Investigating the role of Class-1 Phosphoinositide 3 Kinases (PI3Ks) in insulin signaling and obesity

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Cover illustration: The PI3Ks suspects illustration provided by Giovanni Solinas and adapted by Angela Molinaro.

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To my grandfather Giovanni

"*It always seems impossible until it is done*"

-Nelson Mandela

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ABSTRACT

Obesity and obesity related diseases such as type 2 diabetes, cardiovascular disorders, and different types of cancer are leading causes of mortality and morbidity in modern society. However, the mechanism that links obesity to these diseases remains largely unresolved. Class 1 phosphatidylinositide 3 kinases (PI3Kα; PI3Kβ; PI3Kδ and PI3Kγ) play a major role in several physiological processes such as the immune response, the metabolic insulin action, and tissues homeostasis. This thesis aims at a better understanding of the role of the different PI3K isoforms in obesity and insulin signaling.

PI3Kγ plays an important role in leukocyte recruitment during inflammation, in the inhibition of classical macrophage activation and in promoting diet-induced obesity and insulin resistance. In **PAPER I** we have investigated the PI3Kγ mechanisms of action and we have found that the activity of PI3Kγ in hematopoietic cells is dispensable in hepatic inflammation, liver steatosis, adiposity and macrophage recruitment in adipose tissue. However, PI3Kγ activity promotes insulin resistance, the pro-inflammatory M1 macrophage phenotype and neutrophils recruitment in the adipose tissue of obese mice. This observation challenges the concept that PI3Kγ activity is a general inhibitor of classical macrophage activation.

In **PAPER II**, we aim to define the role of class-1 PI3K isoforms and RAS in insulin signaling in hepatocytes. Our data lead to a new and improved mechanism for insulin signaling where insulin-driven PI3K-AKT signaling is mediated by the activities of PI3Kα and PI3Kβ, with RAS promoting PI3Kα-dependent insulin signaling. We conclude that PI3K inhibitors discriminating between PI3Kα and PI3Kβ should be used at doses below their hyperglycemic threshold to preserve isoform specificity and achieve optimal therapeutic index.

In **PAPER III**, we have found that compared to primary hepatocytes, three most commonly used hepatoma cell lines display aberrant insulin signaling, gluconeogenic genes expression, glucose production and different electrophoretic profiles, but similar among the hepatoma cell lines. We conclude that, because the hepatoma cell lines appear to converge to a common aberrant phenotype, these cells can be a valuable tool to study the metabolic aberrations in hepatocellular carcinoma.

General conclusion: Altogether this thesis supports the concept that the therapeutic effects of PI3K inhibitors on obesity, insulin resistance and tumor promotion could be largely dissociated from their deleterious effects on glucose homeostasis by using isoform-selective inhibitors discriminating between PI3Kα and PI3Kβ.

Keywords: Obesity, insulin signaling, PI3Ks, PI3K isoform-selective inhibitors.

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SAMMANFATTNING PÅ SVENSKA

Fetma och fetma-relaterade sjukdomar som typ 2-diabetes, hjärt-kärlsjukdomar och olika typer av cancer är de främsta orsakerna till dödlighet och sjuklighet i det moderna samhället. Mekanismerna som kopplar fetma till dessa sjukdomar kvarstår dock i stort olösta. Fosfatidylinositid 3-kinaser, av klass 1 (PI3Kα; PI3Kβ; PI3Kδ and PI3Kγ), har viktiga roller i flera fysiologiska processer. De är involverade i immunsvaret, vid metabol insulinsignalering samt i vävnadshomeostas. Denna avhandling syftar till en bättre förståelse för de olika PI3K-isoformernas roll i fetma och insulinsignalering.

Det är känt att PI3Kγ har en betydelsefull roll i leukocytrekrytering vid inflammation, samt i inhibering av klassisk makrofagaktivering. PI3Kγ är dessutom kopplad till dietinducerad fetma och insulinresistens. I **ARKITEL I** undersökte vi PI3Kγs verkningsmekanismer och fann att aktiviteten av PI3Kγ i hematopoetiska celler inte kan vara den enda mekanismen i leverinflammation, leversteatos, fetma och rekrytering av makrofager i fettvävnad. Dock främjade PI3Kγ-aktivitet insulinresistens, den pro-inflammatoriska M1-makrofagfenotypen samt rekrytering av neutrofiler i fettvävnad hos feta möss. Denna observation utmanar konceptet att PI3K3-aktivitet är en allmän inhibitor av klassisk makrofagaktivering.

I **ARTIKEL II** ville vi definiera klass 1 PI3K-isoformers och RAS roll i insulinsignalering i hepatocyter. Våra resultat ger en ny och bättre mekanism för insulinsignalering där insulindriven PI3K-AKT-signalering förmedlas av aktiviteterna PI3Kα och PI3Kβ, med RAS som främjar PI3Kα-beroende insulinsignalering. Vi drar slutsatsen att PI3K-inhibitor, som skiljer mellan PI3Kα och PI3Kβ, bör användas i doser under deras hyperglykemiska trösklar. Detta för att bevara isoform-specificitet och för att uppnå ett optimalt terapeutiskt index.

I **ARTIKEL III** fann vi jämfört med primära hepatocyter att tre mest använda hepatomcellinjer uppvisar avvikande insulinsignalering, genutryck av glukoneogenes samt glukosproduktion och olika proteinelektroforetiska profiler, men liknande bland hepatomacellinjer. Sammanfattningsvis, eftersom hepatomcellinjer verkar konvergera till en allmän avvikande fenotyp, kan dessa cellinjer vara ett värdefullt verktyg för att studera metabola avvikelser i hepatocellulära carcinom.

SLUTSATS: Sammantaget stöder resultat i den här avhandlingen att användning av isoform-selektiva inhibitorer, som skiljer mellan PI3Kα och PI3Kβ, vid behandling med PI3K-inhibitor mot fetma, insulinresistens och tumörtillväxt skulle kunna reducera dess negativa effekter på glukosmetabolismen.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Breasson L., Becattini B., Sardi C., Molinaro A., Zani F., Marone R., Botindari F., Bousquenaud M., Ruegg C., Wymann M. P., Solinas G. **PI3Kγ activity in leukocytes promotes adipose tissue inflammation and early-onset insulin resistance during obesity.** *Science Signaling. 2017 Jul 18;10(488).*

II. Molinaro A., Becattini B., Mazzoli A., Bleve A., Radici L., Maxvall I., Rotter Sopasakis V., Molinaro A., Bäckhed F., Solinas G. **Insulin-driven PI3K-AKT signaling in the hepatocyte is Mediated by Redundant PI3Kα and PI3Kβ Activities and is promoted by RAS.** *Cell Metabolism. 2019 Jun 4;29(6):1400-1409.e5.*

III. Molinaro A., Becattini B. and Solinas G. **Insulin Signaling and Glucose Metabolism in Different Hepatoma Cell Lines Deviate from Hepatocyte Physiology Toward a Convergent Aberrant Phenotype.** *Manuscript submitted and under revision.*

CONTENT

ABBREVIATIONS

Ad	Adenovirus
AP1	Activator Protein 1
ATM	Ataxia-telangiectasia mutated protein
BMDM	Bone marrow derived macrophages
BMI	Body Mass Index
CCR2	CC-chemokine receptor 2
CNS	Central Nervous System
CREB	c-AMP response element binding protein
DNA-PK	DNA-dependent protein kinase
FAD	Food and Drug Administration
FAS	Fatty acid synthase
FFA	Free Fatty Acids
FOXO 1	Forkhead family box 1
G6P	Glucose 6 phosphatase
GEF	Guanine nucleotide exchange factor
GLUT4	Glucose transporter 4
GPCRs	G protein-coupled receptors
GRB2	Growth factor receptor bound protein 2
GSK3	Glycogen synthase kinase 3
GTT	Glucose tolerance test

HCC	Hepatocellular carcinoma
HER2	Human epidermal growth
HFD	High fat diet
HPG	Hepatic glucose production
HR	Hormone receptor
HSL	Hormone sensitive lipase
IGF-1	Insulin-like growth factor 1
IKK	IkB Kinase
IL-1β	Interleukin 1β
IL-6	Interleukin-6
INF-γ	Interferon γ
iNOS	Inducible nitric oxide synthase
IRS1/2	Insulin receptor substrate 1/2
ITT	Insulin tolerance test
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein Kinases
MCP1	Monocyte chemotactic protein 1
mTORC2	Mechanistic target of rapamycin complex 2
MYD88	Myeloid differentiation primary response 88
NFKB	Nuclear factor-KB
NO	Nitric oxide

PD3B	Phosphodiesterase 3B
PDK1	Phosphoinositide-dependent kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PH	Pleckstrin homology domain
PI3K	Phosphoinositide 3 Kinase
PIP_2	Phosphoinositide 4,5 biphosphate
PIP_3	Phosphoinositide 3,4,5 triphosphate
PKA	Protein Kinase A
PKB	Protein kinase B
PROS	PI3KCA-related overgrowth spectrum
PTB	Phosphotyrosine binding protein B
PTEN	Phosphatase and tensin homolog
RTK	Receptor tyrosine kinase
SH2	SRC-homology 2 domain
SHIP	SH2-containing Inositol 5'-Phosphatase
SNS	Sympathetic Nervous System
SVF	Stromal vascular fraction
T2D	Type 2 Diabetes
TAMs	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TG	Triglyceride

TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor α
WHO	World Health Organization

1 INTRODUCTION

1.1 OBESITY IS A MAJOR BIOMEDICAL BURDEN OF MODERN SOCIETIES

Obesity is affecting over 650 million people worldwide and is the major risk factor for several chronic diseases including type 2 diabetes, cardiovascular diseases, stroke, asthma and cancer [1-3]. Obesity has become a global burden, whose numbers doubled in more than 70 countries since 1980 and is constantly increasing all over the world [4]. In 2015, the deaths caused by obesity-related diseases reached 4 million; 2.3 million were due to cardiovascular diseases in obese patients (70% of the total deaths) and 0.6 million to diabetes, the second leading cause of obesity-related deaths [4]. The most recent epidemiological studies from 2016 showed that 39% of the worldwide adult population, and 18% of children and adolescents from 5 to 19 years old were overweight or obese [5]. Overweight and obesity are described by the World Health Organization (WHO) as “excessive fat accumulation that presents a risk to health” [5]. The most common way to assess underweight, overweight and obesity is the body mass index (BMI), which is a simple weight-to-height ratio calculated as the weight in kilograms divided by the square of the height in metres (kg/m2) [5]. A person with a BMI equal or more than 25 is considered overweight, while a person with a BMI of 30 or more is generally considered obese [5]. Despite the growing recognition of the problem, the mechanisms triggering the link between obesity and obesity-associated diseases remain unsolved.

1.1.1 OBESITY IS CAUSED BY CHRONIC POSITIVE ENERGY BALANCE

Excessive energy intake or insufficient energy expenditure over time contribute to the development of obesity. [6]. Energy intake is intended as food intake and absorption, while energy expenditure represents the energy required to maintain important physiological functions also known as basal metabolism, the energy used to generate heat (thermogenesis) and to perform physical activities [7]. When the energy intake is equal to energy expenditure an energy balance is reached [8] and the body weight is stable. The energy intake and the

energy consumption are co-regulated. In fact, in response to a reduced energy intake the body decreases the energy expenditure; making the weight-loss process a challenge in the long run. If the energy intake rate is higher than the energy expenditure rate, the energy balance is compromised leading to an increase in body weight [6,7]. Indeed, slight changes of the energy balance of only 1-2% in favor of energy intake compare to energy expenditure could cause increase in body weight of ~20 kg in long run [9]. Food intake is regulated by both physiological and external signals that act directly on the brain and alter the secretions from other organs. The sympathetic nervous system (SNS) intervenes in the regulation of body weight integrating signals from central nervous system and from peripheral levels [10, 11]. The SNS is part of the autonomic nervous system and acts by β-adrenergic system on adipose tissue, increasing the lipolysis of triglycerides (TG) and the release of free fatty acids [12] and glycerol in the blood [13]. SNS also increases lipolysis of TG in brown adipose tissue, followed by the oxidation of FFA as part of adaptive thermogenesis [14].

It is well established that body weight regulation depends on signals from different organs for example ghrelin, produced by the stomach; leptin, from adipocytes; insulin, released by the pancreas. The gastric ghrelin is a key regulator of nutrient sensing and meal initiation acting on the specific receptor on hypothalamus, substantia nigra and other central nervous system (CNS) sites inducing appetite and its secretion is inhibited by food intake [15]. Leptin is instead the product of the *ob* gene in adipocytes and its action on the CNS and especially on the hypothalamus reduces appetite and increases the energy expenditure via the sympathetic activation [16-18]. Indeed, mice homozygous *ob/ob* show hyperphagia, hyperglycemia, hyperinsulinemia and hypothermia leading to obesity [19]. Insulin is a hormone, secreted by β-cells of pancreatic islets of Langerhans, which maintains normoglycemia in the bloodstream, triggers lipogenesis and decreases lipolysis [20]. Additionally, some studies indicate that insulin is also involved in the appetite regulation and in energy intake [21].

Understanding the physiological processes that regulate energy homeostasis represents an important challenge in order to combat the global obesity epidemic.

1.2 OBESITY IS A MAJOR RISK FACTOR FOR SEVERAL CHRONIC DISEASES

Increasing evidence indicate that overweight and obesity are major risk factors for several diseases such as type-2 diabetes, cardiovascular diseases, and different types of cancer. However, the mechanisms that link obesity to these chronic diseases are still largely unsolved.

1.2.1 TYPE-2 DIABETES

Type 2 diabetes (T2D) is the most common form of diabetes. In 2016, diabetes was the direct cause of 1.6 million deaths [5]. The pathogenesis of T2D is characterized by insulin resistance and failure of pancreatic β-cells to compensate for such resistance by increasing insulin levels. Insulin regulates blood glucose and lipid levels through different physiological mechanisms such as increasing glucose uptake in muscle and fat, inhibiting hepatic glucose production, stimulating hepatic glycogen synthesis, promoting lipogenesis and reducing lipolysis in adipose tissue. In response to increased blood glucose levels, and certain amino acids such as leucine [22], normal pancreatic β-cells produce insulin, which stabilizes the glucose levels in the blood. Insulin resistant cells are not capable of responding properly to insulin, leading over time to hyperinsulinemia as result of compensatory production of insulin to reduce hyperglycemia. After an initial compensatory insulin secretion, pancreatic β-cells become over time unable to produce enough insulin to compensate high blood glucose levels, which leads to hyperglycemia and diabetes. Different hypotheses have been proposed to explain the mechanism that causes β -cells failure.

1.2.2 ECTOPIC LIPIDS AS A MECHANISM FOR β-CELLS FAILURE

One of the hypotheses to define the cause of β -cells failure is based on the correlation between obesity, type 2 diabetes and the expansion of adipose tissue in other organs. Indeed, obesity was defined by Vague in 1950 as not only the excess of adiposity but also as growing adipose tissue onto proximal organs [23]. Adipose tissue can be divided in two main components:

adipocytes, which represent the 20-40% of the cellular component of the fat pads [24] and stromal-vascular fraction (SVF), which is composed by leukocytes, adipocyte precursor cells and endothelial cells [25] and it is involved in adipose tissue remodeling, angiogenesis [26], cell proliferation [27], apoptosis and extracellular matrix remodeling [28]. Adipose tissue has the capacity to store enormous amounts of energy in form of triglycerides. Furthermore, adipose tissue secrets hormones and cytokines (known as adipokines) which are involved in the regulation of glucose and lipid metabolism and energy homeostasis [29]. In lean adults the 20% of the whole body consists of fat mass, while it is more than 50% in class III obese adults [30]. However, a study showed that the number of cells in the adipose tissue is stabilized in adults [29]. Their results indicate that the adipose precursor cells are recruited during adolescence, while in adulthood adipocytes mainly change their size as a consequence for their transformation in fat mass [29]. Adipocytes can increase in number by a process called hyperplasia and/or in size by a process called hypertrophia. It has been shown that, whereas insulin is inhibiting lipolysis in normal adipocytes, hypertrophic adipocytes become less sensitive to insulin [31]. Additionally, the increase in diameter of the adipocytes resulted in worsen metabolic function of adipose tissue but also in insulin resistance and accumulation of FFA in non-adipose tissue [32, 33]. In 1995, Unger combined the two observations formulating the hypothesis of the expandability of the adipose tissue [34]. This hypothesis was based on the concept that normally lipids are stored in adipocytes in a certain capacity, and when this limit is reached lipids move to non-adipose tissues overcoming their oxidation capacities and causing ectopic accumulation of FFA. The ectopic lipid accumulation in non-adipose tissues and especially in liver and in pancreas during obesity could alter the insulin sensitivity of the target tissues and impair β-cells function [33]. Indeed, the impaired insulin sensitivity induces continuous stimulation of β-cells in producing insulin to compensate hyperglycemia, this may lead to β-cell damage over time, contributing to type 2 diabetes development [33]. Shimabkuro also proposed a mechanism for β-cells failure where the FFA accumulation in pancreatic islet triggers DNA fragmentation and cell apoptosis by the activation of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production [35].

Overall, these evidence lead to the conclusion that obesity is characterized by ectopic accumulation of FFA, which over time can cause severe damages and impaired organ functions. In particular, the fat deposits in the pancreatic islets can reduce the insulin sensitivity and cause β-cell damage, β-cell apoptosis and type 2 diabetes.

1.2.3 OBESITY-DRIVEN METABOLIC INFLAMMATION

Although the mechanisms linking the pathogenesis of obesity with insulin resistance and type-2 diabetes are still under investigation, several lines of evidence have revealed a close relationship between energy intake and the activation of innate immune system in multiple organs involved in energy homeostasis, including adipose tissue, liver, skeletal muscle, heart and brain. The adipose tissue is composed by both adipocytes and SVF, which maintains the integrity and hormonal sensitivity of the adipocytes. SVF is composed of a heterogeneous population of cells, mainly: T cells, mast cells, B cells, neutrophils and macrophages [36]. Macrophages are specific type of white blood cells responsible for phagocytosis and tissue remodeling during innate immune response [25]. The quantity of macrophages present in different tissues can be modulated during obesity development. In fact, several studies showed that the population of macrophages in the adipose tissue of obese mice is increased 4 to 6 time than in control mice [24, 37]. It has been hypothesized that the massive accumulation of macrophages might be a consequence of two processes. First, hypertrophic adipocytes are involved in the secretion of monocyte chemotactic protein 1 (MCP-1), a chemokine that recruits macrophages from the bloodstream [38-40]. Furthermore, the proliferation of monocytes *in situ* due to MCP-1 consequently results in the accumulation of macrophages in white adipose tissue [41, 42]. Adipocyte size does not only influence macrophage number in the white adipose tissue but also their phenotype. In obese mice, adipose tissue macrophages displayed an M1 classical pro-inflammatory phenotype which expresses pro-inflammatory genes such as: interleukin 6 (IL-6), and interleukin-1-beta (IL-1β), and tumor necrosis factor alpha (TNF-α) [43]. M1 macrophages can be activated by inflammatory stimuli such as interferon γ (INF-γ) alone or together with lipopolysaccharide (LPS) or with TNF-α [44]. This increase number of M1-polarized macrophages represents a hallmark of the adipose tissue inflammation and is associated with the development of insulin resistance and metabolic diseases [24, 36]. Indeed, the absence of macrophages by the disruption of macrophages trafficking receptor CC-chemokine receptor 2 (CCR2), or by selective ablation of M1 macrophages, displayed a reduction of inflammation and improved insulin sensitivity [38, 45].

There are also evidence of the contribution of neutrophils in obesity-induced inflammation. Neutrophils are part of the innate immunity and they play a key role in the acute inflammation thanks to their ability to migrate in the

inflammatory sites and produce granulocyte enzymes to break down bacterial or virulence factors causing the inflammation [46] . Neutrophils are recruited to the inflammatory site before the macrophages and they use their granulocyte enzymes to kill bacterial or fungi [45]. Neutrophils also promote the M1 macrophage phenotype through the activation of Toll Like Receptor 4 (TLR4) present on the macrophage membranes [47, 48]. The TLR4 absence in adipose tissue and macrophages has been observed to restore the insulin sensitivity [47]. The activation of TLR4 by LPS leads to the recruitment of myeloid differentiation primary response protein 88 (MYD88) followed by the release of nuclear factor-kB (NF-kB) from the inhibitory complex of IkB. The interaction between the TLR4 and MYD88 activates the IkB kinase (IKK) complex, which is formed by three subunits: IKKα, IKKβ and IKKγ and is responsible for the phosphorylation of IkB. The phosphorylation of IkB frees NF-kB and allows its translocation in the nucleus triggering the activation of different factors involved in inflammation such as: TNF-α, IL-6, IL-1β [49, 50].

Altogether these evidence reveal a role of obesity in promoting metabolic inflammation. Furthermore, the accumulation of ectopic lipids can trigger FFA spillover and subsequent lipotoxicity leading to infiltration of proinflammatory macrophages initiating the inflammatory response. Therefore, both the ectopic hypothesis and the metabolic inflammation represent two distinct but interrelated processes that correlate with the development of insulin resistance and β-cells failure in type 2 diabetes.

1.2.4 OBESITY-MEDIATED TUMOR PROMOTION

In 2003 it was reported that in both men and women, body-mass index was significantly correlated with higher mortality rates due to either one of the following types of cancer: esophagus, or colon and rectum, or liver, or gallbladder, or pancreas, or breast or kidney cancer, or non-Hodgkin's lymphoma or multiple myeloma [51]. Patients with a BMI of 40 kg/m^2 had three times higher risk to die from liver cancer than adults with a lower BMI [50]. Higher body mass index values were observed also in stomach and prostate cancers in men and in uterus, cervix, and ovary cancers in women. The cancer risk in women seemed to be associated with the estrogen receptor alpha, which triggers cell proliferation and it has been associated with breast cancer in obese-postmenopausal women [52].

It has been proposed that insulin and Insulin-like growth factor 1 (IGF-1) might play a role in cancer promotion through the phosphorylation of IRS1 and IRS2 proteins and the activation of the cell proliferation pathway. Gunter and colleagues examined the correlation between insulin, IGF-1 and breast cancer in a cohort of nondiabetic postmenopausal women [53]. Their findings revealed that hyperinsulinemia was an independent risk factor for breast cancer [52]. Another study showed the correlation between hyperinsulinemia and increased cancer risk in non-obese patients, suggesting the possible role of insulin and IGF-1 in cancer development [54].

Furthermore, it has been reported that obese adults have also high levels of circulating FFA [55] which is linked to the secretion in the bloodstream of proinflammatory cytokines such as: TNF-α and IL-6 [42, [56] , which are also involved in obesity-induced tumor-promotion [57]. It was also observed by Leujeune et al. a dual role of TNF-α on tumor growth. TNF-α acted as a tumor suppressor when administrated locally in high dose, or as a tumor promoter in chronic production [58].

This scenario highlights the importance to better understand the mechanisms linking obesity to cancer in order to develop novel therapeutic target for their treatment.

1.3 INSULIN SIGNALING

Insulin is a hormone circulating in the bloodstream which binds with the insulin receptor (IR) on the plasma membrane of the target cell and activates intracellular signals which are involved in the regulation of glucose levels. The insulin receptor binds insulin and becomes active by its auto phosphorylation on tyrosine residues [59]. Ullrich and Rutter cloned the insulin receptor confirming the need of the autophosphorylation of the insulin receptor on the tyrosine to start the intracellular pathways [60, 61]. The insulin receptor is a heterotetrameric glycoprotein composed by two extracellular α subunits that binds insulin and two β subunits with transmembrane domain and intracellular tyrosine-kinase activity domain. When insulin binds the extracellular α subunits of the IR different conformational changes occur resulting in the autophosphorylation of the IR β subunits on the tyrosine residues [62, 63] . The autophosphorylation of the IR generates a docking site for proteins containing phosphotyrosine binding domains (PTB), for example the insulin receptor substrate (IRS) [64]. There are four different isoforms of IRS proteins in mammals and IRS1 and IRS2 are the main isoforms responsible for metabolic

homeostasis [63]. IRS proteins that bind the IR are subjected also to phosphorylation on multiple tyrosine residues which creates binding sites for proteins that contain SRC-homology 2 (SH2) domain [63]. The interaction between IR and IRS activates two different downstream signaling the phosphoinositide 3 kinase/AKT pathway and the Ras-mitogen-activated protein kinase (MAPK) signaling. Regarding the first pathway, the phosphoinositide-3-kinases (PI3Ks) represent a key node in insulin signaling. They bind IRS1/2 through their SH2 domain allowing the release of the catalytic subunit of PI3Ks, p110, from the inhibitory effect of the PI3Ks regulatory subunit, p85, and their consequential activation [65, 66]. The PI3Ks phosphorylate phosphoinositide 4,5 bisphosphate (PIP_2) to generate the second messenger phosphoinositide 3,4,5 triphosphate (PIP_3). PIP_3 is a key node in transduction networks and its intracellular levels of PIP_3 are also controlled by the lipid phosphatases SH2-containing inositol 5' phosphatase (SHIP) or Phosphatase and tensin homolog (PTEN), which dephosphorylate PIP_3 respectively in position 5 and 3 [67, 68]. PIP_3 is responsible for the recruitment at the plasma membrane and the activation of several proteins containing pleckstrin homology (PH) domains, such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (AKT/PKB). The recruitment of PDK1 and AKT/PKB to the plasma membrane allows PDK1 to phosphorylate Akt on Threonine 308 [69]. To be fully activated, Akt has to be phosphorylated also on its Serine 473 residue by the mechanistic target of rapamycin complex 2 (mTORC2) [70], or by DNA-dependent protein kinase (DNA-PK) or by ataxia–telangiectasia mutated protein (ATM) at its C-terminal regulatory domain [71] (Fig. 1).

The phosphorylation of IRS activates a second pathway, the MAP kinase pathway, by the binding of the growth factor receptor bound protein 2 (GRB2), which contains a SH2 domain. GRB2 is then activated and its role is to bind through its SH3 domain and activate SOS, a guanine exchange factor (GEF). The complex GRB2/SOS promotes the interaction between SOS and Ras and exchanges GDP with GTP transforming Ras from an inactive to an active state. The activation of Ras leads to a downstream cascade with first the activation of the mitogen activated protein kinase kinase kinase, Raf. Raf is then responsible for the phosphorylation of the mitogen activated protein kinase kinase, MEK1/2 which triggers the phosphorylation of the mitogen activated protein kinase, ERK1/2 (Fig. 1). In the cytoplasm ERK1/2 dimerizes c-fos and c-jun activating in the nucleus the transcription of Activator Protein 1 (AP1) motifs involved in cellular proliferation, and apoptosis. ERK1/2 also activates ribosomal S6 kinase which regulates the transcription factor c-AMP response element binding protein (CREB) and inhibits SOS, functioning as a negative

feedback for the MAP kinase signaling. ERK 1/2 can also regulate directly in the nucleus the transcription of Myc and other genes involved in cell proliferation and differentiation [72].

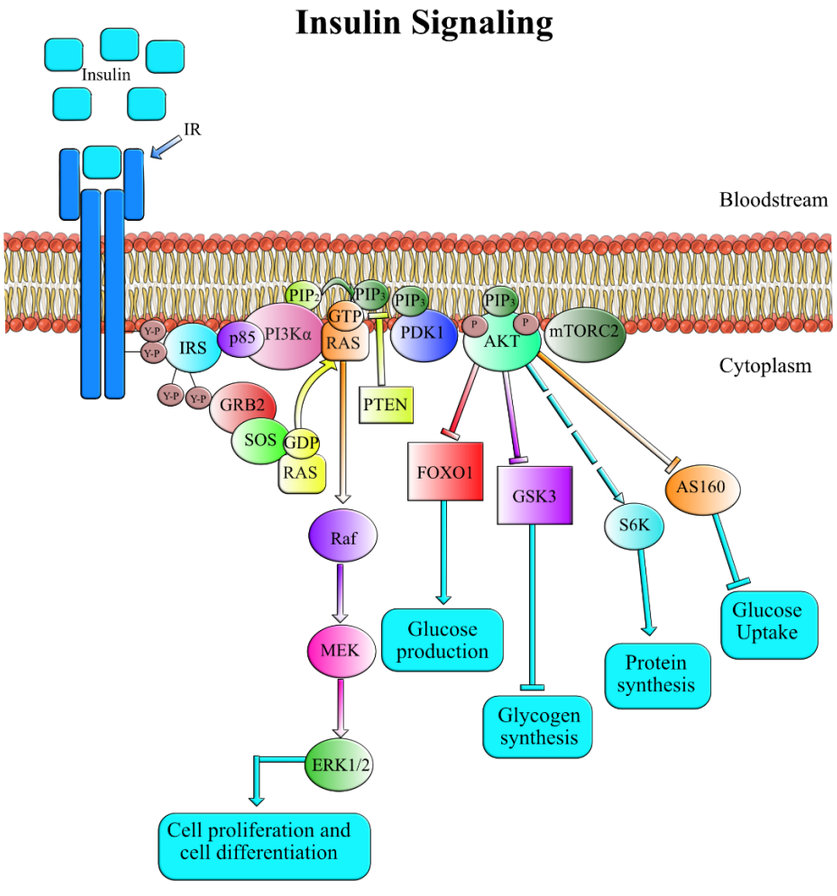

Figure 1: Insulin signaling cascade. Insulin binds the insulin receptor (IR) which autophosphorylates on tyrosine residues. The activation of the insulin receptor allows the recruitment and the phosphorylation on tyrosine sites of the insulin receptor substrate (IRS) proteins. The activated IRS binds the lipid kinase phosphoinsitide 3 kinase (PI3K) releasing the its catalytic subunit (p110) from the inhibitory effect of its regulatory subunit (p85). PI3Kα is considered to be the main Class 1 PI3K isoform responsible to drive the insulin signaling and it requires Ras-GTPase to be fully activated at the plasma membrane. PI3Ks phosphorylate phosphoinositide 4,5 bisphosphate (PIP_2) in phosphoinositide 3,4,5 triphosphate (PIP_3) at the plasma membrane. PIP_3 recruits Akt and phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates Akt on Theonine 308 residue at the plasma membrane. Akt to be fully activated

requires a second phosphorylation on Serine 473 site triggered by mTOR complex 2 (mTORC2) or DNA-dependent protein kinase (DNA-PK) (not shown) or ataxia–telangiectasia mutated protein (ATM) (not shown). The activated Akt regulates glucose production, glycogen synthesis and glucose uptake by phosphorylating and inhibiting respectively forkhead family box O 1 (FOXO1), glycogen synthase kinase 3 (GSK3) and AS160, negative regulator of GLUT4 (not shown). Akt is also indirectly involved in the phosphorylation of S6K and in the protein synthesis process. A second pathway is induced by insulin and its binding with insulin receptor and insulin receptor substrate, the MAP kinase pathway. Indeed, the activated IRS binds the growth factor receptor bound protein 2 (GRB2). GRB2 is activated and binds a guanine exchange factor, SOS forming a complex GRB2/SOS. This complex induces the interaction between SOS and Ras and the exchange of GDP with GTP activating Ras. Ras-GTP activates the mitogen activated protein kinase kinase kinase, Raf. Raf then phosphorylates the mitogen activated protein kinase kinase, MEK1/2. MEK1/2 drives the phosphorylation of the mitogen activated protein kinase, ERK1/2, which is involved in cell proliferation and differentiation.

1.3.1 PHYSIOLOGICAL ACTION OF INSULIN

The main insulin targets are: liver, where insulin mainly suppresses hepatic glucose production and increases glycogen synthesis; muscle, where it promotes glucose uptake; white adipose tissue, where insulin promotes glucose uptake and inhibits lipolysis (Fig. 2); and neurons, where insulin conveys information on nutritional state supporting anorexigenic and locomotor signals [63]. Akt is the key node of insulin signaling and activates different downstream pathways in different cell types. In hepatocytes insulin suppresses gluconeogenesis by the inhibitory phosphorylation of an important transcriptional factor downstream of Akt, the forkhead family box O 1 (FOXO1) and the regulation of gluconeogenic genes expression such as: glucose 6 phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK) [73], responsible for the release and the synthesis of glucose. Moreover, Akt induces glycogen synthesis in hepatocytes by inhibiting glycogen synthase kinase 3 (GSK3), which is a negative regulator of glycogen synthesis [70]. In muscle and adipose tissue insulin is responsible for modulating glucose uptake increasing the translocation of glucose transporter type 4 (GLUT4) from intracellular to the plasma membrane by inhibiting AS160 that is a negative regulator of GLUT4 translocation [70]. In adipose tissue insulin also regulates lipolysis by converting cAMP in 5'AMP which cannot anymore positively regulate PKA and the phosphorylation of HSL, enzyme responsible for the hydrolysis of lipid triglycerides into glycerol and three fatty acids (Fig. 2). This mechanism is subjected to the phosphorylation of the phosphodiesterase 3B (PD3B) by IRS/Akt signaling [14].

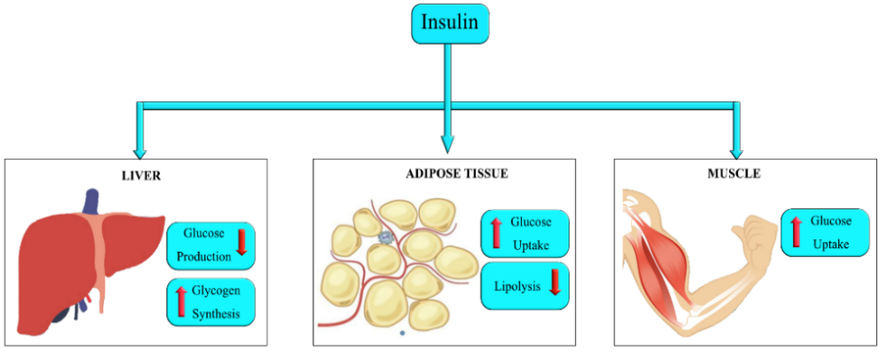

Figure 2: Insulin regulation of glucose homeostasis in different tissues. Insulin regulates glucose homeostasis by reducing glucose production and increasing glycogen synthesis in the liver, or decreasing lipolysis in adipose tissue, or inducing glucose uptake in both adipose tissue and muscle.

1.3.2 PHOSPHATIDYLINOSITOL 3 KINASES FAMILY

The phosphoinositide-3-kinase enzymes are divided in three classes based on their substrate specificity. Class-I PI3K family is divided in two subclasses: class-IA (PI3Kα, PI3Kβ, and PI3Kδ) and the class-IB PI3Kγ. Class-II PI3Ks is instead composed by PI3K-C2α, β, γ, and it can be activated by tyrosine kinases, integrins and regulates membrane trafficking and receptor internalization. Class-III PI3K is composed by VPS34 which is involved in autophagy.

PI3Ks type I family is involved in several mechanisms such as in the control of the immune system, in insulin pathway and growth factor signaling [74]. PI3Ks class I are composed by a catalytic subunit p110 and a regulatory subunit. The catalytic subunit p110α, p110β, p110γ and p110δ are encoded respectively by the genes PIK3CA, PIK3CB, PIK3CG and PIK3CD. While the class-IA PI3Ks interacts with different regulatory subunits: p85α (encoded by PIK3R1 gene) which is spliced into two other isoforms p55α and p50α, p85β (encoded by PIK3R2) and p55γ (encoded by PIK3R3); p110γ binds two different regulatory subunits p84/p87 or p101 (encoded respectively by PIK3R6 and PIK3R5 genes) (Fig. 3).

All class-IA PI3Ks are activated by receptor tyrosine kinases (RTKs), and PI3Kβ is also activated by G protein-coupled receptors (GPCRs) [75].

Moreover, it has also been reported in structural and binding studies that a direct interaction between Ras and in PI3Kα is required for PI3Kα to be fully activated at the plasma membrane [76-78]. Class-IB PI3Kγ is typically activated by G protein-coupled receptors implicated in immunity (eg. chemokines) and metabolism (eg. the β-adrenergic receptors) [79]. PI3Kγ plays a central role in leukocyte recruitment in acute inflammation, and is implicated in the polarization of macrophage activation, and mast cell degranulation [80-83].

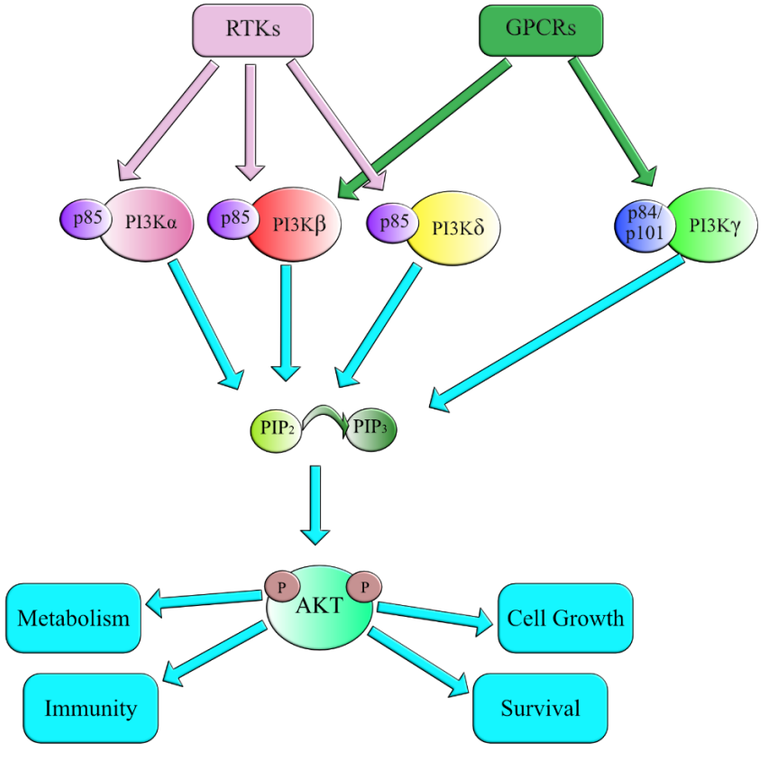

Figure 3: The phosphoinositide-3-kinase family Class-IA PI3Ks (PI3Kα, PI3Kβ, PI3Kδ) are activated by receptor tyrosine kinases (RTKs), and PI3Kβ is also activated by G protein-coupled receptors (GPCRs). Class-IB PI3Kγ is activated by GPCRs. All Class-I PI3Ks phosphorylate phosphoinositide 4,5 bisphosphate (PIP_2) in phosphoinositide 3,4,5 triphosphate (PIP_3), inducing the insulin-driven Akt phosphorylation and the downstream pathways which are involved in metabolism, immunity, survival and cell growth.

Additionally, to these differences in interactions, PI3Ks isoforms are located in different tissues in the human body, for example p110α and p110β are ubiquitously expressed, however p110δ and p110γ are most abundant in hematopoietic cells [84]. p85α is also ubiquitously expressed, [73, 85] and it has been shown that the deletion of p85α triggers the reduction of p110 expression levels confirming the role of p85 in the stability of p110 isoforms [66]. In addition, it has been reported that the homozygous deletion of either p110α or p110β (the two most widely expressed PI3Ks) caused early embryonic lethality in mice [86, 87], indicating the important role of both isoforms in embryogenesis. Mice with heterozygous deletion of p110α or p110β were still viable but they displayed no effect on insulin signaling as a complication of a compensatory down-regulation of the p85 regulatory subunit [88]. It has also been shown that a kinase-dead homozygote mutation allele for p110α ($p110\alpha^{D933A/D933}$) was lethal in mice due to embryonic angiogenic defects [89]. However, the heterozygote $p110\alpha^{D933A/WT}$ mice were viable and showed a reduction in insulin-induced PI3K activity, indicating that p110α is a key intermediate in IGF-1, insulin and leptin signaling and the specificity of IRS1 towards p110α [89]. Knight and colleagues also performed a pharmacological mapping of the different class I PI3Ks in insulin signaling using cell lines [90], showing a main role of PI3Kα in the insulin signaling. Additionally, liver-specific conditional knock-out mice for p110α displayed impaired insulin action and glucose homeostasis. The overexpression of p110β did not ameliorate the phenotype of these mice, confirming the major role of p110α in the insulin signaling [91]. These studies lead to the dogma that PI3Kα is the predominant lipid kinase required for insulin signaling.

1.3.3 THE ROLE OF PI3Ks IN METABOLIC INFLAMMATION

The class-I enzymes do not respond equally to same stimuli, for example the inhibition of PI3Kγ causes a drastic reduction of the inflammatory response, suggesting a major role of PI3Kγ in inflammation [16, 20, 21]. Indeed, the null-PI3Kγ mice showed impaired inflammatory response by a reduced mobility of neutrophils and macrophages to chemotactic stimuli [14]. In addition, it was shown that PI3Kγ is required for PIP_3 chemoattractant-induced production, mediated by T cells and neutrophils [92].

It has been independently reported by our group and by Kobayashi's that PI3Kγ plays an important role in the progression of diet-induced obesity and metabolic inflammation [93, 94]. In their study, Kobayashi et al. proposed that

in mice lacking PI3Kγ the improvement in hepatic steatosis, insulin sensitivity and obesity was due to the direct action of PI3Kγ in myeloid cells, driving the metabolic inflammation and insulin resistance [94]. On the contrary, Becattini et al. proposed that the improved metabolic phenotype of the mice lacking PI3Kγ placed on high fat diet is due to the inhibitory action of PI3Kγ on diet-induced thermogenesis in the non-hematopoietic compartment. PI3Kγ might act as an inhibitor of PKA-mediated hormone-sensitive lipase (HSL) activation and adaptive thermogenesis [93].

1.3.4 PHOSPHATIDYLINOSITOL 3 KINASES AS DRUG TARGET FOR CANCER AND PROS THERAPY

It is well established that Phosphatydilinositide-3 kinase (PI3K) activity plays a major role in tumor promotion and growth [95]. A tumor is a very complex environment where different type of cells such as tumor-associated macrophages (TAMs), neutrophils (TANs), and dendritic cells are communicating among them and express high levels of PI3Kγ [82, 92, 96, 97].

Analysis of the tumor myeloid environment revealed that it follows the inflammatory response classification. It has been shown that proinflammatory macrophages M1 and cytotoxic T cells (CD8+ T-cells) can reduce tumor development. On the contrary, the anti-inflammatory M2 macrophages promote tumor growth and immune evasion through their immunosuppressive action [44, 98]. Kaneda and colleagues observed an association between PI3Kγ mRNA levels and inflammatory gene expression [83]. Furthermore, in solid tumors characterized by important inflammatory cell infiltration, such as pancreatic ductal adenocarcinoma; it has been shown that the inhibition of PI3Kγ enhanced an anti-tumor inflammatory response [99].

Other proteins involved in the insulin signaling and in tumor promotion are PTEN and PI3Kα which are indeed frequently mutated in human cancer and in overgrowth syndromes [100]. PTEN is well known to be a tumor suppressor and a negative regulator of insulin signaling [101]. PIK3CA, encoding for PI3Kα is frequently mutated in many tumors including: colon, ovary, breast, brain, liver, stomach, endometrial and lung cancers [102-104]. Several somatic mutations of PIK3CA have been identified but three hot-spot mutations E542K, E545K and H104R represent the 80% of all the PIK3CA mutations found in tumors. E542K and E545K mutations are located in the helical

domain, while H104R is present in the kinase domain of the catalytic subunit p110α. It has been shown with *in vitro* experiments that these mutations enhanced cellular proliferation and invasion by increasing PI3K activity [105, 106]. Somatic PIK3CA activating mutation, in isolation, are not sufficient to cause cancer but lead to a spectrum of overgrowth syndromes named PIK3CA-related overgrowth spectrum (PROS) [101]. PROS is a group of diseases characterized by post-zygotic gain-of-function mutations in the PIK3CA gene, resulting in constitutive activation of PIK3CA. Patients with a diagnosis of PROS appear to have tissue-specific overgrowth, while for other patients could be more pleiotropic [101].

There is a wide medical interest on PI3K inhibitors as potential cancer therapeutics, but it is necessary to achieve a better understanding of the PI3K action. Different pan-inhibitors have been used as cancer therapies showing limited success due to side effects from the treatments such as hyperglycemia and compensatory hyperinsulinemia [95, 100]. Nowadays, four PI3K inhibitors have been used in clinic: Idelalisib (PI3Kδ inhibitor), Copanlisib (Pan-PI3K inhibitor), Pictilisib (PI3Kα/δ inhibitor) BYL719 (Alpelisib, PI3Kα inhibitor). Especially regarding BYL719, a recent study showed a reduction of tissues overgrowth and strong improvements of the physical conditions of PROS patients which were treated with a low dose of the PI3Kα selective inhibitor. The low dose of BYL719 did not cause hyperglycemia, a result which cannot be easily explained by the current model for insulin signaling [107].

In the normal state, when the blood glucose levels are increased, the pancreatic β-cells respond to hyperglycemia by producing insulin, which blocks the hepatic glucose production (HGP) and enhances the glycogen synthesis in liver by the activation of the PI3Ks. During the inhibition of PI3Ks, the pancreatic β-cells are still producing insulin but the liver is not able to block the hepatic glucose production any further due to the inactivation of PI3Ks, causing both hyperglycemia and consequently hyperinsulinemia. In response to constant increased levels of insulin, the PI3Ks activity is partially reactivated in metabolically relevant tissues and also in insulin sensitive cancer cells, reducing the effects of the PI3Ks inhibitors [95, 108].

All the findings enounced previously enlighten the need for a better mechanistic understanding of the role of each PI3Ks isoform in insulin signaling and metabolic homeostasis to be able to discriminate the beneficial effect of the inhibition of PI3Ks on cancer from the deleterious effect on glucose homeostasis. The acquisition of this knowledge will help in developing more efficient treatments for cancer, PROS, obesity and obesity-related

diseases using the already developed isoform-specific PI3Ks inhibitor or new compounds.

2 AIM

The aim of this thesis is to gain new knowledge on the specific role of phosphoinositide 3 kinase isoforms in insulin signaling and obesity.

PAPER I: the aim of this study is to clarify the role of PI3Kγ in diet-induced obesity and insulin resistance. To achieve our goal, we used mice models that have a systemic ablation or a tissue specific knockout of PI3Kγ and both dietary or genetic mouse models of obesity.

PAPER II: this study aims at identifying the role of Ras and the different PI3K isoforms in insulin signaling. The current model of insulin signaling defines PI3Kα as the responsible isoform for the insulin pathway in a Ras independent manner. This concept is in contrast with the evidence that PI3Kα requires Ras to be fully activated. The two observations show a contradiction in the current model of insulin pathway. Therefore, we decided to further investigate the involvement of other PI3K isoforms in insulin signaling.

PAPER III: The aim of this study is to compare the insulin signaling and gluconeogenesis in hepatoma cell lines derived from different species with primary hepatocytes.

3 METHODOLOGICAL CONSIDERATIONS

In this session, I will briefly discuss the methods used in the three papers included in this thesis and their pros and cons.

3.1 MICE MODELS OF OBESITY

Mice are the most commonly used research models. The use of these animals in research has several advantages. Mice have small body size, they have a short life span, they reproduce quickly and share similarities with human anatomy and genome. Indeed, mice genome shares 95% similarity with human DNA, which confirms the possibility to use mice as model for metabolic studies [109].

However, animal models present also different disadvantages. They might show some differences especially in the translation of findings from animal models to humans. This could be due to the different pathogenesis of the metabolic disorders between mice and humans or to the not complete similarity between the two species. There are also other factors that have to be taken in consideration when a study is performed on animal models which are: the age, the gender, the laboratory environment including water, food, temperature, circadian cycle, noise and also the stress conditions that the mice are facing in the housing, bedding and breeding. Therefore, it is very important to plan ahead if and how to use the animal model in scientific studies.

Furthermore, it is essential to consider the ethical implications in using animal models and to respect the 3R principle of replacement, reduction and refinement. This ethical guideline encourages researchers to avoid or replace the use of animal models if possible, to minimize the number of animals needed for a study and to improve animal welfare reducing or alleviating potential suffering, stress and pain for the animals.

In this thesis we have used C57BL/6 and 129/SvJ-C57BL/6J mixed genetic background mice. The animals were kept at 22°C under 12-hour light and 12-hour dark cycles in the animal facility. The mice were weaned at 4 weeks of age and kept on chow diet for the genetical modified mice and on high fat diet (60% of calories from fat from Bio-Sery, Diet F3282) for the diet-induced obesity model. The mice were kept on high fat diet from 5-6 weeks until 23 weeks of age. All animal experiments were approved by cantonial veterinary

Committee in Switzerland and the Animal Ethical Committee in Göteborg, Sweden.

3.1.1 TRANSGENIC MICE

In **PAPER I** we used total body knockout mice for PI3Kγ (PI3Kγ$^{-/-}$) from Prof. Matthias P. Wymann.

The *ob/ob*-PI3Kγ$^{-/-}$ mice were obtained by crossing the *ob/+* mice with PI3Kγ$^{-/-}$mice which present a systemic ablation of PI3Kγ.

The PI3Kγ$^{F/F}$ mice from the European Conditional Mouse Mutagenesis Program (EUCOMM) were generated by crossing mice expressing the Flippase (FLP) recombinase under the *actin* promoter with Pik3cg<tm1a(EUCOMM)Wtsi>/Wtsi mice. These mice were characterized by the exons 3 and 4 flanked by *LoxP* sites and were used in the study as control group for the conditional knockout mice for PI3Kγ.

We obtained the conditional knockout mice lacking PI3Kγ in myeloid cells (PI3Kγ^{LysM}) by crossing the PI3Kγ$^{F/F}$ mice with transgenic mice (from Jackson laboratory) expressing the Cre recombinase under the control of the *lysozyme-M* promoter. To generate the conditional knockout mice for the PI3Kγ in hematopoietic and endothelial cells (PI3Kγ^{HE}), we crossed the PI3Kγ$^{F/F}$ mice with mice expressing the Cre recombinase under the *TEK* (*Tie2*) promoter.

In **PAPER II** we used C57BL/6 wild type and PI3Kα^{Hep} and PI3Kα$^{F/F}$ mice which were 129/SvJ C57BL/6J mixed background mice and provided by Vittoria Rotter Sopasakis [91]. The PI3Kα^{Hep} were characterized by the ablation of PI3Kα specifically in the hepatocytes and we used the PI3Kα$^{F/F}$ as control LoxP floxed group.

In **PAPER III** we used C57BL/6 wild type mice.

3.1.2 *IN VIVO* STUDIES

Here I would like to reflect on how we performed our *in vivo* analysis and the reasoning behind our choice.

All the *in vivo* experiments were performed comparing each cohort of mice with a control group. All the mice cohorts were generated from the same strain and were age match and littermates. These experimental conditions allowed us

to avoid the possible complications derived from different backgrounds or different age or different gender of the mice. The fasting conditions were maintained exactly the same for each group (4 hours) and performed during the same range of circadian cycle (from 7:00 to 11:00 AM), avoiding the possible variation in glucose levels occurring in longer or shorter fasting times at different hours of the day. Several researchers also prolonged the fasting hours to 14-18 hours to reach a more fasting condition especially in terms of low glucose levels and depletion of glycogen levels [110, 111]. However, prolonged duration of fasting can cause metabolic stress. Therefore, to avoid the metabolic stress and to follow the ethical guideline in our facility we performed 4 hours of fasting, which was a suitable time to see differences in glucose tolerance and insulin sensitivity between the different mice models without causing any alterations in the analysis or any pain to the animals.

We performed mainly glucose tolerance test (GTT) and insulin tolerance test (ITT) which are the most frequently used analysis to determine glucose metabolism in mice [112, 113]. GTT provides general information on the glucose homeostasis in the whole body, while ITT gives also information regarding the ability of specific tissues to respond to insulin. In fact, insulin administration reduces the glucose levels in the blood mainly due to the insulin action on liver and muscle. The results of these tests can variate according to the concentration of glucose or insulin administered to the mice. In our experiments we injected peritoneally 2g of glucose per kg of body weight and 0.5 I.U. of insulin per kg of body weight. After the injection blood was collected from the tail, glucose levels were measured with a glucometer and serum was prepared for insulin measurements. The procedure could be stressful for the mice, which then were kept in their cages for a week before performing any other procedure giving the animals the possibility to release the stress avoiding any alterations or complications in the following *in vivo* analysis.

In **PAPER II**, we also injected peritoneally a PI3Kβ specific inhibitor, TGX221, in three months old PI3Kα^{Hep} and PI3K$\alpha^{F/F}$ mice. We used the 2mg of the inhibitor per kg of body weight as suggested from the company (Sellekchem). TGX221 solution was prepared under laminar flow using 1,6% TGX221 (from 32,92 mM stock in DMSO), 30% PEG, 1% Tween-80 in PBS. We used control vehicle composed by the same solution without the inhibitor. The injections were performed as for the GTT and ITT and blood was collected from the tail to measure the glucose levels with a glucometers.

All mice were anesthetized with Isofluorane (Baxter KDG9623) and sacrified by bleeding following the approved protocols from the cantonial veterinary Committee in Switzerland and the Ethics Committee on Animal Care Use in Gothenburg, Sweden.

3.2 HUMAN SAMPLES FROM DONORS

In **PAPER II** we performed the analysis on insulin signaling on hepatocytes derived from human donors from a commercial provider approved by AstraZeneca, BioreclamationIVT complying with the company Human Biological Samples policies.

3.3 *IN VITRO* MODELS OF OBESITY FOR METABOLIC STUDIES

In vitro studies are important and frequently used model to study obesity and metabolic diseases [114, 115]. The advantages of using a cell model is the possibility to test from the same source different experimental conditions and it is a simpler tool than *in vivo* analysis. However, it is essential to determine ahead which type of cells are more suitable for metabolic studies and how to culture them to not alter in anyway their responsiveness and their metabolic homeostasis.

In **PAPER I** we produced primary bone marrow derived macrophages (BMDM) from WT and PI3K$\gamma^{-/-}$ mice. The cells were extracted from proximal tibia bone marrow from mice and differentiated in macrophages *in vitro* in RPMI medium containing L-glutamine, 10% fetal calf serum, 1% penicillin/streptomycin and 10% L929 cell-conditioned medium for 6 days. We used the BMDM because we wanted to investigate the cell-autonomous role of PI3Kγ in macrophage activation.

In **PAPER II** our aim was to identify if another PI3K isoform was involved in the insulin signaling, therefore we performed all our experiments in primary hepatocyte, which are the main responsive cell type to insulin.

The preparation of primary hepatocyte and BMDM are long and very delicate techniques which require to scarify mice and to repeat the procedure from

different mice to validate the results. The time, the cost and number of mice used to finalize the results can become a disadvantage. However, primary cells preserve the tissue characteristics and functions without occurring in mutations, therefore they represent a suitable *in vitro* model for metabolic studies.

In several studies researchers used cell lines to perform their experiments. The most commonly used cell line for metabolic studies is the HepG2. The HepG2 cells derived from the liver cancer tissue of a 15 years old Caucasian male in 1983. The advantage of using the cell lines is their availability, their price and their capacity to proliferate very easily. On the other hand, the disadvantage is that they are immortalized cells that can survive unlimited time but to be able to do so they accumulate a series of mutations in key genes involved in metabolic pathways such as insulin signaling [116]. To assess the limits of the cell lines for metabolic studies and especially for insulin signaling, gluconeogenic gene expression, glucose production and electrophoretic protein profile, we compared in **PAPER III** three hepatoma cell lines to primary hepatocytes. The cell lines used in this study were HepG2, human hepatoma, McARH77777, rat hepatoma and Hepa 1-6, mouse hepatoma compared to primary hepatocytes from WT mice and human hepatocyte lysate.

3.3.1 ADENOVIRUS VECTORS

In **PAPER II** and **PAPER III** we investigated the insulin signaling using adenoviruses from Prof. Luciano Pirola Unitè 1060 INSERM CarMen Lyon France. Adenoviruses are nonenveloped icosahedral protein capsids with approx. 70-100 nm diameter and a linear double-stranded DNA of about 36 Kb. Over 100 different mammalian and foul adenovirus (Ad) serotypes have been characterized and type 5-based vectors are the most extensively studied [117]. One of the advantages in using the adenoviruses as gene vectors is the high efficiency and fidelity of gene transfer in terms of delivery of many genome copies per target cells either in terms of delivery of large genes or in the genetical stability of vector genomes. Furthermore, adenoviruses could be used for insertional mutagenesis, could be produced in large scale and administrated *in vivo*. On the other hand, the pre-existing immunity against adenoviruses might alter the transgene delivery and expression [117]. Adenoviruses can also be lethal at high doses and are immunogenic and can cause inflammation which can interfere which metabolism homeostasis [117].

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 MAIN RESULTS PAPER I

Obesity is associated with low-grade metabolic inflammation and anti-inflammatory interventions might be considered also as treatments for obesity-related diseases. Phosphoinositide 3-kinase γ, PI3Kγ, is an important signal transducer activated by innate immunity signals such as chemokines and bacterial lipopolysaccharides [12, 97, 118-120], but also by the adrenergic signaling which is essential in several metabolic processes such as lipolysis and adaptive thermogenesis [20, 21]. It has been shown by our group and by other laboratories that, mice lacking PI3Kγ are protected by diet-induced obesity, metabolic inflammation and insulin resistance [93, 94]. Two mechanisms have been proposed to explain the action of PI3Kγ in obesity progression. The first mechanism suggests PI3Kγ as a promoter of obesity-driven metabolic inflammation in myeloid cells and consequently cause of insulin resistance, altered hepatic gene expression for lipid metabolism and fatty liver. The second mechanism focuses on the role of PI3Kγ both as inhibitor of PKA-driven HSL activation and adaptive thermogenesis and as promoter of excessive adiposity, steatosis, metabolic inflammation and insulin resistance in a nonhematopoietic cell type [22]. However, no cause-effect mechanism of PI3Kγ action in diet-induced obesity, metabolic inflammation and insulin resistance was defined. Our results elucidate the role of PI3Kγ in diet-induced inflammation and insulin resistance.

We first reproduced the key observations from the two studies above that in the same cohort of mice, PI3Kγ ablation protects from diet-induced obesity, insulin resistance and hepatic steatosis. We also observed an increase of HSL phosphorylation in adipose tissue of knockout mice for PI3Kγ (PI3Kγ$^{-/-}$), indicating an increased PKA-induced lipolysis. PI3Kγ$^{-/-}$ mice also showed protection from metabolic inflammation in liver and in white adipose tissue reduced macrophage accumulation, reduced pro-inflammatory M1 macrophage phenotype activation markers expression and increased expression of anti-inflammatory M2 macrophage phenotype activation markers.

It has been shown that PI3Kγ ablation in genetically obese mice (*ob/ob)* ameliorates obesity-induced insulin resistance and metabolic inflammation independently from changes in body weight [94]. To further investigate if PI3Kγ action in obesity, metabolic inflammation and insulin resistance is due to its role in adiposity, we generated mice lacking PI3Kγ in *ob/ob* mice background (*ob/ob*-PI3Kγ$^{-/-}$mice). Compared to control *ob/ob* mice at two different time points, *ob/ob*-PI3Kγ$^{-/-}$ showed similar body weight, body composition, similar hepatic steatosis, and number of adipose tissue crown-like structures. The *ob/ob*-PI3Kγ$^{-/-}$ mice displayed a mild and transient improvement in insulin and glucose tolerance tests. Moreover, the loss of PI3Kγ shifted the gene expression of macrophages activation markers from M1 profile to M2 in white adipose tissue (Fig. 4).

Furthermore, to investigate the role of PI3Kγ in adipose tissue macrophages in obesity, we generated conditional knockout mice for PI3Kγ in myeloid cells (PI3Kγ^{LysM}). However, due to a partial deletion of PI3Kγ in adipose tissue myeloid cells from PI3Kγ^{LysM} mice and due to the involvement of leukocytes in promoting classical M1 activation of adipose tissue macrophages during obesity [30, 31, 33], we generated another conditional knockout mice model lacking PI3Kγ in hematopoietic and endothelial cells (PI3Kγ^{HE}). These mice model placed in high fat diet (HFD) also revealed similar body weight and HSL phosphorylation levels as well as adipocyte size distribution, hepatic steatosis, hepatic inflammation, and adipose tissue crown-like structures to the control group. However, PI3Kγ^{HE} mice displayed a transient improvement of glucose and insulin tolerance tests. In addition, we observed again a shift of macrophage polarization towards M2 phenotype in adipose tissue which increased even more with age (Fig. 4).

We then produced primary BMDM from knockout mice for PI3Kγ and WT mice and cultured them in presence of LPS and IFNγ to drive an M1 macrophage activation, and IL4 and IL13 to drive an M2 macrophage activation to investigate the cell autonomous role of PI3Kγ. Loss of PI3Kγ showed a significant reduction of AKT phosphorylation, indicating that the activity of PI3Kγ is required to have an efficient AKT phosphorylation in BMDM in response to both M1 and M2 activation stimuli. However, we could observe only small but significant differences in BMDM gene expression of M1 and M2 markers and especially MCP-1 in macrophages exposed to M1 activation medium for 4 hours or MGL-1 after 24 hours in M1 activation medium, supporting the idea that PI3Kγ has a role in promoting M1 macrophage phenotype. We then treated the BMDM with macrophage-activation signals to analyze the role of PI3Kγ in classical or alternative

macrophage activation pathways. Our data from BMDMs indicate that PI3Kγ activity reduces STAT6 phosphorylation after exposure to IL13 in macrophages. Moreover, our findings reveal that the promotion of the M2 macrophage phenotype in adipose tissue in absence of PI3Kγ is most probably the result of a non cell-autonomous mechanism.

Since adipose tissue neutrophils were implicated in obesity-driven M1 polarization and PI3Kγ plays a major role in neutrophil chemotaxis in inflammation [35], we evaluated PI3Kγ role in the recruitment of neutrophils to adipose tissue. PI3Kγ^{HE} on high fat diet and *ob/ob*-PI3Kγ$^{-/-}$ on chow diet mice had fewer neutrophils in the adipose tissue compared to their control mice at two different time points. Our data indicate that PI3Kγ activity in the hematopoietic and endothelial cells does not alter INFγ, IL13 and IL4 concentrations in the adipose tissue but is necessary in the recruitment of neutrophils (Fig. 4).

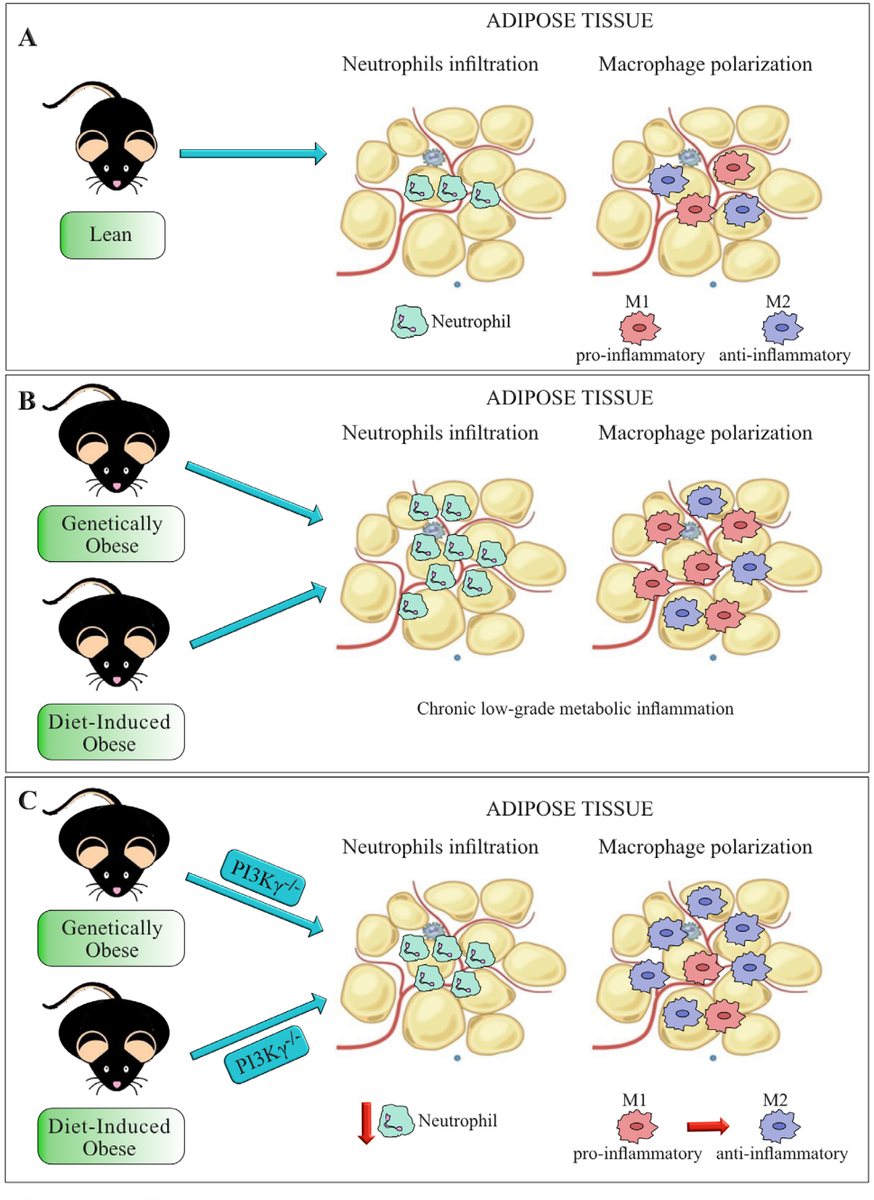

Figure 4: PI3Kγ activity in leukocytes promotes adipose tissue inflammation and early-onset insulin resistance. (A)Compared to lean mice, (B) obese mice show chronic low-grade metabolic

inflammation. However, (C) loss of PI3Kγ in genetically obese mice or diet-induced obese mice showed reduced neutrophils infiltration and a shift of macrophage polarization from M1 to M2 phenotype in adipose tissue.

4.1.2 DISCUSSION PAPER I

Our data indicate that the action of PI3Kγ in obesity-driven inflammation and insulin resistance is mostly dependent on its role in promoting diet-induced adiposity. Furthermore, our findings on *ob/ob*-PI3K$\gamma^{-/-}$, conditional knockout mice for PI3Kγ fed with HFD and BMDM, highlight an additional role of PI3Kγ activity in leukocytes in promoting obesity-driven insulin resistance and a shift of gene expression of macrophage markers in obese adipose tissue from a M2 status to an M1 phenotype. This observation is challenging the concept that PI3Kγ is a major general inhibitor of classical macrophage activation and a promoter of immunosuppressive gene expression helping tumors to evade from immune response [16, 17, 19].

Altogether our data demonstrate that PI3Kγ activity in macrophage activation is not cell-autonomous mechanism and depends on the context. Moreover, the ablation of PI3Kγ reduced neutrophils accumulation in adipose tissue, which are important promoter of M1 macrophage polarization suggesting a role of PI3Kγ in macrophage inflammatory gene expression in adipose tissue through its action on the recruitment of neutrophils.

4.2 PAPER II

4.2.1 MAIN RESULTS PAPER II

There are four PI3Ks isoforms [118], however, several studies draw the conclusion that the insulin action is mainly mediated by PI3Kα [90, 91, 121-125] in a Ras independent manner [64, 121, 126-128]. However, PI3Kα requires an active Ras GTPase to be fully activated at the plasma membrane [76-78]. In this paper we aimed to clarify the role of different PI3K isoform in insulin signaling.

We performed all our analysis in WT mice or mice lacking the PI3Kα specifically in hepatocytes ($PI3K\alpha^{Hep}$) and their control group, $PI3K\alpha^{F/F}$ mice. We have phenotyped a cohort of weight matched mice and we observed that the loss of PI3Kα in hepatocytes did not cause hyperglycemia but only a moderate insulin intolerance, which was compensated by mild hyperinsulinemia and a mild glucose intolerance only in male mice. Furthermore, we showed that the genetical or somatic ablation of PI3Kα through adenoviral-delivery of Cre recombinase did not affect AKT phosphorylation and downstream signaling, indicating that the loss of PI3Kα is widely compensated by another PI3K isoform.

We performed a pharmacological mapping of the PI3K isoform compensating for PI3Kα ablation using isoform-specific PI3Ks inhibitors in cultured hepatocytes from $PI3K\alpha^{Hep}$ mice and $PI3K\alpha^{F/F}$ mice using the phosphorylation on AKT as readout. We observed redundant activities of PI3Kα and PI3Kβ in insulin signaling. Indeed, in presence of PI3Kβ specific inhibitor, TGX221, insulin-driven AKT phosphorylation was completely blunted together with relevant metabolic downstream targets of AKT only in hepatocytes lacking PI3Kα. Furthermore, we performed an *in vivo* experiment where we observed no effect of the inhibition of PI3Kβ on $PI3K\alpha^{F/F}$ control mice but a remarkable hyperglycemia in $PI3K\alpha^{Hep}$ mice. Overall, these results confirmed that the insulin signaling is mediated by redundant activities of PI3Kα and PI3Kβ in hepatocytes and that only a combined inhibition of PI3Kα and PI3Kβ can cause hyperglycemia (Fig. 5A).

Insulin-induced PI3Kα activity should be reduced by the absence of an active Ras, which is responsible for the activation of PI3Kα at the plasma membrane [76-78]. Therefore, we have investigated the role of Ras in primary hepatocytes infected with different doses of adenovirus expression the dominant negative mutant for Ras, HRAS17N, or control adenovirus GFP. The blockage of Ras,

already at low dose of HRAS17N, blunted ERK phosphorylation levels in murine hepatocytes. Moreover, the higher dose of 100 MOI of HRAS17N virus revealed again blunted signal of ERK but also a reduced insulin-driven AKT phosphorylation without affecting the phosphorylation of the insulin receptor and without showing an obvious toxicity (Fig. 5B). The reason behind the different sensibility to the adenovirus HRAS17N between AKT and ERK is related to their different mechanisms of activation. ERK requires active Ras dimers at the plasma membrane becoming more sensitive mechanism to the Ras inhibition than PI3Kα which does not require the formation of Ras dimers but a direct binding with active Ras monomers to functionally interact to the plasma membrane and be activated [76-78]. However, the inhibition of Ras in the hepatocyte did not impair insulin signaling downstream AKT or AKT phosphorylation in primary hepatocytes lacking PI3Kα.

We observed the same blunted signaling of ERK phosphorylation and reduced AKT phosphorylation in human hepatocytes derived from four different donors, indicating that in insulin-driven AKT phosphorylation Ras role is conserved between mouse and human.

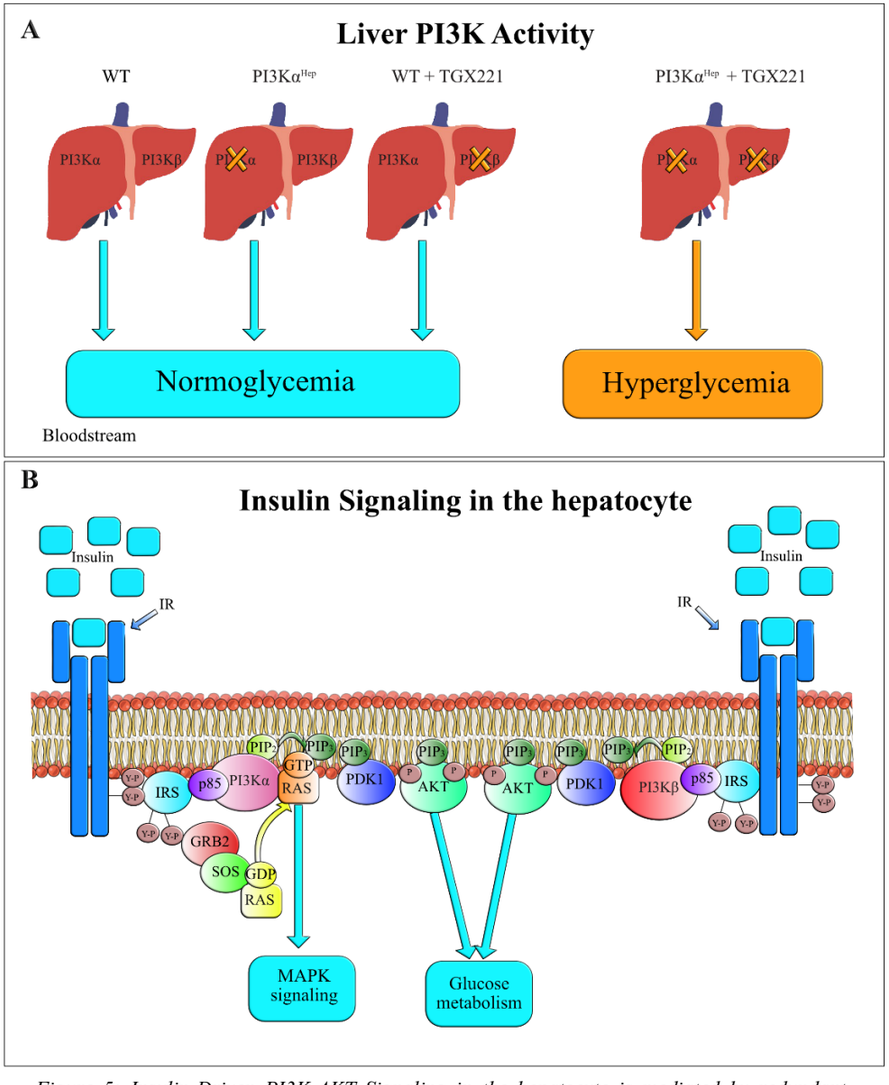

Figure 5: Insulin-Driven PI3K-AKT Signaling in the hepatocyte is mediated by redundant PI3Kα and PI3Kβ activities and is promoted by Ras. (A) Mice lacking PI3Kα specifically in hepatocytes (PI3Kα^{Hep}) were injected with TGX221, selective inhibitor of PI3Kβ, showing severe hyperglycemia. On the contrary, in WT mice or PI3Kα^{Hep} or WT mice injective with TGX221, where only one isoform was lacking either PI3Kα or PI3Kβ, we could not observe any changes in glucose levels in the bloodstream. (B) This observation together with our data on

primary hepatocytes where the blockage of Ras reduced Akt phosphorylation and blunted ERK signaling, led us to define a new and improved model of insulin signaling in the hepatocyte where the insulin-driven Akt signaling is mediated by redundant activities of both PI3Kα and PI3Kβ, and PI3Kα is Ras dependent.

4.2.2 DISCUSSION PAPER II

Our findings from **PAPER II** suggest a paradigm shift from a current model of insulin signaling where PI3Kα is the only responsible PI3K isoform to drive the insulin pathway [90, 91, 122-125], to a new and improved mechanism where insulin-driven PI3K-AKT pathway in hepatocyte is mediated by a redundant activity of both PI3Kα and PI3Kβ. Furthermore, PI3Kα activity in the insulin signaling has been considered to be Ras independent [64, 121, 126-128]. However, Ras is required to fully activate PI3Kα at the plasma membrane. Our results show that the blockage of Ras through an adenovirus infection blunted ERK and only at higher concentration of the adenovirus also reduced the AKT phosphorylation levels in murine hepatocytes but not in the functional downstream pathway. This observation indicates that Ras action on AKT phosphorylation is PI3Kα dependent and that the residual PI3Kα and PI3Kβ activities were sufficient to induce the insulin-induced AKT downstream pathway in hepatocytes.

Our model for insulin signaling is consistent with the results from a recent study showing improvement in the conditions of patients with PI3KCA-related overgrowth syndrome (PROS) treated with a low dose of the PI3Kα isoform-specific inhibitor, BYL719, without causing any alteration of glycemia [107]. On May 24th 2019, the Food and Drug Administration (FAD) approved BYL719 also known as Apelisib in combination with fulvestrant (a selective estrogen receptor degrader) to treat postmenopausal women, and men, with hormone receptor (HR) positive, or human epidermal growth factor receptor 2 (HER2)-negative, or PIK3CA-mutated, or advanced, or metastatic breast cancer [129]. Indeed, it has been reported a significant prolonged progression-free survival and a great response in breast cancer patients treated with Alpelisib-fulvestrant [130].

This recent finding cannot be justified by the current insulin signaling model where PI3Kα is the main PI3Ks isoform involved in the insulin pathway. Our new model for insulin signaling, that considers the redundant activity of PI3Kα and PI3Kβ, proposes that BYL719 can cause hyperglycemia only at a high dose where it is no longer selective for PI3Kα. In fact, the hyperglycemia threshold of BYL719 is at twenty-thirty times higher concentration than the

IC_{50} of BYL719 for PI3Kβ. BYL719 has a higher hyperglycemia threshold than pan-PI3Ks inhibitors, which can be a possible explanation for the limited effects of the pan-inhibitors used as cancer therapies [131]. Indeed, our new model for insulin signaling suggests that isoform-specific PI3K inhibitors discriminating between PI3Kα and PI3Kβ in order to preserve their isoform selectivity should be used at doses below their hyperglycemic threshold.

4.3 PAPER III

4.3.1 MAIN RESULTS PAPER III

To better understand hepatic metabolism both in a physiological state and in obesity is essential to define a solid cell culture model to study insulin signaling, glucose metabolism and disease progression. Primary hepatocyte preparation requires the sacrifice of mice and it is a very delicate technique, while immortalized cell lines are easily available. However, the insulin action in hepatoma cell lines still remains largely unexplored. Here we compared three hepatoma cell lines from three different species: HepG2, human hepatoma, Hepa 1-6, murine hepatoma and McARH7777, rat hepatoma to primary hepatocytes for insulin signaling, glucose production and protein expression profile.

We have found that the dominant negative Ras mutant, HRAS17N, blunted the ERK signal and reduced the AKT phosphorylation in primary hepatocytes but not in the hepatoma cell lines. The hepatoma cell lines instead showed a constitutive activated RAS-MAPK signaling, and elevated basal levels of AKT phosphorylation on Thr 308, both resistant to the presence of the adenovirus HRAS17N. Insulin-driven AKT phosphorylation on Ser 473 was also not affected by the blockage of Ras. However, the phosphorylation of the insulin receptor triggered by insulin was not impaired in all the hepatoma cell lines, suggesting that HepG2, Hepa 1-6 and McARH7777 displayed post receptor aberrant insulin signaling (Fig. 6).

The observations of aberrant insulin signaling in different hepatoma cell lines led us to further investigate the effects of such aberrant insulin signaling on gluconeogenic genes expression and glucose production. Whereas we could measure an increase of both glucose 6 phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK) in primary hepatocytes in presence of cAMP analog dbcAMP and reduction to basal levels in presence of insulin, in all the hepatoma cell lines we could not measure any significant changes of gene expression for both G6P and PEPCK. Due to the same origin, we directly compared Hepa 1-6 to primary hepatocytes and the Hepa 1-6 cells displayed undetectable levels of gene expression of gluconeogenic genes and absence of insulin-driven GSK phosphorylation compared to primary hepatocytes. Furthermore, HepG2, Hepa 1-6 and McARH7777 displayed dramatically reduced glucose production rates compared to primary hepatocytes (Fig. 6).

To evaluate if the hepatoma cell lines use intracellular glucose for *de novo* lipogenesis, we measured the lipogenic enzyme fatty acid synthase (FAS) protein levels and we observed that all the hepatoma cell lines showed a significant reduction of FAS compared to primary hepatocytes. All our observations showed not only remarkable differences between the hepatoma cell lines and primary hepatocytes, but also some similarities between different cell lines derived from different species. Therefore, we analyzed the electrophoretic protein profile of extracts from a human donor, from three independent primary hepatocyte preparation from mice and three different passages in plate of HepG2, Hepa 1-6, McARH7777. Our results showed that all murine hepatocyte preparation possess a similar electrophoretic protein profile compared to human hepatocytes. On the other hand, all the hepatoma cell lines displayed a distinct protein pattern compared to primary hepatocytes but similar among them, revealing a convergent aberrant phenotype between different species (Fig. 6).

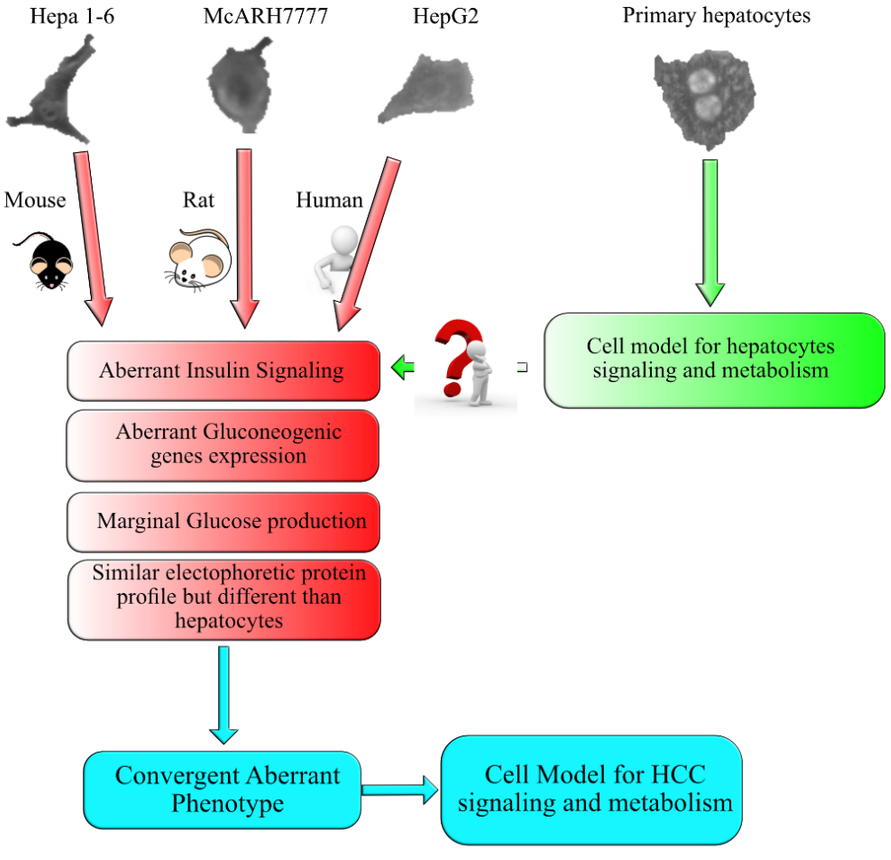

Figure 6: Insulin Signaling and Glucose Metabolism in Different Hepatoma Cell Lines Deviate from Hepatocyte Physiology Toward a Convergent Aberrant Phenotype. The hepatoma cell lines: Hepa 1-6, murine hepatoma, McARH7777, rat hepatoma and Hepg2, human hepatoma, were compared to primary hepatocytes to validate them as cell model for metabolic studies. The hepatoma cell lines displayed aberrant insulin signaling, aberrant gluconeogenic genes expression, marginal glucose production and distinct electrophoretic protein profiles. The hepatoma cell lines appeared to have a convergent aberrant phenotype between the different species which makes them a cell model for hepatocellular carcinoma (HCC), but not of hepatocyte insulin signaling or action.

4.3.2 DISCUSSION PAPER III

Our data showed that the hepatoma cell lines HepG2, Hepa 1-6, McARH7777 displayed aberrant insulin signaling, altered expression of gluconeogenic genes, GSK unresponsiveness to insulin, and reduced glucose production. The hepatoma cell lines also displayed low FAS protein levels and dramatically different electrophoretic profiles compared to primary hepatocytes but similar between them. This aberrant phenotype can be considered as a hallmark of hepatocellular carcinoma. Indeed, it was reported that gene expression profile of several hepatoma cell lines is similar to primary tumors ones [132]. It was also shown that G6P and PEPCK expression levels were dampened in murine and human HCC compared to healthy liver [133]. From our research of 100 random articles on insulin-driven AKT phosphorylation, only seven studies reported AKT phosphorylation on Thr 308 and the others showed only AKT phosphorylation on Ser 473 or not specified phosphorylation site, suggesting that most likely also others before us observed a similar pattern on AKT Thr 308 in hepatoma cell lines. The aberrant phenotype that these hepatoma cell lines revealed might be related to the mutations present in the genome of these cell lines. The hepatoma cell line that was characterized by the Broad Institute Cancer Cell Line Encyclopedia is HepG2, which presents 386 mutations in their genome. Between these mutations, we found a well-known NRAS activating mutation Q61L, which could explain the constitutively active ERK and the resistance to HRAS17N phosphorylation; S265R missense mutation on MAPK4, a kinase that directly phosphorylates AKT on Thr 308; G573S, a nonsense mutation on PI3KR2; D69N mutation for FOXO1 which might affect gluconeogenic gene expression.

These results reveal the need of a new and more careful interpretation of the thousands of metabolic studies published on the hepatoma cell lines considering the new emerged evidence. In addition, it is important to point out that all the cell lines used in this study derive from different clones from different species. This information leads us to another observation regarding our findings that all of the hepatoma cell lines derangements appear to be conserved between different species. Therefore, it is possible to conclude that these cell lines could be considered as a valuable tool to study the derangements in insulin signaling and the metabolic transformation occurring in HCC and especially at proteomic level.

5 CONCLUSIONS AND FUTURE PRESPECTIVES

This thesis aimed at improving our understanding of the role of the different PI3Ks isoforms in obesity and insulin signaling.

In **PAPER I**, we examined the role of PI3Kγ in obesity and metabolic inflammation. We have found that most of the beneficial effects of PI3Kγ ablation in obesity are due to its role on adiposity. However, we have also found that the PI3Kγ activity promotes adipose tissue inflammation and insulin resistance during obesity. These results clarify the role of PI3Kγ in obesity and insulin resistance and challenge the dogma that in PI3Kγ is a major inhibitor of classical macrophages activation.

In **PAPER II**, we investigated the role PI3Ks isoforms in insulin signaling. Our results lead to a new improved model for insulin signaling, where the insulin pathway is driven by both PI3Kα and PI3Kβ activities and for PI3Kα in Ras dependent manner. Our data suggest that selective-PI3K isoform inhibitors, which can discriminate between PI3Kα and PI3Kβ, might dissociate the beneficial effect of PI3K inhibition on cancer therapy from the deleterious effects on glucose homeostasis.

In **PAPER III**, we described that compared to primary hepatocytes, three commonly used hepatoma cell lines showed aberrant insulin signaling, aberrant gluconeogenic gene expression, marginal glucose production and distinct protein expression profile. We conclude that insulin signaling and metabolism in these hepatoma cell lines is representative of HCC but not of the hepatocyte.

Altogether the data in this thesis indicate that PI3K isoform-selective inhibitors, discriminating between PI3Kα and PI3Kβ, could display optimal therapeutic index by minimizing the effects of PI3K inhibition on insulin action leading to hyperglycemia.

In the future, it would be important to identify which PI3Ks isoforms are involved in adiposity to exploit PI3K inhibition as anti-obesogenic therapy, and in the progression of specific tumors to achieve optimal therapeutic index in cancer therapies targeting selected PI3Ks isoforms. Finally, the use of PI3K inhibitors in the treatment of cancer in the obese might be promising but it requires a better understanding on the role of specific PI3Ks isoforms in obesity-mediated tumor promotion.

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APPENDIX

Appendix I -Paper I

PI3Kγ activity in leukocytes promotes adipose tissue inflammation and early-onset insulin resistance during obesity.

Breasson L., Becattini B., Sardi C., Molinaro A., Zani F., Marone R., Botindari F., Bousquenaud M., Ruegg C., Wymann M. P., Solinas G.

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Appendix II-Paper II

Insulin-driven PI3K-AKT signaling in the hepatocyte is Mediated by Redundant PI3Kα and PI3Kβ Activities and is promoted by RAS.

Molinaro A., Becattini B., Mazzoli A., Bleve A., Radici L., Maxvall I., Rotter Sopasakis V., Molinaro A., Bäckhed F., Solinas G.

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Appendix III-Paper III

Insulin Signaling and Glucose Metabolism in Different Hepatoma Cell Lines Deviate from Hepatocyte Physiology Toward a Convergent Aberrant Phenotype

Molinaro A., Becattini B. and Solinas G.

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