucose handling, reinforcing the functional relevance of the molecular changes described.

Beyond glucose transport, inflammatory stress profoundly affected cellular energy metabolism. Both adipocytes and hepatocytes exhibited a decline in mitochondrial oxidative capacity, as evidenced by reduced basal respiration, ATP production and maximal respiratory capacity. The concomitant impairment of oxidative phosphorylation and glycolytic responses revealed a state of metabolic inflexibility, in which cells are unable to adapt efficiently to changes in energetic demand. In hepatocytes, this bioenergetic dysfunction was accompanied by reduced glycogen accumulation and increased lipid droplet deposition, indicating a broader reprogramming of metabolic pathways toward lipid storage and ectopic lipid accumulation. These findings align with the growing recognition of mitochondrial dysfunction and altered lipid handling as integral components of inflammation driven metabolic disease.

A key contribution of this study is the identification of a consistent upregulation of CXCR1, CXCR2 and their ligand CXCL1 in both adipocytes and hepatocyte like cells exposed to TNF-α. The coordinated induction of receptors and ligand suggests the establishment of an autocrine or paracrine chemokine loop capable of sustaining inflammatory signalling within metabolically active cells. This observation is particularly relevant because it positions CXCR1/2 signalling not merely as a marker of inflammation, but as a potential amplifier of metabolic dysfunction. The concordance of these changes across two distinct insulin responsive cell types underscores the systemic relevance of the CXCR1/2 axis in inflammation associated insulin resistance.

Importantly, functional inhibition of CXCR1 and CXCR2 consistently mitigated the molecular and metabolic defects induced by TNF-α. Pharmacological blockade with Ladarixin partially restored IRS expression, improved insulin stimulated Akt activation and attenuated JNK signalling. At the metabolic level, Ladarixin enhanced glucose uptake, improved mitochondrial bioenergetic parameters, promoted glycogen storage and reduced lipid accumulation. The breadth of these effects indicates that CXCR1/2 signalling intersects multiple layers of metabolic regulation, linking inflammatory cues to defects in insulin signalling, energy metabolism and lipid homeostasis.

Taken together, the results of this study define a coherent mechanistic framework in which TNF-α induced inflammation disrupts insulin signalling and metabolic function through coordinated activation of stress pathways, suppression of insulin responsive cascades and engagement of the CXCR1/2 chemokine axis. By establishing this integrated molecular and functional landscape, the present work provides a strong foundation for evaluating the contribution of CXCR1 and CXCR2 to insulin resistance and sets the stage for

a more focused discussion of their potential as therapeutic targets in metabolic disease.

6 CONCLUSIONS

The work presented in this thesis provides an integrated characterization of the molecular and metabolic mechanisms through which inflammatory stress promotes insulin resistance in adipocytes and hepatocyte like cells. Using TNF-α as a model of chronic low grade inflammation, and combining transcriptional, biochemical and functional approaches, this study demonstrates that inflammation induces a coordinated disruption of insulin signalling, energy metabolism and substrate handling in multiple insulin responsive cell types.

A central conclusion of this work is that TNF-α driven insulin resistance is initiated by early defects in the insulin signalling cascade. In both adipocytes and hepatocytes, inflammatory stress markedly reduced the expression of IRS1 and IRS2, compromising signal transmission from the insulin receptor to downstream pathways. This impairment was compounded by the activation of stress responsive kinases, particularly JNK, which further interfered with insulin signalling through inhibitory mechanisms acting at the post translational level. The convergence of reduced IRS availability and enhanced stress kinase activity establishes a robust inhibitory environment that severely limits insulin responsiveness.

Downstream of these proximal defects, TNF-α induced a consistent attenuation of Akt activation in response to insulin stimulation. Given the central role of Akt in regulating glucose transport, glycogen synthesis and lipid metabolism, its impaired phosphorylation provides a mechanistic basis for the widespread metabolic alterations observed in insulin resistant cells. These signalling defects translated into functional impairments in glucose handling, including

reduced GLUT4 expression and translocation in adipocytes and diminished GLUT2 abundance in hepatocytes, ultimately resulting in a marked decrease in insulin stimulated glucose uptake.

Beyond alterations in glucose metabolism, this study demonstrates that inflammatory stress profoundly affects cellular energy homeostasis. Both adipocytes and hepatocytes exhibited impaired mitochondrial oxidative capacity, characterized by reduced basal respiration, lower ATP production and diminished maximal respiratory capacity. These bioenergetic defects were accompanied by metabolic inflexibility and, in hepatocytes, by altered glycogen storage and increased lipid accumulation. Together, these findings highlight mitochondrial dysfunction and dysregulated lipid handling as integral components of inflammation driven insulin resistance.

A key outcome of this work is the identification of the chemokine receptors CXCR1 and CXCR2, together with their ligand CXCL1, as consistently upregulated elements of the inflammatory response in insulin resistant cells. The coordinated induction of receptors and ligand in both adipocytes and hepatocytes suggests the activation of an autocrine or paracrine chemokine axis that integrates inflammatory signalling with metabolic dysfunction. This observation positions CXCR1 and CXCR2 not merely as markers of inflammation, but as active contributors to the maintenance and amplification of insulin resistance.

Importantly, functional inhibition of CXCR1 and CXCR2 demonstrated that this chemokine axis plays a causal role in sustaining metabolic impairment. Pharmacological blockade with Ladarixin partially restored insulin signalling, improved glucose uptake, enhanced mitochondrial bioenergetic performance and attenuated lipid dysregulation in both cellular models. These findings

provide direct evidence that targeting CXCR1 and CXCR2 can counteract key molecular and functional defects induced by inflammatory stress.

In conclusion, this thesis demonstrates that inflammation driven insulin resistance arises from the integration of signalling defects, stress pathway activation, mitochondrial dysfunction and altered substrate metabolism. The consistent involvement of CXCR1 and CXCR2 across multiple levels of this process identifies the chemokine axis as a relevant molecular link between inflammation and metabolic disease. By delineating the mechanisms through which CXCR1/2 signalling contributes to insulin resistance, this work provides a strong experimental foundation for considering chemokine receptor targeting as a potential strategy for restoring metabolic homeostasis in inflammatory metabolic disorders.

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