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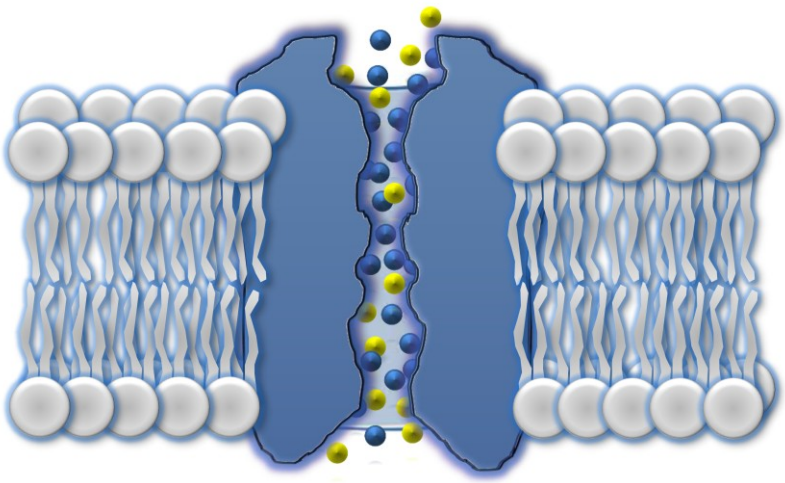


Leire Méndez Giménez de los Galanes

Universidad de Navarra

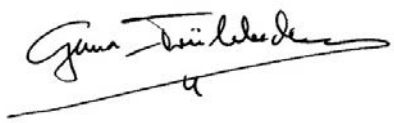
SCHOOL OF SCIENCES

ROLE OF AQUAPORINS IN THE IMPROVEMENT OF ADIPOSITY AND NON-ALCOHOLIC FATTY LIVER DISEASE AFTER BARIATRIC SURGERY

Submitted by **Leire Méndez Giménez de los Galanes** in partial fulfillment of the requirements for the Doctoral Degree in Biology of the University of Navarra

This dissertation has been written under our supervision at the Metabolic Research Laboratory of the University of Navarra and we approved its submission to the Defense Committee.

Signed on May, 2017



Prof. Gema Frühbeck Martínez **Dr. Amaia Rodríguez Murueta-Goyena**

ACKNOWLEDGEMENTS

I would like to thank the University of Navarra and the CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN) of the Instituto de Salud Carlos III for the financial support of this thesis (PI12/00515) and their grants for the International PhD programme. In addition, this work has been financially supported by Fondo de Investigación Sanitaria-FEDER (PI10/01677, PI13/01430 and PI16/01217) of the Instituto de Salud Carlos III, the Department of Health of the Gobierno de Navarra (61/2014) and the PIUNA project of the University of Navarra (PIUNA 2011-2014).

First and foremost, I would like to express my warmest gratitude and appreciation to my supervisors. Prof. Gema Frühbeck, who trusted in me for the first time, for her inestimable help in carrying out this work encouraging me to go even further with her constant intellectual support and enthusiasm for science. She is an epitome of the hard work towards perfection in research and effort. I also want to thank Dr. Amaia Rodríguez, for her patience and total availability to help me in the achievement of this thesis. She has provided invaluable guidance through this entire scientific assignment. I thank her for the logistical support and for being a great motivation. Furthermore, I am always going to be grateful for her suggestions, conversations, and wisdom that she provides me on a daily basis. I appreciate all of her time and ideas that made my PhD experience productive and stimulating. Without the support of these two scientific titans, the present thesis would never have seen the light of day.

I would further like to thank my current and former-colleagues of the Metabolic Research Laboratory of the Clínica Universidad de Navarra, an awesome team with whom I am proud to work with. I owe you my gratitude for your friendship, support and expertise during my learning process. My deepest recognition to each and every member of the team, I wish you all a fruitful future in this wonderful laboratory. It has been also a pleasure to meet the students of the School of Medicine and international PhD exchanges working in our lab during these years. Thank you for all the nice things that we have learned together. We are lucky to have you.

I am also grateful to all the staff of the breeding house of the Centro de Investigación Farmacológica Aplicada (CIFA) of the University of Navarra for their invaluable work and help with the experimental animals.

I would also like to specially thank the researchers that I met at the Faculty of Pharmacy of the University of Lisbon, a city that became my home during my international PhD exchange. I would like to deeply thank Dr. Graça Soveral for giving me the opportunity to learn new and interesting techniques in her research group as well as for her continuous encouragement and guidance. I would further like to thank her research team for the help, advice and invaluable support during the experiments. Thank you all for your kindness, sympathy and the hospitality, making me to feel part of your group. Thank you, for science and above all, for humanity.

In summary, I would like to point out that although only my name appears on the cover of this work, many people have contributed to its elaboration. I owe my gratitude to all those who made this thesis possible and have offered an unforgettable experience to me.

Finally, to life and science, for inspiring us to learn more and more.

ABBREVIATIONS

Adipo-IR: adipose tissue insulin resistance index

AQP: aquaporin

ATGL: adipose triglyceride lipase

BAT: brown adipose tissue

BMI: body mass index

CD36: fatty acid translocase

DBP: diastolic blood pressure

DEXA: dual-energy X-ray absorptiometry

EWAT: epididymal white adipose tissue

EWL: excess weight loss

FABP: fatty acid binding protein

FATP: fatty acid transporter protein

FFA: free fatty acids

FGF: fibroblast growth factor

FXR: farnesoid X receptor

G6Pase: glucose-6-phosphatase

GH: growth hormone

GHS-R: growth hormone secretagogue receptor

GIP: gastric inhibitory peptide

GK: glycerol kinase

GLP-1: glucagon-like peptide-1

GOAT: ghrelin *O*-acyltransferase

HFD: high-fat diet

HOMA: homeostasis model assessment

HSL: hormone-sensitive lipase

IL: interleukin

IPITT: intraperitoneal insulin tolerance test

IRE: insulin response element

IRS: insulin receptor substrate

HFD: high-fat diet

LPL: lipoprotein lipase

mTOR: mechanistic target of rapamycin

NAFLD: non-alcoholic fatty liver disease

NASH: non-alcoholic steatohepatitis

ND: normal diet

NO: nitric oxide

NPA: asparagine-proline-alanine motif

OGTT: oral glucose tolerance test

PEPCK: phosphoenolpyruvate carboxykinase

Pf: water permeability

Pgly: glycerol permeability

PI3K: phosphatidylinositol 3-kinase

PKA: protein kinase A

PP: pancreatic polypeptide

PPAR: peroxisome proliferator-activated receptor

PPRE: peroxisome proliferator-activated receptor response element

PRWAT: perirenal white adipose tissue

PYY: peptide YY

RYGB: Roux-en-Y gastric bypass

SBP: systolic blood pressure

SCWAT: subcutaneous white adipose tissue

SREBF1: sterol regulatory element-binding transcription factor 1

SVFC: stroma-vascular fraction cells

TGR5: G-protein-coupled receptor bile acid receptor 5

T2D: type 2 diabetes

TG: triacylglycerols

TNF-α: tumor necrosis factor α

UCP1: uncoupling protein 1

VLDL: very-low density lipoprotein

VRAC: volume-regulated anion channel

WAT: white adipose tissue

WHO: World Health Organization

Index

Introduction

1. OBESITY

1.1. Classification and prevalence of obesity

Obesity has become one of the leading causes of disability and death in the last decades (Frühbeck *et al*, 2013a). In this regard, the American Medical Association declared obesity as a disease in 2013 (Atkinson, 2014). Obesity is a complex multifactorial disorder characterized by the accumulation of excess body fat due to a chronic imbalance between energy intake and energy expenditure (Kopelman, 2000). Body mass index (BMI) is the most commonly used tool to define the ponderal categories of the individuals in the clinical practice. The BMI was initially described by Quetelet in 1869 and it is calculated as the weight (in kilograms) divided by the height (in meters) squared. According to the cut-off points established by the World Health Organization (WHO), overweight is defined as a BMI ranging from 25.0 to 29.9 kg/m^2, whereas obesity is diagnosed with a BMI ≥ 30.0 kg/m^2 (**Table 1**).

Table 1. Classification of ponderal categories according to BMI cut-off points.

Classification	BMI (kg/m^2)	
	Principal cut-off points	Additional cut-off points
Underweight	**<18.5**	**<18.5**
Severe thinness	<16.0	<16.0
Moderate thinness	16.0-16.9	16.0-16.9
Mild thinness	17.0-18.4	17.0-18.4
Normal range	**18.5-24.9**	**18.5-22.9**
		23.0-24.9
Overweight	**≥25.0**	**≥25.0**
Pre-obese	25.0-29.9	25.0-27.4
		27.5-29.9
Obese	**≥30.0**	**≥30.0**
Obese class I	30.0-34.9	30.0-32.4
		32.5-34.9
Obese class II	35.0-39.9	35.0-37.4
		37.5-39.9
Obese class III	**≥40.0**	**≥40.0**

Despite its wide use, BMI is only a surrogate measure of body fat and does not provide an accurate measurement of body composition (Blundell *et al*, 2014) having a high rate of misclassification of obesity (Gómez-Ambrosi *et al*, 2012). In this regard, the direct measurement of body fat with precise techniques including dual-energy X-ray absorptiometry (DEXA), air-displacement plethysmography or bioimpedance, has enabled the classification of individuals according to the degree of their real adiposity (Gallagher *et al*, 2000). The cut-off points according to the percentage of body fat have been established between 20.1-24.9% for men and 30.1-34.9% for women for overweight, and ≥25.0% for men and ≥35.0% for women for obesity (Gómez-Ambrosi *et al*, 2012).

During the past few decades the prevalence of obesity and overweight has reached epidemic proportions worldwide with this condition being a major contributor to the global burden of disease (Ng *et al*, 2014). The prevalence of obesity is increasing not only in industrialized countries, but also in non-industrialized ones, particularly in those undergoing economic transition (Scully, 2014). Globally, more than 2.1 million adults are overweight with 671 million of them being obese (Ng *et al*, 2014). Based on the latest estimates from the WHO in the European Union countries, the prevalence of obesity has tripled since the 1980s with overweight and obesity affecting 50% of the European population (Frühbeck *et al*, 2013a). The prevalence of childhood obesity is also rising in low-income and middle-income countries with the global number of overweight children under the age of 5 years being 42 million, 31 million of them living in developing countries in 2014 (Farpour-Lambert *et al*, 2015). In Spain, the world's fifty-second and Europe's fourth largest country, the prevalence of obesity in the adult population is estimated in 22.9% (22.4% men and 21.4% women) while that of overweight is 39.4% (46.4% in men and 32.5% in women) according to the data of the ENRICA study (Gutiérrez-Fisac *et al*, 2012).

1.2. Obesity as a health problem

Overweight and obesity are the 5th leading risk for global deaths according to the WHO and, hence, the prevention of obesity has been declared as a major public health priority in many countries (Uerlich *et al*, 2016). In this regard, obesity is commonly associated with the onset of several pathologies, including insulin resistance, cardiovascular diseases, sleep apnea and several types of cancer, among others (**Figure 1**) (Kopelman, 2000, Kanneganti *et al*, 2012). Upper-body or visceral obesity,

characterized by the accumulation of fat in the abdominal region, is a major contributor to the development of hypertension, insulin resistance, dyslipidemia and premature death (Yusuf *et al*, 2005, Kuk *et al*, 2006). By contrast, individuals with lower-body or gynoid obesity, characterized by the accumulation of fat in the subcutaneous gluteo-femoral depots, exhibit a lower morbidity and mortality risk than subjects with visceral obesity (Rodríguez *et al*, 2007a). Obesity increases the risk of cardiovascular disease (Rimm *et al*, 1995) as a result of obesity-related dyslipidemia and atherosclerosis (Grundy, 2004).

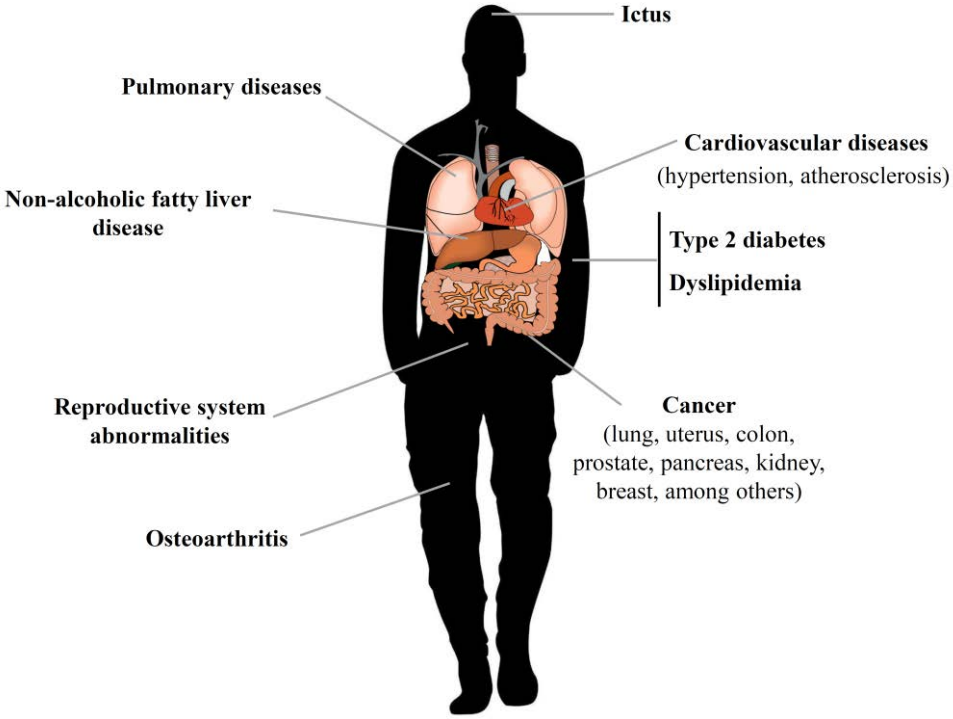


Figure 1. Diagram of some of the co-morbidities associated with obesity.

Excess adiposity is particularly associated with increased risk of developing type 2 diabetes (T2D) with overweight and obesity probably accounting for about 80-90% of T2D cases (Astrup *et al*, 2000). This disorder is determined by two main alterations: i) insulin resistance in target peripheral tissues such as liver, skeletal muscle and adipose tissue; and, ii) a dysfunction of β-cells in the pancreas leading to insufficient insulin production (Catalán *et al*, 2009). Obesity is characterized by elevated fasting plasma insulin, exaggerated insulin response to an oral glucose load as well as impaired insulin sensitivity (Kopelman, 2000). Moreover, the incidence of macrovascular (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications

(diabetic nephropathy, neuropathy, and retinopathy) of T2D is aggravated in the obese state (Sjöstrom *et al*, 2014).

Non-alcoholic fatty liver disease (NAFLD) is a pathology characterized by intrahepatic triacylglycerol (TG) overaccumulation, which is commonly associated with obesity, dyslipidemia, insulin resistance and T2D (Chalasani *et al*, 2012). NAFLD encompasses a spectrum that ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), which can result ultimately in liver fibrosis and cirrhosis (European Association for the Study of the Liver *et al*, 2016). The severity of steatosis is closely associated with the amount of visceral fat, BMI and body fat percentage, but is weakly associated with the amount of subcutaneous fat (Kelley *et al*, 2003). Advanced forms of NAFLD appear more frequently in obese patients with associated comorbidities such as insulin resistance and central obesity (Dixon *et al*, 2001). The prevalence of NAFLD and NASH increases from around 20% and 3%, respectively, in the general population, to 75% and 25-70%, respectively, in morbid obesity (Boza *et al*, 2005, Machado *et al*, 2006).

1.3. Biological and morphological adipose tissue changes in obesity

The adipose tissue is mainly composed by adipocytes, but also contains heterogeneous cell populations in the stroma-vascular fraction (SVF), such as mesenchymal stem cells, preadipocytes, endothelial cells, pericytes or immune cells, among others (Frühbeck, 2008). Traditionally, two types of adipose tissue have been distinguished depending on their morphological and functional characteristics: white (WAT) and brown (BAT) adipose tissue (Giralt *et al*, 2013). White adipocytes are spherical cells of 20-200 μm of diameter, formed by a large lipid droplet occupying most of the cell that displaces the nucleus and the cytoplasm to the periphery (Frühbeck, 2008). The main functions of WAT are the storage of energy in the form of TG, thermal insulation and secretion of adipocyte-derived factors termed "adipokines" that regulate diverse biological processes in an autocrine, paracrine and endocrine fashion (**Figure 2**). WAT is located in different regions, including subcutaneous, abdominal or visceral, retroperitoneal, inguinal and gonadal fat depots (Cinti, 2012). Brown adipocytes are smaller cells (15-60 μm) with polygonal shape, multilocular lipid droplets, multiple mitochondria and a central nucleus (Giordano *et al*, 2014). The main biological function of BAT is adaptive thermogenesis by the activation of uncoupling protein 1 (UCP1), but it can also act as a TG reservoir and secrete adipokines, although to a lesser extent than

WAT (Frühbeck *et al*, 2009). In 2010 the existence of a third type of fat cells, termed beige adipocytes, was described, which have morphology of brown adipocytes inside the WAT (Wu J. *et al*, 2012). The acquisition of this phenotype similar to brown adipocytes occurs after the exposure to cold, stimulation of β-adrenergic receptors or treatment with peroxisome proliferator-activated receptor γ (PPARγ) agonists, in a process called "fat browning" (Nedergaard *et al*, 2013).

The pathological expansion of the adipose tissue in obesity induces profound biological and morphological changes in WAT and BAT, ultimately leading to obesity-associated pathologies (Rodríguez *et al*, 2015b), as explained below.

1.3.1. Hypertrophy and hyperplasia of adipocytes

Adipose tissue growth is a strictly regulated process, since both excess adipose tissue (overweight or obesity) and total or partial absence of adipose tissue (congenital or acquired lipodystrophies) are associated with severe metabolic disorders (Arner E. *et al*, 2010). The precursors of adipocytes originate in the prenatal period. During childhood the adipose tissue is expanded mainly by increasing the number of adipocytes with two peaks of hyperplasia, after birth and prepuberty. In adolescence the adipocyte proliferation rate decreases and remains relatively constant in the adult period, a stage in which adipose tissue grows by an increase in adipocyte size (Spalding *et al*, 2008, Arner P. *et al*, 2013). In this sense, overweight individuals initially exhibit hypertrophy of adipocytes with no relevant changes in the number of fat cells. However, a positive energy balance condition perpetuated over time translates into an increase in both number and size of adipocytes, leading to further hyperplasia in the obese state. Adipocyte hypertrophy in obese patients is associated with alterations in mitochondrial function, changes in membrane proteins, increased degree of apoptosis and inflammation of adipose tissue, which contribute to the development of obesity-associated pathologies (Heinonen *et al*, 2014). These alterations are more evident in patients with visceral obesity.

1.3.2. Alterations in adipocyte lipolysis

In circumstances of negative energy balance such as fasting or exercise adipocytes hydrolyze TG into free fatty acids (FFA) and glycerol to meet physiological needs (Frühbeck *et al*, 2014). Adipocyte lipolysis is mainly controlled by catecholamines, insulin and natriuretic peptides (Langin, 2006). Circulating FFA and

glycerol concentrations are elevated in obesity, suggesting an increase in overall lipolysis during fasting (Rodríguez *et al*, 2011b). Several impairments in adipocyte lipolysis have been reported in obese individuals, including an altered responsiveness to catecholamines that regulate lipolysis via the lipolytic β-receptors (β_1, β_2 and β_3) and anti-lipolytic α_2-receptors (Jocken *et al*, 2008). These abnormalities in catecholamine function promote the release of FFA from the visceral adipocytes through the portal system providing FFA as a substrate for hepatic lipoprotein metabolism or glucose production (Unger, 2002). Moreover, the overload of FFA reduces hepatic degradation of apolipoprotein B and insulin, which may contribute to the dyslipidemia, hyperinsulinemia and insulin resistance observed in visceral obesity (Bergman *et al*, 2000, Després, 2006). The release of FFA is a limiting step for hepatic synthesis of very-low density lipoproteins (VLDL) that may further contribute to the dyslipidemia of visceral obesity (Carr *et al*, 2004). Therefore, regional variations in the lipolytic rate and the production of FFA underlie, in part, the metabolic disorders (insulin resistance, hyperinsulinemia and dyslipidemia) linked to an increased visceral adiposity (Rodríguez *et al*, 2007a).

1.3.3. Adipose tissue inflammation and fibrosis

Obesity is a chronic low-grade inflammatory state. In this sense, the pathological expansion of adipose tissue in obesity is associated with an increased recruitment of macrophages and other immune cells, higher secretion of proinflammatory adipokines, as well as alterations in extracellular matrix components of the adipose tissue, which aggravate the systemic inflammation of obese individuals (Ouchi *et al*, 2011, Kanneganti *et al*, 2012). In physiological conditions adipose tissue inflammation is suppressed by anti-inflammatory interleukins (IL-4, 10 or 13) secreted by eosinophils and Th2 and Treg cells embedded in the adipose tissue. However, excess adiposity favors the infiltration of macrophages, neutrophils, foam cells, T and B lymphocytes, mast and dendritic cells into adipose tissue (Weisberg *et al*, 2003, Elgazar-Carmon *et al*, 2008, Liu J. *et al*, 2009, Wu D. *et al*, 2011, Shapiro *et al*, 2013). A characteristic feature of inflammation associated with obesity is the polarization of macrophages into a proinflammatory M1 profile (Lumeng *et al*, 2007) as well as the transformation of anti-inflammatory Th2 lymphocytes into Th1 and Th17 inflammatory cells, particularly in visceral fat (Kintscher *et al*, 2008, Eljaafari *et al*, 2015). Another histological feature of adipose tissue inflammation is an increased adipocyte apoptosis surrounded by

macrophages, forming "crown-like" structures (Cinti *et al*, 2005). Adipocytes themselves favor this inflammatory microenvironment by the secretion of proinflammatory cytokines, chemokines and alarmins (Catalán *et al*, 2007).

Obese subjects present a lower production of elastin and an increased synthesis of collagen type I, III, V and VI, fibronectin and laminin in the adipose tissue, which favors the appearance of fibrotic zones that are more abundant in visceral than subcutaneous fat (Sun *et al*, 2013, Reggio *et al*, 2016). This alteration in extracellular matrix remodeling decreases the flexibility of the adipose tissue contributing to its dysfunction and inflammation (Henegar *et al*, 2008, Khan *et al*, 2009, Mutch *et al*, 2009). Likewise, fibrosis of adipose tissue limits its expansion capacity, which favors the ectopic accumulation of lipids in peripheral tissues such as liver, pancreas, skeletal muscle or heart, generating a phenomenon called lipotoxicity (Virtue *et al*, 2010). Thus, adipose tissue fibrosis contributes indirectly to the development of hyperinsulinemia, hyperglycemia and dyslipidemia associated with visceral obesity. In addition, adipose tissue stiffness is associated with an increase in markers of hepatocellular damage as well as higher liver steatosis and fibrosis (Abdennour *et al*, 2014).

1.3.4. Altered secretion of adipokines

In 1987, adipsin (or complement factor D) was identified as a highly differentiation-dependent gene in 3T3-L1 adipocytes (Cook *et al*, 1987). In 1993, an increased expression of tumor necrosis factor α (TNF-α) was detected in the adipose tissue from rodent models of obesity, providing a functional link between obesity and inflammation (Hotamisligil *et al*, 1993). One year later, leptin was discovered as an adipokine that regulates food intake and energy expenditure in an endocrine manner (Zhang Y. *et al*, 1994). Nowadays, it is well known that adipose tissue produces a huge variety of adipokines involved in the regulation of appetite, glucose and lipid metabolism, cardiovascular homeostasis and reproduction, among other biological functions (Frühbeck *et al*, 2013b, Rodríguez *et al*, 2015b). A further relevant group of adipokines is represented by acute-phase reactants, cytokines, chemokines, damage-associated molecular pattern molecules and pro- and anti-inflammatory factors (**Figure 2**). Obesity is associated with an altered secretion of adipokines with a special upregulation in the secretion of pro-inflammatory adipokines and a downregulation of the anti-inflammatory adiponectin caused by excess adiposity and adipocyte

dysfunction, that promote inflammatory responses and metabolic dysfunction (Ouchi *et al*, 2011).

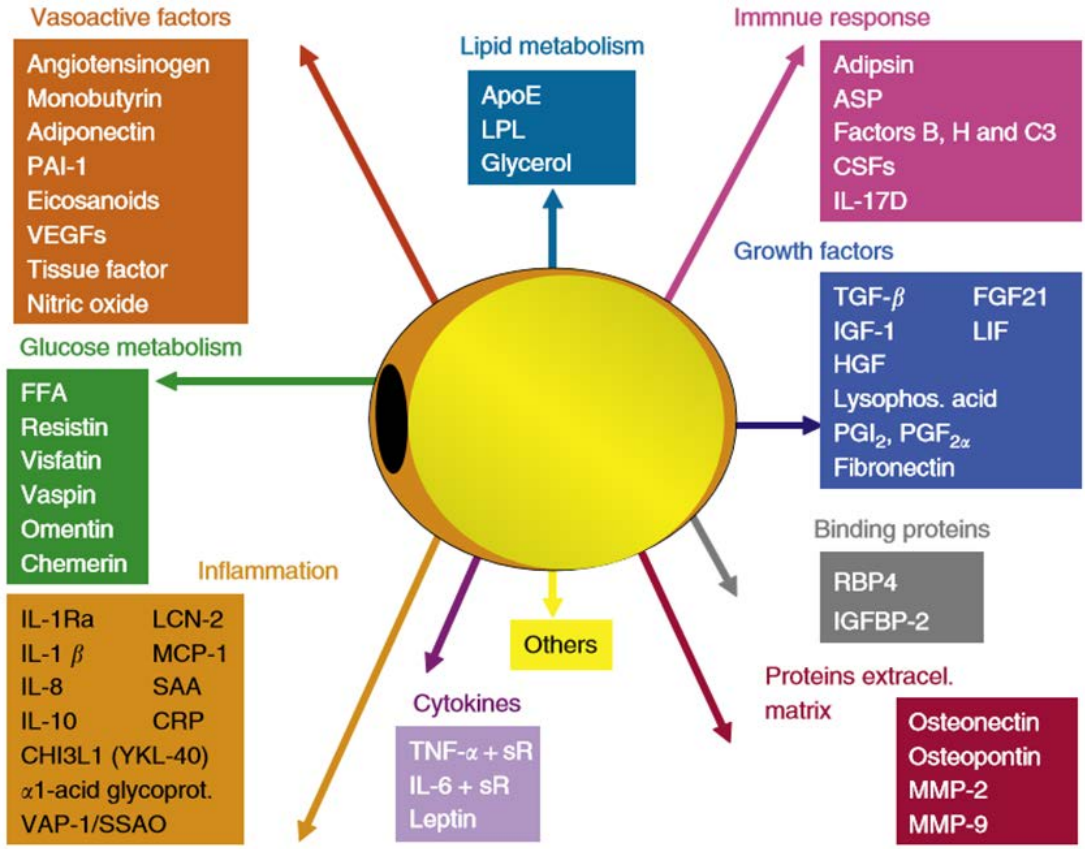


Figure 2. Factors secreted by white adipose tissue [modified from (Frühbeck *et al*, 2013b)].

1.3.5. Reduced BAT mass and/or activity

BAT represents a small fraction of total adiposity (~0.1%), but it has a great ability to dissipate energy through the clearance of FFA, glucose consumption and the generation of heat during the thermogenesis process (Frühbeck *et al*, 2009, Nedergaard *et al*, 2011, Lee P. *et al*, 2015). BAT activity is induced in response to cold and by activation of the sympathetic nervous system (Virtanen *et al*, 2009). Likewise, brown fat activity is higher in women than in men, and shows a progressive decline with age (Bauwens *et al*, 2014). In this sense, obese subjects present a reduction in the size and/or activity of brown and beige fat depots, which could contribute to the development of deleterious complications of obesity. An inverse correlation between BAT and total adiposity, BMI and fasting glycemia has been observed (Cypess *et al*, 2009, Saito *et al*, 2009, Ouellet *et al*, 2011). Interestingly, the interscapular region presents a greater expression of a classic marker of brown adipocytes, ZIC1 (Lidell *et*

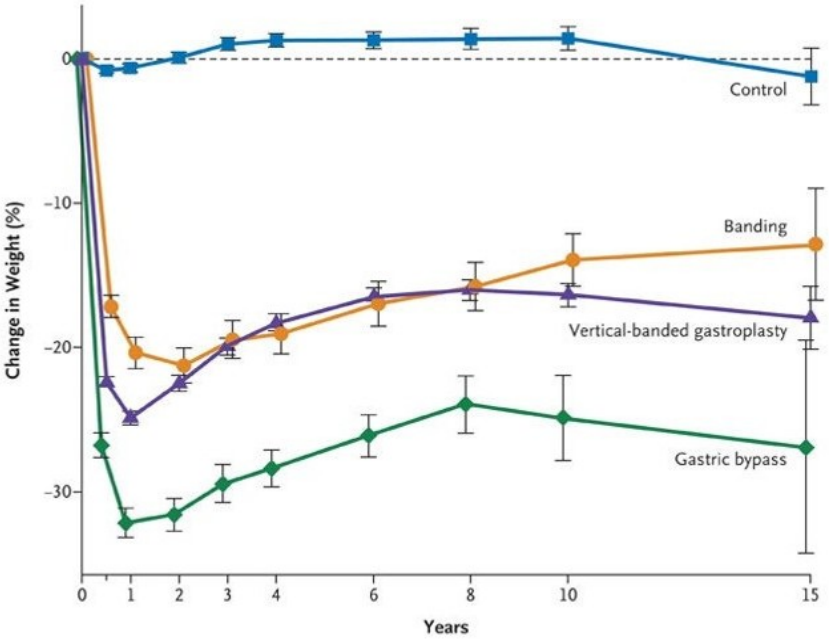
al, 2013), whereas the supraclavicular zone presents a greater expression of the marker of beige adipocytes TBX1 (Wu J. *et al*, 2012). Thus, the different populations of brown and beige adipocytes in these anatomical regions respond differentially to external stimuli thereby constituting different potential targets for therapeutic interventions. The discovery of brown and beige fat in adult individuals has opened a wide field of research focused on these tissues as a potential therapeutic target for developing anti-obesity drugs, given the thermogenic capacity of both fat depots (Lidell *et al*, 2013).

2. BARIATRIC SURGERY

The management of overweight and obesity starts with lifestyle interventions consisting of a high-quality diet accompanied by an exercise prescription describing frequency, intensity, type, and time with a minimum of 150-minute moderate weekly activity (Bray *et al*, 2016). The benefits of this approach include appreciable reductions in visceral obesity and cardiometabolic risk factors (Ross *et al*, 2009). However, lifestyle modification alone has failed to ameliorate the obesity epidemic due to longer-term weight maintenance difficulty, needing the implementation of effective pharmacological treatment (Anthes, 2014). The indications for adding pharmacotherapy include a history of failure to achieve clinically meaningful weight loss (>5% of total body weight) and to sustain lost weight, for patients with a BMI≥30 kg/m^2 or a BMI ranging from 27.0-29.9 kg/m^2 with one major obesity-related comorbidity (i.e. hypertension, diabetes, obstructive sleep apnea, among others) (Toplak *et al*, 2015). Nowadays, approved medications for obesity treatment worldwide are orlistat, naltrexone/bupropion, and liraglutide in Europe; in the USA, lorcaserin and phentermine/topiramate are also available. Current treatments for weight loss with lifestyle interventions (diet and exercise) and pharmacotherapy have a high failure rate (Anthes, 2014, Toplak *et al*, 2015).

Bariatric surgery has been shown to be the most effective treatment to achieve sustained weight loss and to improve the morbidity and mortality in severe obese patients (Buchwald *et al*, 2009, Schauer *et al*, 2012, Sjöström, 2013). Most of the initial evidence is derived from the non-randomized, prospective, controlled Swedish Obese Subjects (SOS) study, which was the first long-term clinical trial to provide information on the beneficial effects of bariatric surgery (Sjöström *et al*, 2004, Sjöström *et al*, 2007). The SOS study started in 1987, enrolled 2,037 obese individuals, and is following them

up for over 20 years. Among the surgery group, patients underwent vertical banded gastroplasty (69%), gastric banding (19%), or Roux-en-Y gastric bypass (RYGB) (12%) (**Figure 3**). The SOS study was the first study to demonstrate a sustained long-term weight loss of up to 40%, a 24% reduction in overall mortality, mainly because of the reduced risk of myocardial infarction and cancer (in women), as well as a significant improvement in T2D compared with an observational control group (Sjöström *et al*, 2004, Sjöström *et al*, 2007, Sjöström, 2008, Sjöström *et al*, 2012, Sjöström, 2013, Sjöstrom *et al*, 2014). In 2006, the multicenter observational cohort study Longitudinal Assessment of Bariatric Surgery (LABS) enrolled 2,458 patients undergoing bariatric surgery for the first time and largely included mixed surgical procedures (70.7% RYGB, 24.8% gastric banding and 4.5% other procedures) compared with the SOS study (Courcoulas *et al*, 2013, Courcoulas *et al*, 2015). Three years after the surgical interventions, percentages of weight loss from baseline were 31.5% and 15.9% for RYGB and gastric banding, respectively.



No. Examined									
Control	2037	1768	1660	1553	1490	1281	982	886	190
Banding	376	363	357	328	333	298	267	237	52
Vertical-banded gastroplasty	1369	1298	1244	1121	1086	1004	899	746	108
Gastric bypass	265	245	245	211	209	166	92	58	10

Figure 3. Long-term weight loss according to the bariatric surgery technique in the SOS study [modified from (Sjöström *et al*, 2007)].

Currently, the selection criteria for bariatric surgery for morbid obese patients between 18-60 years include a BMI≥40 kg/m^2 or BMI≥35 kg/m^2 with associated comorbidities susceptible to improve with weight loss (Fried *et al*, 2013, Fried *et al*,

2014). These BMI thresholds should be reduced by 2.5 points for individuals of Asian genetic background (Fried *et al*, 2014). Moreover, the positive effects of bariatric surgery, especially with respect to improvements in T2D have expanded the eligibility criteria for metabolic surgery to obese patients with T2D and a BMI of 30-35 kg/m^2 (Moncada *et al*, 2016b, Rubino *et al*, 2016).

Given the global obesity burden it is not surprising that bariatric surgery has increased in popularity due to its ability to produce long-term weight loss that is superior to traditional weight loss treatments in both magnitude and durability (Frühbeck, 2015).

2.1. Types of bariatric surgery procedures

Traditionally, surgical procedures are classified in three major categories: restrictive, malabsorptive and mixed techniques (**Figure 4**). Restrictive procedures limit food intake by reducing the size or capacity of the stomach, and include adjustable gastric banding, sleeve gastrectomy and gastric plication techniques. Malabsorptive procedures, such as duodenal-jejunal bypass or jejuno-ileal bypass, are based on the removal of portions of small intestine, thereby decreasing nutrient absorption (Tack *et al*, 2014) and require the supplementation with vitamins and minerals in order to avoid deficiency diseases, such as anemia or osteoporosis (Alvarez-Leite, 2004). Finally, mixed techniques are a combination of restrictive and malabsorptive techniques and include the RYGB, biliopancreatic diversion and duodenal switch (DeMaria, 2007).

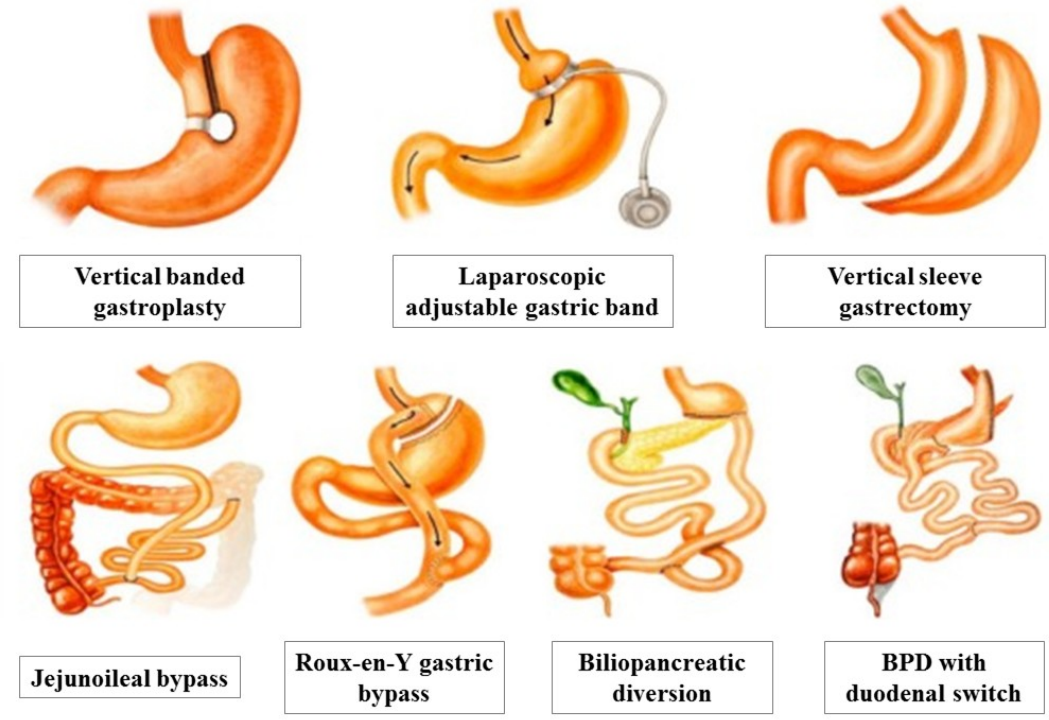


Figure 4. Types of bariatric surgery [modified from (Algahtani *et al*, 2016)].

Nowadays, the most commonly used bariatric surgery techniques are sleeve gastrectomy and RYGB, while gastric plication constitutes a relatively recent restrictive bariatric surgery procedure to induce weight loss in morbid obesity (Talebpour *et al*, 2012). The type of bariatric procedure performed depends on patient characteristics and surgeon's preferences.

2.1.1. Sleeve gastrectomy

Sleeve gastrectomy is a restrictive procedure that leaves a tube-like portion after excising the fundus and greater curvature of the stomach (Deitel *et al*, 2008, Katz *et al*, 2011) (**Figure 5**). In recent years, it has emerged as a widely applied technique due to simplicity of the surgical technique and improvement of obesity and its associated comorbidities. Sleeve gastrectomy constitutes an effective technique for weight loss in humans (Deitel *et al*, 2008, Gagner *et al*, 2013) and in experimental models of genetic and diet-induced obesity (de Bona Castelan *et al*, 2007, Valentí *et al*, 2011, Rodríguez *et al*, 2012b, Rodríguez *et al*, 2012a) as well as for the improvement of β-cell dysfunction, insulin resistance and remission of T2D after weight loss (Eickhoff *et al*, 2015).

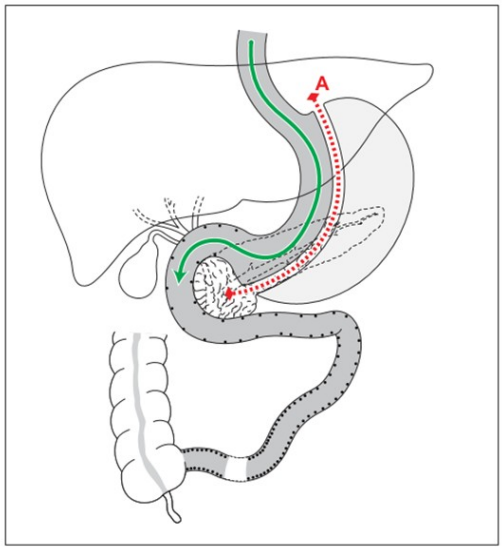


Figure 5. Diagram of the sleeve gastrectomy procedure [modified from (Scott *et al*, 2011)]. Red lines indicate surgical manipulations, and green arrows indicate nutrient flow. The stomach is transected from the greater curvature (A) in a cephalad direction in parallel to the sleeve until the angle of His is reached.

The percentage of excess weight loss (%EWL) is around 63-75% within the first year being supposedly similar to that achieved after RYGB (Scott *et al*, 2011) not only in the first year, but also beyond 5 years (Braghetto *et al*, 2012). It also has a low complication rate with leaks, suture-line hemorrhage, post-operation gastroesophageal reflux, vomiting and dumping syndrome being the most common associated disturbances (Deitel *et al*, 2008, Scott *et al*, 2011, Tack *et al*, 2014). The mortality rate of this bariatric surgery technique in experimented hands is typically of 0.1-0.5%, similar to cholecystectomy and hysterectomy (Rubino *et al*, 2016). Growing evidence suggests that proficiency of the operating surgeon is an important factor determining mortality, complications, reoperations, and readmissions (Birkmeyer *et al*, 2013).

Sleeve gastrectomy can offer a durable solution for the control of T2D with the improvement and resolution of T2D being 75% and 47%, respectively (Frühbeck, 2015). Sleeve gastrectomy elicits an improvement in insulin sensitivity comparable to that observed after RYGB (Ribaric *et al*, 2014, Schauer *et al*, 2014). More specifically, changes in fasting blood glucose and homeostasis model assessment (HOMA) index are comparable as early as 1 week and persist for at least 52 weeks after sleeve gastrectomy and RYGB (Woelnerhanssen *et al*, 2011). Sleeve gastrectomy also improves liver morphology as well as the spectrum of liver diseases of NAFLD (including NASH) (Mattar *et al*, 2005, Stratopoulos *et al*, 2005, Mathurin *et al*, 2009, Taitano *et al*, 2015). Sleeve gastrectomy also induces an improvement in hyperlipidemia of 44% leading to a

reduced risk of cardiovascular disease in obese patients. In line with these observations, sleeve gastrectomy reduces systolic (SBP) as well as diastolic (DBP) blood pressure with a resolution of hypertension of 66% (Sjöström *et al*, 2004, Vidal *et al*, 2008, Iannelli *et al*, 2011, Sjöström *et al*, 2012, Frühbeck, 2015). The beneficial effects of sleeve gastrectomy on insulin sensitivity and blood pressure values are independent of surgical trauma, aging and food intake reduction as evidenced in experimental models of genetic and diet-induced obesity (Rodríguez *et al*, 2012b, Rodríguez *et al*, 2012a, Moncada *et al*, 2016c).

2.1.2. Gastric plication

Gastric plication resembles sleeve gastrectomy without gastric resection, since it is performed by invagination of the greater gastric curvature creating a narrow gastric tube (Ramos *et al*, 2010, Brethauer *et al*, 2011, Broderick *et al*, 2014, Ji *et al*, 2014) (**Figure 6**). After gastric plication, obese patients experience appetite reduction, food intake restriction and early satiety with relatively rapid weight loss (Talebpour *et al*, 2007, Ramos *et al*, 2010, Brethauer *et al*, 2011, Huang *et al*, 2012). Gastric plication induces weight loss in morbid obese patients (Talebpour *et al*, 2012, Niazi *et al*, 2013, Verdi *et al*, 2015, Chouillard *et al*, 2016) and animal models of obesity (Fusco *et al*, 2006, Guimarães *et al*, 2013), with all authors reporting a significant %EWL around 50% the first 6 months and 60-65% in the first year (Kourkoulos *et al*, 2012). New studies with longer follow-up periods indicate a durable result for up to 36 months (Talebpour *et al*, 2007). As compared to laparoscopic sleeve gastrectomy, gastric plication could offer advantages including technical simplicity, low complication rate such as gastric perforation and bleeding, similar weight loss patterns, preservation of the integrity of the stomach, potential reversibility and lower operative cost (Talebpour *et al*, 2007, Ramos *et al*, 2010). Recent reports demonstrate that gastric plication is associated with a 30-day mortality of 0% in morbid obese patients (Kourkoulos *et al*, 2012, Talebpour *et al*, 2012). The disadvantages include higher risk of post-operative nausea and vomiting, a non-zero risk of perforation and bleeding, and likely unsustainable weight loss (Broderick *et al*, 2014).

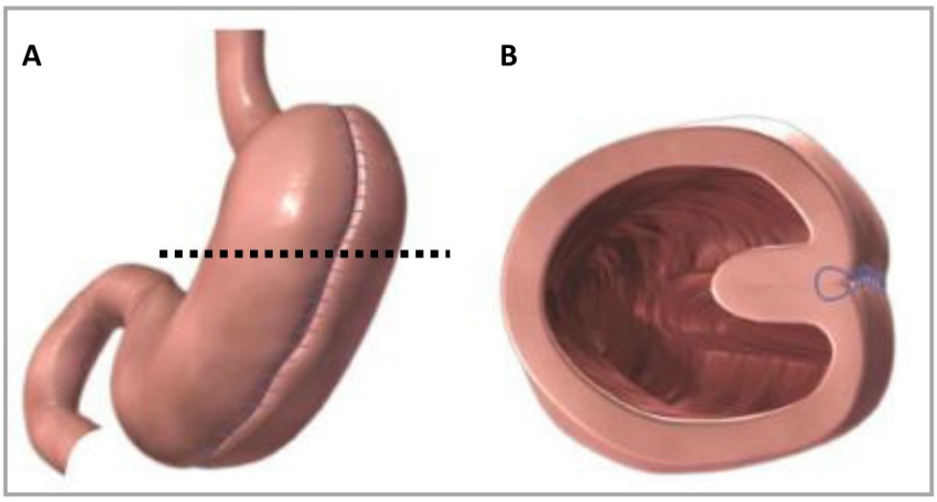


Figure 6. Diagram of gastric plication procedure [modified from (Ramos et al, 2010)]. External view (A) and cross-section (B) of the stomach. The dotted line indicates the cut-off point.

The remission rate of T2D after gastric plication is 57% (Buchwald *et al*, 2009). However, a recent study (Talebpour *et al*, 2015) showed that gastric plication achieved T2D remission in 92% of the studied patients, with improvement of blood glucose levels in the remaining 8%. The observed improvement in glycemia was also accompanied by substantial improvements in hypertension and dyslipidemia. Due to its recent appearance, more studies are needed in order to demonstrate the tissue impact of gastric plication in long-term weight loss and improvement of obesity-related diseases.

2.1.3. Roux-en-Y gastric bypass

RYGB involves the reduction of the gastric size by creating a 15-30 mL stomach pouch and bypassing the duodenum and part of the jejunum, therefore, decreasing the absorption of nutrients (DeMaria, 2007, Scott *et al*, 2011) (**Figure 7**). It constitutes one of the most frequently used bariatric surgery techniques, given its effectiveness for weight loss and improvement of obesity-related comorbidities, such as T2D (Dirksen *et al*, 2012). The weight loss induced by RYGB is, on average, 25–30% of total body weight at 12–18 months post-operatively, and is maintained for at least 10 years after surgery in most patients (Sjöström *et al*, 2007). Studies of body composition with DEXA and air-displacement plethysmography have demonstrated a reduction of total body fat, especially visceral adipose tissue, following RYGB (Olbers *et al*, 2006, Gómez-Ambrosi *et al*, 2015). The major complications of RYGB include bowel obstructions, cholelithiasis, leakage of enteric contents, internal hernia and gastric fistulas (Higa *et al*, 2000, Blachar *et al*, 2002). In addition, one important disadvantage of RYGB constitutes the chronic malabsoption of calcium, iron, folate and vitamin D,

among others, that can lead to nutrient deficiency-related diseases, such as anemia or osteoporosis (DeMaria, 2007). In this sense, the European Guidelines on metabolic and bariatric surgery recommend the prescription of daily oral vitamin and micronutrient supplements to compensate for their possible reduced intake and absorption (Fried, 2013). Wound or respiratory infections also constitute minor complications of RYGB (Blachar *et al*, 2002). Nowadays, the perioperative mortality of laparoscopic RYGB has decreased to 0.2% (Longitudinal Assessment of Bariatric Surgery *et al*, 2009).

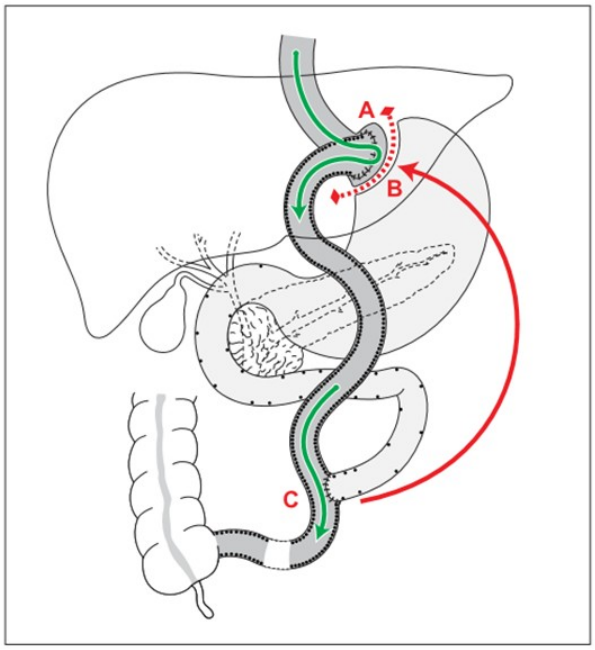


Figure 7. Diagram of Roux-en-Y gastric bypass technique [modified from (Scott *et al*, 2011)]. Red lines indicate surgical manipulations, and green arrows indicate nutrient flow. Small gastric pouch is created by division of the stomach at (A). The jejunum is divided 30-75 cm from the ligament of Treitz, and the distal end is anastomosed to the gastric pouch (B), creating the roux limb. The incongruent proximal end is reanastomosed to the alimentary limb 75-150 cm from the gastrojejunostomy (C).

RYGB results in a significant and rapid improvement of glycemia in patients with impaired glucose tolerance or T2D with an 80-95% remission of hyperglycemia and an 80% T2D resolution, which allows the discontinuation of anti-diabetic medications (Pories *et al*, 1995, Schauer *et al*, 2003, Buchwald *et al*, 2009). The reversal of hyperglycemia is superior to diet alone (Laferrère *et al*, 2008) and, remarkably, this improvement can be seen within days after the operation, prior to any significant weight loss (Rubino *et al*, 2004). In addition, RYGB restores the pancreatic β-cell responsiveness to glucose at short-term (Kashyap *et al*, 2010) and 2 years after surgery (Kashyap *et al*, 2013).

RYGB ameliorates all the characteristic morphological features of NAFLD-NASH, namely steatosis, ballooning, necroinflammation, and fibrosis, after significant weight reduction (Clark *et al*, 2005, Barker *et al*, 2006). These beneficial changes occurred despite significant NAFLD histopathology at baseline and significant weight loss, a combination that has been shown in some studies to worsen hepatic inflammation and fibrosis. In line with this observation, RYGB can also correct plasma lipid derangements associated with obesity (97% hyperlipidemia improvement) (Frühbeck, 2015) leading to a reduced risk of cardiovascular disease in patients, producing a reduction of SBP and DBP values (Sjöström *et al*, 2004, Iannelli *et al*, 2011) with a resolution of hypertension of 68% (Frühbeck, 2015).

2.2. Mechanisms involved in the metabolic effects of bariatric surgery

The precise mechanisms involved in the improvement of the beneficial effects of bariatric surgery remain unclear (Frühbeck, 2015). The reduction of the gastric size and the subsequent decrease in food intake contribute to the resolution of obesity-related comorbidities (Ashrafian *et al*, 2011). However, changes in gastrointestinal hormones, such as ghrelin, glucagon-like peptide 1 (GLP-1), gastric inhibitory peptide (GIP) or peptide YY (PYY) also play a role in the rapid metabolic changes observed after bariatric surgery (Zhu *et al*, 2016). In addition, growing evidence supports the important contribution of: i) bile flow alterations; ii) vagal manipulation; iii) anatomical rearrangement and altered flow of nutrients; iv) modifications of gut microbiota in the resolution of diabetes and other pathologies associated to obesity (Ashrafian *et al*, 2011, Frühbeck, 2015). These metabolic outcomes are achieved through weight-independent and weight-dependent mechanisms. None of the currently available hypotheses (foregut, midgut and hindgut hypotheses as well as gastric center hypothesis; all explained below) is able to fully explain the improved whole-body metabolism achieved by different bariatric surgical procedures (Ashrafian *et al*, 2011, Zhu *et al*, 2016). Further research elucidating the precise metabolic mechanisms of diabetes resolution after surgery can lead to improved operations and disease-specific procedures.

2.2.1. Foregut hypothesis

The foregut hypothesis (**Figure 8**) proposes that food bypassing the duodenum and proximal jejunum leads to a weight-independent decrease in supposedly unknown anti-incretin hormones, which improves insulin sensitivity (Rubino *et al*, 2006). In this sense, the duodenal-jejunal bypass greatly improves diabetes in Goto-Kakizaki rats, a

non-obese animal model of T2D (Rubino *et al*, 2006). Analogously, strong support for the foregut hypothesis has come from the use of the EndoBarrier, an endoluminal device designed to mimic the duodenal-jejunal bypass achieved with RYGB, which produces significant weight loss, resolution of T2D and improvement in the cardiovascular risk factor profile (Patel *et al*, 2013). The major proposed mechanism whereby bypass of the foregut improves glucose tolerance is via an enhanced incretin response (Preitner *et al*, 2004). The two major incretins, GIP and GLP-1, enhance glucose-dependent insulin release. GIP is mainly secreted from duodenal K-cells, whereas GLP-1 is primarily secreted by L-cells of the ileum, although both incretins are detected throughout the intestine (Mortensen *et al*, 2000). The exclusion of the foregut with RYGB enables a rapid reach of ingested food to the hindgut, leading to an increased postprandial secretion of GLP-1 and the subsequent insulin secretion (Salehi *et al*, 2011). In addition to this "incretinic effect", the passage of nutrients through the intestinal foregut activates a negative feedback mechanism ("anti-incretin" or decretin) to balance the effects of incretins aimed to prevent hypoglycemia (Rubino *et al*, 2004, Alfa *et al*, 2015). Anti-incretins interfere with pathways of incretins to inhibit insulin action. In predisposed individuals, chronic stimulation with particular nutrients may create an imbalance between incretin and anti-incretin signals, resulting in insulin resistance and T2D (Rubino *et al*, 2006, Kamvissi *et al*, 2015). However, the foregut hypothesis cannot be the sole explanation for the marked weight loss and improvement in glucose metabolism, since sleeve gastrectomy and gastric banding, two bariatric procedures that reduce the volume of the stomach without bypassing of the small intestine, also induce significant weight loss and sustained improvement in the control of glycemia (Zhu *et al*, 2016).

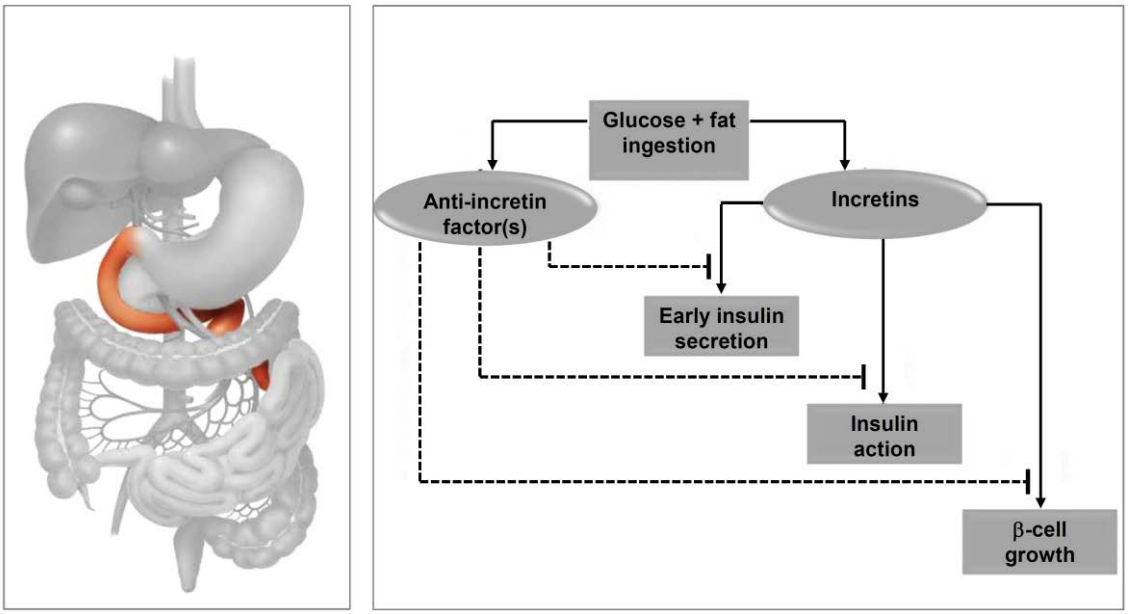


Figure 8. Diagram of the foregut hypothesis postulating that, in addition to the well-known incretin effect, nutrient passage through the proximal small intestine (duodenum and jejunum) could also activate negative feedback mechanisms (anti-incretins) to balance the effects of incretins. An imbalance between incretin and anti-incretin signals could result in insulin resistance and T2D [modified from (Rubino *et al*, 2006)]. Dotted lines represent de inhibition of the pathway.

2.2.2. Midgut hypothesis

The "midgut hypothesis" or intestinal/hepatic regulation hypothesis (**Figure 9**) proposes that the shunting of nutrients to the distal small intestine increases the absorption of lipids by increasing bile flow (Pournaras *et al*, 2013). Both fasting and postprandial serum bile acid concentrations increase significantly after RYGB (Nakatani *et al*, 2009, Steinert *et al*, 2013). Bile acids are derived from cholesterol or oxysterols in the liver and they are released postprandially into the duodenum to mix with ingested nutrients. Bile acids promote fat absorption mainly in the ileum, although the colon can contribute to further bile acid absorption. A small percentage of bile acids are deconjugated by gut bacteria, forming secondary bile acids, which are reabsorbed or excreted in the feces (Sayin *et al*, 2013). Over the past few years, bile acids have evolved from being considered as simple lipid solubilizers to complex metabolic integrators. Bile acids can act on the intestinal nuclear receptor FXR (farsenoid-X receptor, also known as NR1H4) and the G-protein-coupled receptor TGR5 to reduce body weight and improve glucose tolerance (Ryan *et al*, 2014). The transintestinal bile acid flux activates intestinal FXR, inducing synthesis and secretion into the circulation of the ileal-derived enterokine fibroblast growth factor (FGF)-19 (FGF-15 in mice), which can improve glucose tolerance by regulating insulin-independent glucose efflux and by repressing bile acid synthesis and gluconeogenesis in mice (Fang *et al*, 2015,

Penney *et al*, 2015). In addition, bile acids promote the secretion of GLP-1 and PYY from intestinal L-cells via the activation of TGR5, which constitutes another mechanism to improve insulin sensitivity and whole-body glucose metabolism (Katsuma *et al*, 2005).

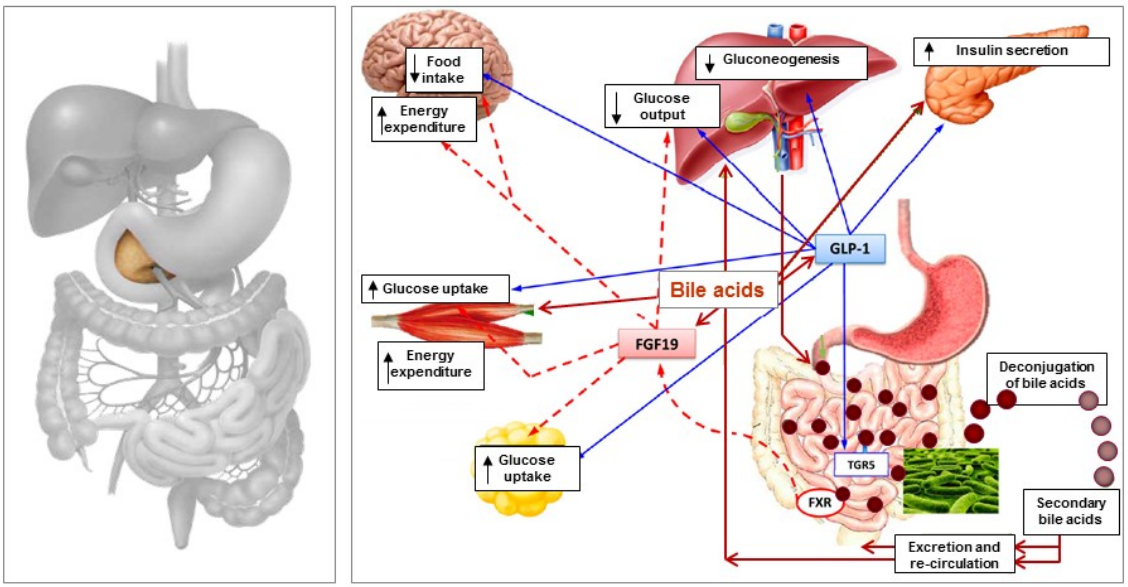


Figure 9. Diagram of the midgut hypothesis proposing that the increase in bile acid secretion after bariatric surgery not only increases the lipid absorption in the distal intestine (ileum), but also exerts beneficial metabolic effects on binding bile acid receptors such as intestinal nuclear receptor FXR or TGR5 (modified from [(Batterham *et al*, 2016)].

The small intestine can produce and release glucose through intestinal gluconeogenesis and release it to the portal vein, leading to the activation of the hepato-portal glucose signaling system, decreasing food intake and suppressing hepatic glucose production (Troy *et al*, 2008). Fasting and the ingestion of proteins induce intestinal gluconeogenesis by increasing the activity of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) gluconeogenic enzymes (Mithieux, 2009). RYGB, but not sleeve gastrectomy, induces the programming of intestinal glucose metabolism, which renders the intestine an important tissue for glucose disposal, contributing to the improvement in glycemic control after surgery in various models of diabetes (Saeidi *et al*, 2013, Mumphrey *et al*, 2015).

2.2.3. Hindgut hypothesis

The hindgut hypothesis (**Figure 10**) states that early exposure of undigested food to the hindgut (distal ileum, colon and rectum) leads to increased secretion of GLP-1 and PYY from intestinal L-cells with subsequent improvement in glycemic control (Cummings B. P. *et al*, 2010). GLP-1 is one of the peptides yielded by post-translational

processing of pre-proglucagon (Dhanvantari *et al*, 1996). Other peptides arising from this process are GLP-2, glucagon, glicentin and oxyntomodulin. GLP-1 promotes postprandial glucose-induced insulin release (Orskov *et al*, 1996, Farilla *et al*, 2003) and also exerts proliferative and anti-apoptotic effects on pancreatic β-cells, thereby improving β-cell function (Drucker, 2003). Interestingly, numerous neuronal populations of the central nervous system express the GLP-1 receptor, including the hypothalamus and the nucleus tractus solitarius, which are crucial for the regulation of energy balance (Shimizu *et al*, 1987). In this regard, stimulation of the central GLP-1 system not only suppresses food intake, but also regulates glucose homeostasis and activates BAT thermogenesis and browning (Sandoval *et al*, 2008, Seo *et al*, 2008, Beiroa *et al*, 2014). Interestingly, it has been shown that sleeve gastrectomy induces weight loss and improves glucose metabolism in two genetic animal models lacking GLP-1 receptor, suggesting that GLP-1 receptor activity is not absolutely necessary for the metabolic effects induced by sleeve gastrectomy (Wilson-Pérez *et al*, 2013a).

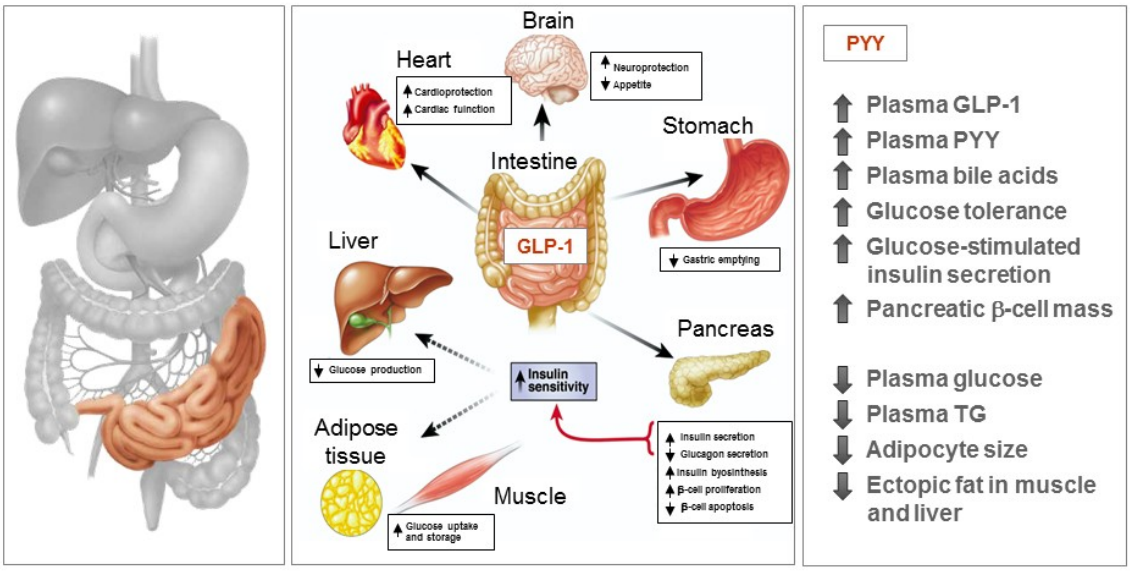


Figure 10. Diagram of the hindgut hypothesis suggesting that the altered anatomy after bariatric surgery induces a rapid transit of nutrients to the distal ileum, colon and rectum that stimulates the synthesis and secretion of GLP-1 and PYY [modified from (Ahima *et al*, 2010, Gigoux *et al*, 2013)]. These gastrointestinal hormones exert pleiotropic effects, including the reduction of food intake and the increase in insulin secretion and sensitivity, thereby contributing to the beneficial metabolic effect observed after bariatric surgery.

On the other hand, two main forms of the 36-amino acid peptide PYY have been described: $PYY_{1\text{-}36}$, the full-length peptide, and $PYY_{3\text{-}36}$, the major circulating form that arises from cleavage of the N-terminal Tyr-Pro residues from the full-length peptide by the enzyme dipeptidyl-peptidase 4 (DPP4) (van den Hoek *et al*, 2004). $PYY_{3\text{-}36}$

decreases food intake and increases thermogenesis, lipolysis, and increased postprandial insulin and glucose responses (le Roux *et al*, 2006, Sloth *et al*, 2007). Thus, elevated post-prandial levels of GLP-1 and PYY_{3-36} could contribute to the improved glucose homeostasis observed after bariatric surgery (Strader *et al*, 2005).

2.2.4. Gastric center or ghrelin hypothesis

The gastric center hypothesis (**Figure 11**) proposes that the greater curvature of the stomach produces several factors involved in the improvement of body weight and whole-body metabolism observed after stomach surgery, such as sleeve gastrectomy, RYGB and total gastrectomy (Zhu *et al*, 2016). In this sense, an obviously candidate is ghrelin which is mainly synthesized by X/A-like cells of the oxyntic glands in the mucosa of the gastric fundus (Frühbeck *et al*, 2004a, Frühbeck *et al*, 2004b). Ghrelin is an orexigenic hormone that was first discovered as the endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R), which stimulates GH release (Kojima *et al*, 1999). Circulating ghrelin exists in two main forms: desacyl ghrelin (95% of total ghrelin) and acylated ghrelin (5% of total ghrelin) that carries an *n*-octanoyl modification at Ser3 catalyzed by the ghrelin *O*-acyltransferase (GOAT) enzyme (Yang J. *et al*, 2008). Ghrelin stimulates appetite and induces a positive energy balance, leading to body weight gain (Tschöp *et al*, 2000, Wren *et al*, 2000, Nakazato *et al*, 2001, Wren *et al*, 2001). In this sense, administration of exogenous ghrelin stimulates appetite and increases food intake by the stimulation of hypothalamic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons expressing its functional receptor, GHS-R 1a (Toshinai *et al*, 2003, Chen *et al*, 2004, López *et al*, 2008). Moreover, ghrelin isoforms can induce adipogenesis in the adipose tissue (Rodríguez *et al*, 2009, Gurriarán-Rodríguez *et al*, 2011) and lipogenesis in the liver (Sangiao-Alvarellos *et al*, 2009, Porteiro *et al*, 2013, Ezquerro *et al*, 2016), further contributing to the increased adiposity.

Circulating ghrelin concentrations are characterized by a preprandial rise and postprandial fall supporting its role in meal initiation (Cummings D. E. *et al*, 2001). Paradoxically, despite the orexigenic and adipogenic actions of ghrelin, obesity, insulin resistance, T2D as well as metabolic syndrome are associated with a decrease in circulating total ghrelin levels (Kojima *et al*, 1999, Tschöp *et al*, 2000, Pöykkö *et al*, 2005). Nevertheless, these pathologies are associated with a dramatic reduction of

plasma desacyl ghrelin levels, while plasma concentrations of acylated ghrelin remain unchanged or increased (Rodríguez *et al*, 2009, Rodríguez *et al*, 2010).

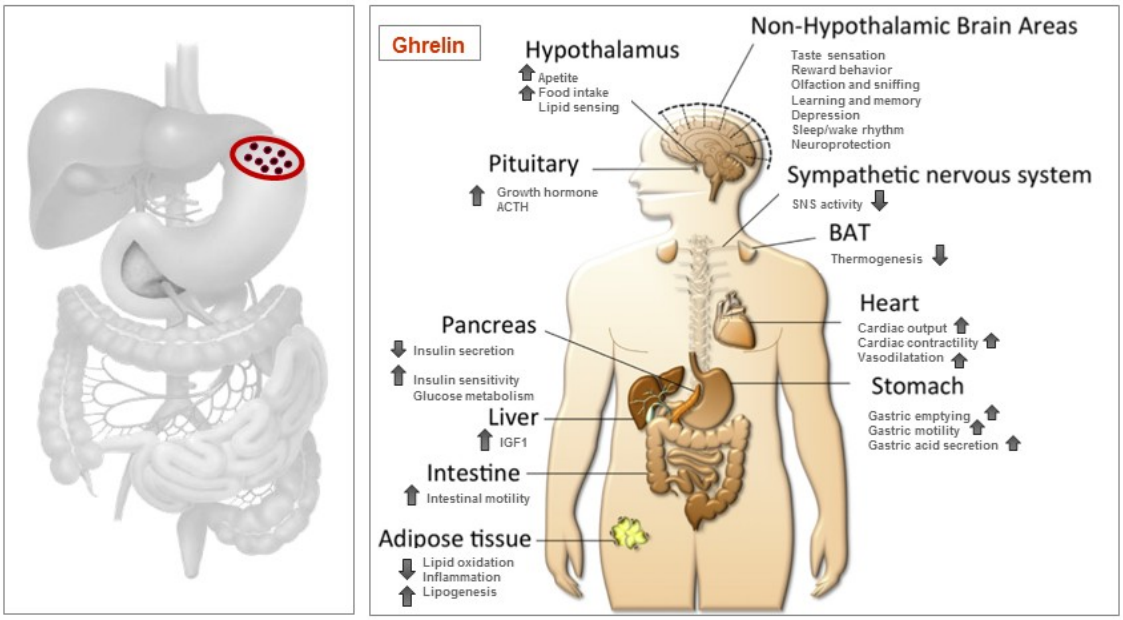


Figure 11. Diagram of the gastric center hypothesis stating that the removal of the greater curvature of the stomach with different techniques contributes to the metabolic effect of bariatric surgery. Ghrelin is an orexigenic and lipogenic peptide hormone mainly produced in the gastric fundus, and its circulating levels are drastically reduced after bariatric surgery [modified from (Müller *et al*, 2015)].

The relationship between bariatric surgery and ghrelin levels is controversial. Plasma ghrelin is markedly reduced after sleeve gastrectomy that removes 70-80% of the stomach, and remained low after 1 year (Frühbeck *et al*, 2004a, Peterli *et al*, 2012). By contrast, patients undergoing RYGB exhibit reduced ghrelin levels, but not as pronounced as those observed with sleeve gastrectomy (Cummings D. E. *et al*, 2002, Frühbeck *et al*, 2004a), with ghrelin levels approaching preoperative values 1 year after surgery (Peterli *et al*, 2012, Malin *et al*, 2014). The decrease in ghrelin levels after sleeve gastrectomy and RYGB depends on the degree to which the surgical technique excludes the gastric fundus (Frühbeck *et al*, 2004a, Frühbeck *et al*, 2004b). Hence, the changes in ghrelin contribute to the initial marked decrease in food intake and weight loss that follow both surgical procedures. Moreover, genetic, immunological, and pharmacological blockade of ghrelin signaling enhances glucose-stimulated insulin secretion and improves peripheral insulin sensitivity (Alamri *et al*, 2016). Thus, it seems plausible that ghrelin changes after stomach surgery also contribute, at least in part, to the post-surgical improvement in insulin sensitivity.

3. AQUAPORINS

Aquaporins (AQPs) are channel-forming integral membrane proteins that belong to the family of the major intrinsic proteins (Agre *et al*, 2003, King *et al*, 2004). AQPs facilitate the rapid transport of water and other small solutes across the cell membranes driven by osmotic or solute gradients (Carbrey *et al*, 2009). So far thirteen aquaporins have been identified (AQP0-AQP12) in mammals, which are ubiquitously expressed in tissues implicated in high rates of active fluid transport (Verkman *et al*, 2014). Within the cellular membranes, AQPs assemble as a tetramer (Verbavatz *et al*, 1993) with each monomer behaving as an independent pore (Jung *et al*, 1994, King *et al*, 2004). AQPs share a common protein fold, with six membrane-spanning helices surrounding the amphipathic channel plus two half-helices with their positive, N-terminal, ends located at the center of the protein and their C-terminal ends pointing towards the intracellular side of the membrane. AQPs also contain two conserved asparagine-proline-alanine motifs (NPA) located at the N-terminal ends of the two half-helices, at the center of the channel (Agre *et al*, 1993), which form a tridimensional "hourglass" structure that allows the movement of solutes through the pore. The aromatic/arginine (ar/R) constriction formed by four defined residues at the extracellular aqueous pore mouth constitutes a major selectivity filter for permeability (Beitz *et al*, 2006, Azad *et al*, 2012).

3.1. Types of aquaporins

According to their permeability and structure characteristics, AQPs are subclassified into three subgroups: orthodox aquaporins, aquaglyceroporins and superaquaporins (King *et al*, 2004, Ishibashi *et al*, 2014). The functional importance of AQPs has been revealed by the analysis of the phenotype of AQP-knockout mice and from humans with loss-of-function mutations in AQPs (King *et al*, 2004, Rodríguez *et al*, 2007b) (**Table 2**).

Table 2. Main phenotypic characteristics derived from aquaporin deficiency in mice and humans.

Type	AQP-knockout mice	AQP-deficient humans	References
AQP0	Cataracts	Congenital cataracts	Francis *et al*, 2000, Shiels *et al*, 2001
AQP1	Polydipsia, defective proximal fluid absorption, impaired angiogenesis and vasodilation	Loss of Colton blood group, decreased urine-concentrating mechanism after water deprivation	Preston *et al*, 1994, King *et al*, 2001, Saadoun *et al*, 2005, Herrera *et al*, 2007
AQP2	Severe urinary concentrating defect	Nephrogenic diabetes insipidus	Deen *et al*, 1994, Rojek A. *et al*, 2006
AQP3	Nephrogenic diabetes insipidus, defective skin hydration	Antibodies against GIL blood group	Ma *et al*, 2000, Hara *et al*, 2002, Ma *et al*, 2002, Roudier *et al*, 2002
AQP4	Reduced brain swelling, mild urine-concentrating defect	Not described	Ma *et al*, 1997, Manley *et al*, 2000, Papadopoulos *et al*, 2004
AQP5	Impaired saliva and sweat secretion, hiperresponsive bronchoconstriction	Non-epidermolytic palmoplantar keratoderma	Ma *et al*, 1999, Krane *et al*, 2001, Nejsum *et al*, 2002, Blaydon *et al*, 2013
AQP6	Not described	Not described	-
AQP7	Adult-onset obesity, increased insulin production and insulin resistance	Impaired increase of serum glycerol during exercise	Kondo *et al*, 2002, Maeda *et al*, 2004, Hara-Chikuma *et al*, 2005, Hibuse *et al*, 2005, Matsumura *et al*, 2007
AQP8	Larger testes	Not described	Yang B. *et al*, 2005
AQP9	Defective glycerol metabolism	Not described	Rojek A. M. *et al*, 2007
AQP10	Murine *Aqp10* is a pseudogene	Not described	Morinaga *et al*, 2002
AQP11	Renal failure with polycystic kidneys	Not described	Ishibashi, 2006, Okada *et al*, 2008
AQP12	Higher susceptibility to caerulein-induced acute pancreatitis	Not described	Ohta *et al*, 2009

3.1.1. Orthodox aquaporins

Aquaporins (AQP0, 1, 2, 4, 5, 6, 8) are considered orthodox or "pure" aquaporins permeated only by water. Nonetheless, AQP1 and AQP4 can transport nitric oxide (NO) (Herrera *et al*, 2006, Wang *et al*, 2010), AQP6 shows conductance to nitrate and other inorganic anions (Yasui M. *et al*, 1999) and AQP8 features high permeability to ammonia and H_2O_2 (Bienert *et al*, 2007, Soria *et al*, 2010). These water channels play important functions in tissues with active fluid transport involved in processes such as crystalline lens transparency (AQP0), angiogenesis and vasodilation (AQP1), urine concentration and acid-base homeostasis in kidneys (AQP1, 2, 3, 6), cerebrospinal fluid secretion (AQP4), saliva, sweat, tears and pulmonary secretions (AQP5), gastrointestinal fluid secretion, hepatic bile formation and secretion, and spermatogenesis (AQP8) (Herrera *et al*, 2007, Madeira *et al*, 2015, Direito *et al*, 2016). In this regard, loss-of-function mutations in human AQPs cause congenital cataracts (AQP0), defective urine concentration (AQP1), nephrogenic diabetes insipidus (AQP2) while autoantibodies against AQP4 cause the autoimmune demyelinating disease neuromyelitis optica (**Table 2**) (Verkman *et al*, 2014).

3.1.2. Aquaglyceroporins

Aquaglyceroporins (AQP3, 7, 9 and 10) are permeated by water and other small solutes, such as glycerol, urea or nitric oxide (Oliva *et al*, 2010). It has been recently demonstrated that aquaglyceroporins also have the ability to transport silicon as orthosilicic acid (Carpentier *et al*, 2016).

AQP3 (also known as glycerol intrinsic protein, GLIP, based on its glycerol transport function) was initially cloned from the rat kidney (Echevarría *et al*, 1994, Ma *et al*, 1994). The principal physiological functions of AQP3 are urine concentration and skin hydration (Echevarría *et al*, 1994, Ma *et al*, 1994), with *Aqp3*-knockout mice showing a reduced skin hydration and elasticity, together with a protection against skin tumorigenesis, impaired wound healing, and nephrogenic diabetes insipidus (Ma *et al*, 2000, Hara *et al*, 2002, Ma *et al*, 2002, Hara-Chikuma *et al*, 2008a, Hara-Chikuma *et al*, 2008b). Although extremely rare, there are cases of homozygous mutations in *AQP3* in humans with the development of antibodies against a new red-blood cell group named GIL and it has been also associated with Menière's disease and higher susceptibility to gallbladder cancer (Roudier *et al*, 2002, Bahamontes-Rosa *et al*, 2008, Candreia *et al*, 2010).

AQP7 (originally named AQPap) was cloned from adipose tissue in 1997 (Ishibashi *et al*, 1997, Ishibashi *et al*, 1998). AQP7 facilitates the transport of water, glycerol and arsenite (Liu Z. *et al*, 2002). *Aqp7*-deficient mice show adult onset obesity, hyperinsulinemia and insulin resistance (Maeda *et al*, 2004, Hara-Chikuma *et al*, 2005, Hibuse *et al*, 2005, Matsumura *et al*, 2007), highlighting the important role of this aquaglyceroporin in the control of fat accumulation as well as glucose homeostasis (Frühbeck, 2005). AQP7 constitutes the main glycerol channel facilitating glycerol transport in adipocytes. The defective glycerol exit in fat cells from *Aqp7*-knockout mice leads to an intracellular glycerol accumulation, resulting in an increased TG biosynthesis and adipocyte hypertrophy (Hara-Chikuma *et al*, 2005, Hibuse *et al*, 2005). In humans, the rare cases of individuals carrying homozygous mutations in the coding region of the *AQP7* gene do not exhibit obesity or diabetes (Kondo *et al*, 2002, Roudier *et al*, 2002, Bahamontes-Rosa *et al*, 2008, Candreia *et al*, 2010). The only apparent consequence of AQP7 deficiency in humans is an impaired glycerol increase in response to exercise, reinforcing the role of this aquaglyceroporin in lipolysis (Kondo *et al*, 2002).

AQP9 was first isolated from rat liver and can transport water, glycerol, urea and arsenite into the hepatocytes (Tsukaguchi *et al*, 1998, Liu Z. *et al*, 2002). AQP9 represents the primary route for glycerol uptake into hepatocytes with transgenic *Aqp9*-knockout mice showing a defective hepatic glycerol metabolism (Rojek A. M. *et al*, 2007). Glycerol is used as a substrate for hepatic gluconeogenesis and, in line with this observation, *Aqp9* deletion in obese diabetic *db/db* mice results in a reduction of 10-40% of circulating glucose levels (Rojek A. M. *et al*, 2007). In human liver, AQP9 also represents the main glycerol channel in hepatocytes, although AQP3, 7 and 10 also facilitate glycerol uptake in these cells (Jelen *et al*, 2011, Lebeck, 2014, Rodríguez *et al*, 2014).

AQP10 is abundantly expressed in human duodenum, jejunum and ileum contributing to intestinal water and glycerol absorption (Hatakeyama *et al*, 2001, Mobasheri *et al*, 2004), although the murine *Aqp10* gene is a pseudogene (Morinaga *et al*, 2002). Two different isoforms are expressed in the human small intestine: i) AQP10v, which is mainly expressed in the capillary endothelial cells of the small intestinal villi to allow water absorption in the intestinal epithelium, and ii) AQP10,

localized in the cytoplasm of the gastro-entero-pancreatic endocrine cells suggesting a role in the secretion of polypeptide hormones from these cells (Li *et al*, 2005).

3.1.3. Superaquaporins

Superaquaporins exhibit very low homology to orthodox aquaporins and aquaglyceroporins due to their unique structure and subcellular localization (Ishibashi *et al*, 2014). In contrast to conventional aquaporins that present two highly conserved NPA (Asn-Pro-Ala) sequence motifs that allow the movement of water through the pore (Agre *et al*, 1993), superaquaporins present a different sequence of the first NPA motif: Asn-Pro-Cys (NPC) in AQP11 and Asn-Pro-Thr (NPT) in AQP12 (Yakata *et al*, 2007, Calvanese *et al*, 2013). Moreover, superaquaporins localize in the membrane of intracellular organelles instead of the plasma membrane (Ishibashi *et al*, 2009).

AQP11 (originally named AQPX1) is highly expressed in rat testis and, to a lesser extent, in kidney, liver and brain (Gorelick *et al*, 2006). It has also been recently described as a novel glycerol channel in human adipocytes being located in lipid droplets (Madeira *et al*, 2014). Transgenic *Aqp11*-knockout mice die before weaning with progressive vacuolization and cyst formation of the proximal tubule, leading to polycystic kidney development (Morishita *et al*, 2005, Tchekneva *et al*, 2008). The vacuoles are also observed in hepatocytes close to the central vein as well as in the epithelium of intestinal villi where water is intensively absorbed (Rojek A. *et al*, 2013). AQP11 seems to play a relevant role in renal intravesicular homeostasis, which is essential for an adequate proximal tubule function.

AQP12 (originally known as AQPX2) is selectively expressed in the acinar cells of the pancreas (Itoh *et al*, 2005) with an intracellular localization in the rough endoplasmic reticulum (RER) and the membranes of zymogen granules near the RER (Itoh *et al*, 2005, Ohta *et al*, 2009). Transgenic mice lacking *Aqp12* gene showed more severe pathological damage in the pancreas and revealed larger exocytotic vesicles (vacuoles) in the pancreatic acini. Thus, AQP12 may participate in the control of the proper maturation and secretion of pancreatic fluid following rapid and intense stimulation (Ohta *et al*, 2009). The literature about AQP12 is scarce and more research is necessary in order to discern further biological functions of this superaquaporin.

3.2. Role of aquaglyceroporins in the onset of obesity and its associated comorbidities

Advances in determining the mechanisms that underlie obesity and obesity-associated pathologies, such as insulin resistance, T2D and NAFLD, have been provided by the discovery and characterization of aquaglyceroporins in the adipose tissue, liver and pancreas (Frühbeck, 2005, Rodríguez *et al*, 2011a). Circulating glycerol results from lipolysis, diet-derived glycerol absorbed in the intestine and glycerol reabsorbed in the proximal tubules (Echevarría *et al*, 1994, Ramírez-Lorca *et al*, 1999, Sohara *et al*, 2005). Glycerol constitutes a key metabolite for lipid accumulation as the carbon backbone of TG (Reshef *et al*, 2003). During fasting hepatic glucose output embodies the main source of plasma glucose, and plasma glycerol becomes the major substrate for hepatic gluconeogenesis (Rojek A. M. *et al*, 2007). Finally, glycerol uptake is accompanied by β-cell swelling, activation of the volume-regulated anion channel (VRAC) and insulin release (Best *et al*, 2009). Thus, the regulation of glycerol transport by aquaglyceroporins contributes to the control of fat accumulation, glucose homeostasis and insulin secretion, among other biological functions.

3.2.1. Aquaglyceroporins in lipogenesis and lipolysis

Adipose tissue constitutes the main source of plasma glycerol (Reshef *et al*, 2003). AQP7 was considered the unique glycerol channel in adipose tissue, but nowadays it has been demonstrated that AQP3, 9 and 10 represent additional pathways for the transport of glycerol in human adipocytes (Miranda *et al*, 2010, Rodríguez *et al*, 2011b, Laforenza *et al*, 2013). In addition, AQP5 and 11 are expressed in adipocytes and can transport glycerol across the biological membranes of fat cells (Madeira *et al*, 2014, Madeira *et al*, 2015). Under physiological circumstances, adipocytes adapt the balance between TG synthesis (lipogenesis) and hydrolysis (lipolysis) in order to meet body energy demands (**Figure 12**) (Rodríguez *et al*, 2007b, Frühbeck *et al*, 2014). In this regard, aquaglyceroporins are necessary for the uptake and release of glycerol, a metabolite that constitutes the carbon backbone of TG (Maeda *et al*, 2008).

Under lipogenic conditions, in a postprandial state, adipocytes synthesize TG from the esterification of FFA and glycerol-3-phosphate. Fatty acid binding protein (FABP), fatty acid translocase (FAT, CD36) or fatty acid transporter protein (FATP), facilitate the FFA transport across the membrane of adipocytes (Kishida *et al*, 2001b, Rodríguez *et al*, 2006). Moreover, lipoprotein lipase (LPL) (located on the surface of

adipocytes and capillaries) removes FFA from chylomicrons and VLDL (Gonzales *et al*, 2007). Glycerol-3-phosphate is the other metabolite required for TG biosynthesis, and derives from three different sources: i) glucose, since glycerol-3-phosphate constitutes a secondary metabolite of glycolysis (Rodríguez *et al*, 2006, Maeda *et al*, 2008); ii) lipolysis-derived glycerol, which is phosphorylated by the glycerol kinase (GK) enzyme (Guan *et al*, 2002); and iii) glycerol uptake mediated by aquaglyceroporins (Rodríguez *et al*, 2011b). Several lipogenic stimuli, such as insulin, ghrelin and dexamethasone control the expression of AQP7 in adipocytes. In this sense, AQP7 is downregulated by acylated and desacyl ghrelin as well as by dexamethasone (Fasshauer *et al*, 2003, Rodríguez *et al*, 2009, Shen *et al*, 2012), thus promoting fat enlargement. Insulin regulates AQP7 expression in a different manner in rodents and humans (Rodríguez *et al*, 2011b). In rodents, insulin decreases AQP7 expression in WAT, since the *Aqp7* gene promoter presents a negative insulin response element (IRE) (Kishida *et al*, 2001b, Kishida *et al*, 2001a). By contrast, insulin increases the expression of AQP3, 7 and 10 in human adipocytes through the phosphatidylinositol 3-kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) signaling pathway, which might reflect the increase in TG content induced by this lipogenic hormone (Rodríguez *et al*, 2011b, Laforenza *et al*, 2013).

In circumstances of negative energy balance, such as fasting or exercise, TG are hydrolyzed to glycerol and FFA by adipose triglyceride lipase (ATGL) as well as hormone-sensitive lipase (HSL) (Lafontan *et al*, 2009, Kolditz *et al*, 2010, Frühbeck *et al*, 2014). Both FFA and glycerol are released into the bloodstream and can be used as energy substrates in different peripheral tissues. FFA is recycled within adipose tissue as well as in peripheral tissues such as liver, skeletal muscle, heart, pancreas or BAT (Reshef *et al*, 2003). Glycerol released from the adipose tissue acts as a substrate for hepatic gluconeogenesis and *de novo* TG biosynthesis (Rodríguez *et al*, 2011b). Several stimuli involved in the regulation of lipolysis, such as catecholamines, leptin, atrial natriuretic peptide (ANP), uroguanylin and guanylin, regulate the expression and translocation of aquaglyceroporins in adipocytes (Yasui M. *et al*, 1999, Kishida *et al*, 2001b, Rodríguez *et al*, 2011b, Rodríguez *et al*, 2015a, Rodríguez *et al*, 2016). The activation of β-adrenoreceptors leads to an increase in cAMP production and activation of protein kinase A (PKA), which in turn induces HSL phosphorylation and translocation of AQP3, 7 and 10 from the cytosolic fraction (AQP3) or lipid droplets

(AQP7 and AQP10) to the plasma membrane of adipocytes (Kishida *et al*, 2000, Walker *et al*, 2007, Yasui H. *et al*, 2008, Rodríguez *et al*, 2011b, Frühbeck *et al*, 2014). A recent study has shown that AQP7 is locked to the lipid droplet by perilipin-1, thereby preventing localization of AQP7 to the plasma membrane where it can exert glycerol efflux activity (Hansen *et al*, 2016). Interestingly, catecholamine-activated PKA phosphorylates the N-terminus of AQP7 reducing the complex formation with perilipin, and, thereby, facilitating its translocation to the plasma membrane and the subsequent glycerol efflux. On the other hand, acute leptin stimulation induces the mobilization of aquaglyceroporins towards lipid droplets (AQP3) and the plasma membrane (AQP7) in murine adipocytes (Rodríguez *et al*, 2015a). Leptin represses AQP7 protein expression in human adipocytes via the PI3K/Akt/mTOR signaling cascade (Rodríguez *et al*, 2011b), suggesting a negative feedback regulation to restrict glycerol release from adipose tissue.

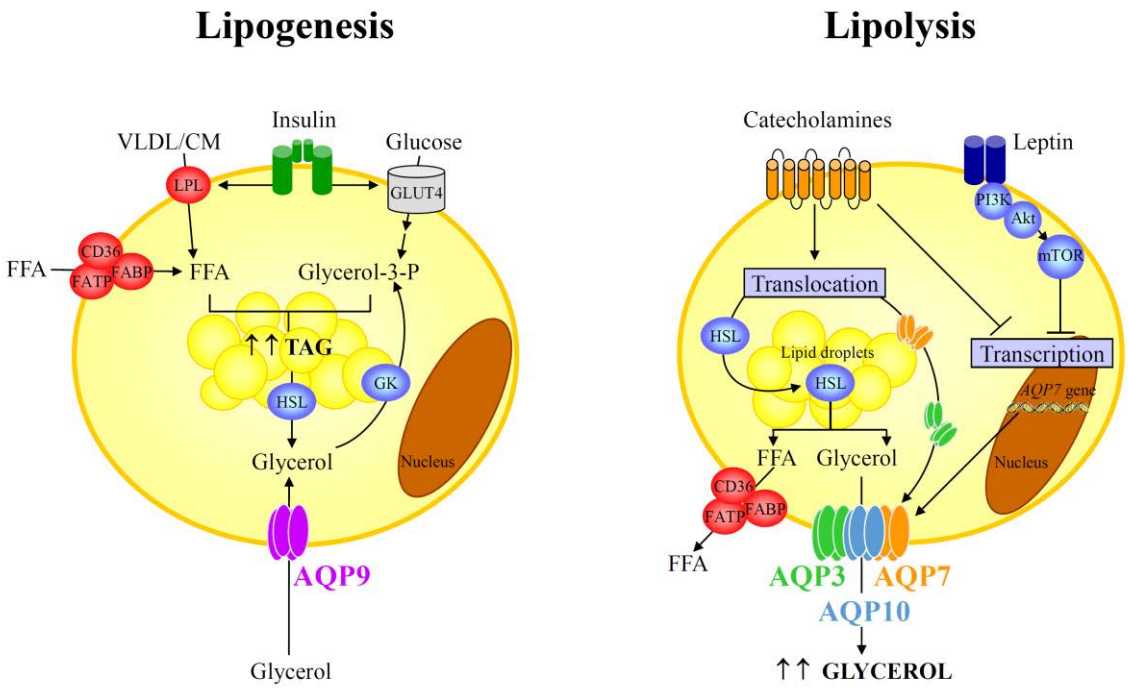


Figure 12. Role of aquaglyceroporins in lipogenesis and lipolysis [modified from (Rodríguez *et al*, 2011a)].

Human obesity is associated with a deregulation in the expression of aquaglyceroporins in adipose tissue (Marrades *et al*, 2006, Prudente *et al*, 2007, Catalán *et al*, 2008, Rodríguez *et al*, 2011b). In this sense, the expression of AQP3 and AQP7 is increased in human visceral WAT, which might be related to the increased lipolytic rate in this fat depot (Rodríguez *et al*, 2011b). However, AQP7 is downregulated in the

subcutaneous WAT (SCWAT) leading to the promotion of an intracellular glycerol accumulation and a progressive adipocyte hypertrophy (Rodríguez *et al*, 2011b).

3.2.2. Aquaglyceroporins in hepatic gluconeogenesis and steatosis

The liver is responsible for about 70-90% of whole-body glycerol metabolism (Peroni *et al*, 1995, Reshef *et al*, 2003). All the aquaglyceroporins are expressed in human liver with AQP9 being the primary route of hepatocyte glycerol uptake (Jelen *et al*, 2011, Calamita *et al*, 2012). AQP9 is mainly localized in the sinusoidal plasma membrane that faces the portal vein (Jelen *et al*, 2011, Lebeck, 2014). Glycerol is phosphorylated to glycerol-3-phosphate by the GK enzyme, and glycerol-3-phosphate constitutes a precursor for gluconeogenesis as well as for the *de novo* TG synthesis (**Figure 13**) (Rodríguez *et al*, 2006, Maeda *et al*, 2008). Insulin inhibits gluconeogenesis by reducing the activity of PEPCK and by blocking glycogenolysis, the breakdown glycogen polymers into glucose monomers (Rodríguez *et al*, 2011a). Moreover, insulin regulates the hepatic expression of AQP9, although this regulation appears to be different in rodent and humans. Insulin downregulates the *Aqp9* gene expression via the negative IRE in the gene promoter (Kishida *et al*, 2001b, Kuriyama *et al*, 2002, Higuchi *et al*, 2007), while in humans insulin upregulates the expression of AQP3, 7 and 9 in hepatocytes via the PI3K/Akt/mTOR pathway (Rodríguez *et al*, 2011b).

The coordinated regulation of adipose and hepatic aquaglyceroporins is required for the control of whole-body glucose homeostasis as well as lipid accumulation in both rodents and humans (Kuriyama *et al*, 2002, Catalán *et al*, 2008, Rodríguez *et al*, 2011b). Under physiological circumstances, insulin regulates the expression of AQP7 and AQP9 channels that supposes the increase or decrease of glycerol release from fat and uptake in the liver with the aim to regulate glucose production depending on the nutritional state (Kuriyama *et al*, 2002, Rodríguez *et al*, 2006). In this sense, it has been demonstrated in rodents that the overexpression of AQP7 in adipose tissue and AQP9 in the liver in the context of insulin resistance leads to elevated circulating glycerol, thus leading to an increase in hepatic glycerol uptake and gluconeogenesis, that supposes an aggravation of hyperglycemia (Kishida *et al*, 2001b, Kuriyama *et al*, 2002). By contrast, a reduced glycerol permeability and AQP9 expression has been identified in the liver of insulin-resistant individuals that might constitute a compensatory mechanism to diminish glycerol availability aimed at reducing the *de novo* synthesis of glucose in

hepatocytes and further enhancing the development of hyperglycemia (Catalán *et al*, 2008, Miranda *et al*, 2009, Rodríguez *et al*, 2011b, Rodríguez *et al*, 2014).

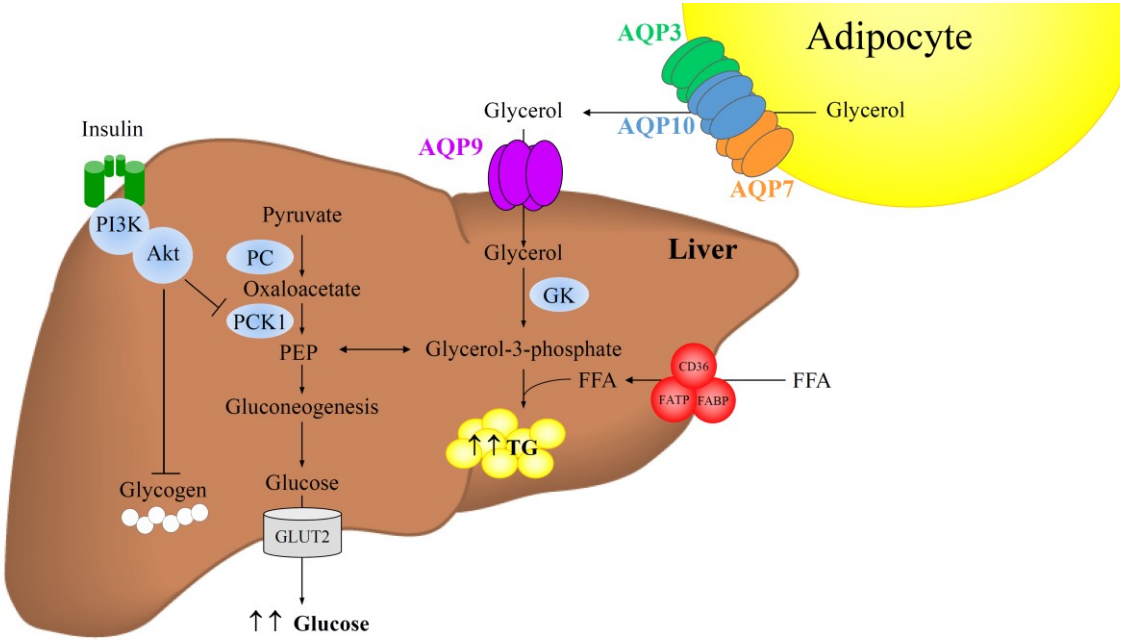


Figure 13. Role of aquaglyceroporins in hepatic gluconeogenesis and steatosis [modified from (Rodríguez *et al*, 2011a)].

The expression and functionality of hepatic AQP9 is also impaired in NAFLD and NASH (Rodríguez *et al*, 2014). Ectopic fat accumulation is strongly associated with insulin-resistant states, such as obesity, metabolic syndrome or T2D (Utzschneider *et al*, 2006, Chalasani *et al*, 2012). In this scenario, the decreased hepatic AQP9 expression in patients with NAFLD and NASH is in direct relation to the degree of steatosis and lobular inflammation, being further reduced in insulin-resistant individuals (Rodríguez *et al*, 2014). The downregulation of AQP9 together with the subsequent reduction in hepatic glycerol permeability in insulin-resistant states emerges as a compensatory mechanism whereby the liver counteracts further TG accumulation within its parenchyma as well as reduces hepatic gluconeogenesis in patients with NAFLD (Calamita *et al*, 2008, Potter *et al*, 2011, Rodríguez *et al*, 2014).

3.2.3. Aquaglyceroporins in pancreatic insulin secretion

Glucose is the primary regulator of insulin synthesis and secretion in pancreatic β-cells (Muoio *et al*, 2008), but glycerol constitutes another metabolite involved in this process (Matsumura *et al*, 2007, Louchami *et al*, 2012). AQP7 has been identified in pancreatic β-cells, but not in the ducts or the acini of the exocrine pancreas of mice and

rats as well as in BRIN-BD11 β-cell line (Matsumura *et al*, 2007, Best *et al*, 2009, Delporte *et al*, 2009). Circulating glycerol is transported into β-cells through AQP7, transformed into glycerol-3-phosphate by the activation of the GK activity and entered into the glycerol-3-phosphate shuttle (**Figure 14**). In this metabolic process glycerol-3-phosphate is converted into dihydroxyacetone phosphate (DHAP) by an inner membrane-bound mitochondrial glycerol-3-phosphate dehydrogenase (GPD) reducing FAD to $FADH_2$ that enters mitochondrial oxidative phosphorylation to generate ATP (Skelly *et al*, 2001, Matsumura *et al*, 2007). The subsequent increase in the cytoplasmic ATP/ADP ratio induces the closure of ATP-sensitive K^+ channels, followed by the plasma membrane depolarization and, finally, the opening of voltage-sensitive Ca^{2+} channels and a rapid influx of Ca^{2+} (Ma *et al*, 1994). The consequent increase in cytosolic Ca^{2+} triggers the exocytosis of insulin-containing secretory granules (Best *et al*, 2009).

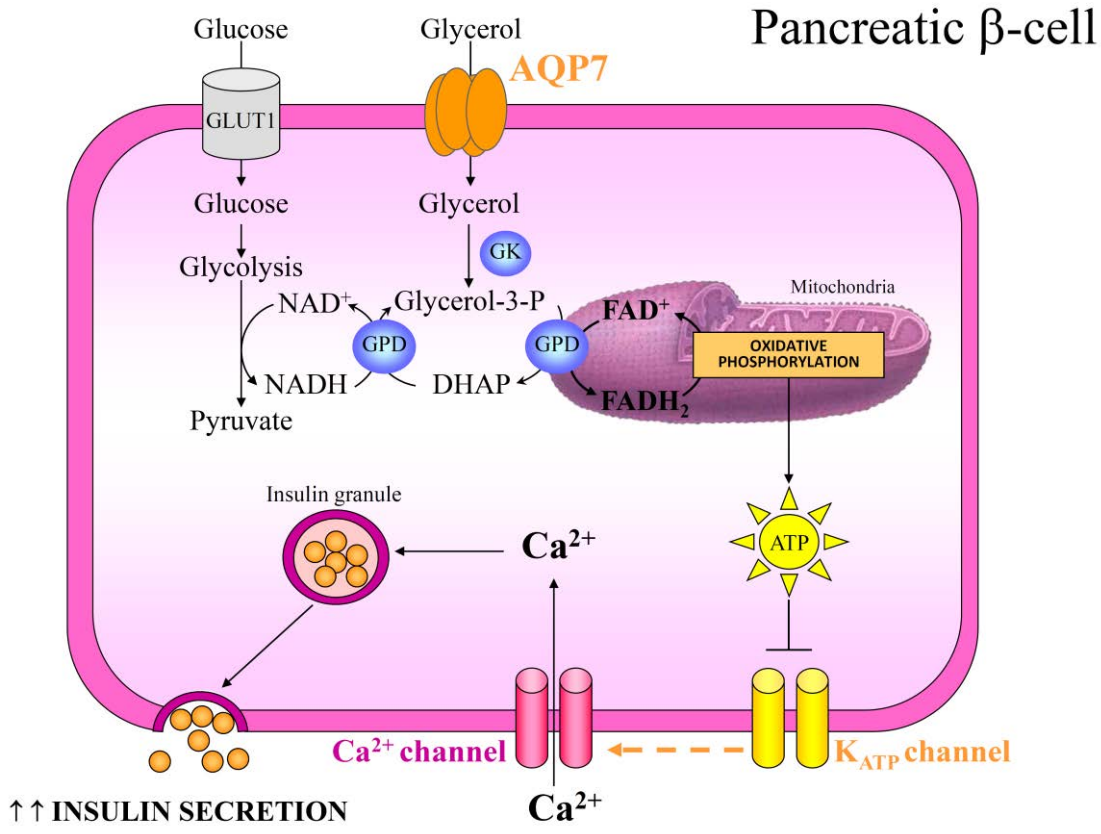


Figure 14. Role of aquaglyceroporins in pancreatic β-cell glycerol uptake and insulin secretion [modified from (Rodríguez *et al*, 2011a)].

Aqp7-knockout mice show increased β-cell glycerol content and GK activity, which result in higher basal and glucose-induced insulin secretion as well as reduced β-cell mass indicating a more efficient insulin biosynthesis and secretion (Matsumura *et*

al, 2007). Moreover, the elevated glycerol content in *Aqp7*-deficient mice promotes an increase in islet TG levels. Obesity-associated T2D is associated with an altered expression profile of AQP7 in insulin-sensitive tissues such as adipose tissue, liver and skeletal muscle (Marrades *et al*, 2006, Matsumura *et al*, 2007, Prudente *et al*, 2007, Catalán *et al*, 2008, Best *et al*, 2009, Delporte *et al*, 2009, Miranda *et al*, 2009, Rodríguez *et al*, 2011b). However, the impact of obesity and insulin resistance on the expression of AQP7 in pancreatic β-cells remains unknown.

Hypothesis and specific aims

HYPOTHESIS

The regulation of aquaglyceroporins in metabolic tissues is important for the control of fat accumulation, glucose homeostasis and insulin secretion. Obesity and insulin resistance are associated with changes in the expression of adipose tissue aquaglyceroporins, one of the main sources of plasma glycerol. Moreover, the coordinated regulation of aquaglyceroporins in adipose tissue and liver is impaired in both pathologies. The overall aim of the present thesis was to analyze whether the altered expression of aquaglyceroporins in adipose tissue, liver and pancreas is recovered in diet-induced obese rats submitted to two different bariatric surgery techniques, namely sleeve gastrectomy or gastric plication.

SPECIFIC AIMS

Specifically, the aims of the present thesis were:

1. To analyze the potential participation of adipose and hepatic aquaglyceroporins in the improvement of adiposity and hepatic steatosis after sleeve gastrectomy in an experimental model of diet-induced obesity which leads to NAFLD.

2. To investigate the potential role of the two known pancreatic aquaporins, AQP7 and AQP12, in the beneficial effect of sleeve gastrectomy on the improvement of glucose tolerance and β-cell function in diet-induced obese rats.

3. To study the effectiveness of gastric plication on weight loss, improvement of the metabolic profile, hepatic gluconeogenesis and steatosis in diet-induced obese rats, focusing on the participation of adipose and hepatic aquaglyceroporins in these effects.

Articles

STUDY I

1. Role of aquaglyceroporins and caveolins in energy and metabolic homeostasis

Article

Méndez-Giménez L, Rodríguez A, Balaguer I, Frühbeck G.
Role of aquaglyceroporins and caveolins in energy and metabolic homeostasis.
Mol Cell Endocrinol 2014;397(1-2):78-92.

Principal objective

The present review focuses on the role as energy and metabolic sensors of aquaglyceroporins and caveolins, two key integral membrane protein families involved in the onset of obesity and lipodystrophies.

Specific objectives

- To review the role of aquaglyceroporins involved in the control of fat accumulation (lipogenesis and lipolysis), hepatic gluconeogenesis and insulin secretion.
- To describe the participation of caveolins on lipid trafficking and insulin signaling.
- To outline the relevance of aquaglyceroporins and caveolins in the development of metabolic diseases such as obesity, congenital lipodystrophies, insulin resistance and dyslipidemia.

Méndez-Giménez L, Rodríguez A, Balaguer I, Frühbeck G. Role of aquaglyceroporins and caveolins in energy and metabolic homeostasis. Molecular and Cellular Endocrinology 2014;397(1-2):78-92.

STUDY II

2. Sleeve gastrectomy reduces hepatic steatosis by improving the coordinated regulation of aquaglyceroporins in adipose tissue and liver in obese rats

Article

Méndez-Giménez L, Becerril S, Moncada R, Valentí V, Ramírez B, Lancha A, Gurbindo J, Balaguer I, Cienfuegos JA, Catalán V, Fernández S, Gómez-Ambrosi J, Rodríguez A, Frühbeck G.

Sleeve gastrectomy reduces hepatic steatosis by improving the coordinated regulation of aquaglyceroporins in adipose tissue and liver in obese rats.

Obes Surg 2015;25(9):1723-34.

Hypothesis

Aquaglyceroporins expressed in the adipose tissue and liver are involved in the improvement of adiposity and hepatic steatosis after sleeve gastrectomy in diet-induced obese rats.

Objectives

- To study the effect of sleeve gastrectomy on body weight, whole-body adiposity, metabolic profile and hepatosteatosis in diet-induced obese rats.
- To analyze the impact of obesity and weight loss achieved by sleeve gastrectomy and pair-feeding on the expression of aquaglyceroporins in EWAT and SCWAT (AQP3 and AQP7) as well as in the liver (AQP9).
- To evaluate the correlation of adipose and hepatic aquaglyceroporins with markers of adiposity, glucose and lipid metabolism as well as hepatic steatosis.

Méndez-Giménez L, Becerril S, Moncada R, Valentí V, Ramírez B, Lancha A, Gurbindo J, Balaguer I, Cienfuegos JA, Catalán V, Fernández S, Gómez-Ambrosi J, Rodríguez A, Frühbeck G. Sleeve gastrectomy reduces hepatic steatosis by improving the coordinated regulation of aquaglyceroporins in adipose tissue and liver in obese rats. Obesity Surgery 2015;25(9):1723-34.

STUDY III

3. Role of aquaporin-7 in ghrelin- and GLP-1-induced improvement of pancreatic β-cell function after sleeve gastrectomy in obese rats

Article

Méndez-Giménez L, Becerril S, Camões SP, Vieira da Silva I, Rodrigues C, Moncada R, Valentí V, Catalán V, Gómez-Ambrosi J, Miranda JP, Soveral G, Frühbeck G, Rodríguez A.

Role of aquaporin-7 in ghrelin- and GLP-1-induced improvement of pancreatic β-cell function after sleeve gastrectomy in obese rats.

Int J Obes 2017; (*in press*).

Hypothesis

Pancreatic AQP7 and AQP12 are involved in the beneficial effect of sleeve gastrectomy on the improvement of glucose tolerance and β-cell function, and steatosis in diet-induced obese rats.

Objectives

- To study the effect of sleeve gastrectomy on glucose tolerance, β-cell mass, apoptosis and steatosis in diet-induced obese rats.
- To analyze the impact of obesity and weight loss achieved by sleeve gastrectomy and pair-feeding on the expression of AQP7 and AQP12 in the rat pancreas.
- To evaluate the correlation of pancreatic AQP7 and AQP12 with markers of glucose metabolism, β-cell function and pancreatic steatosis.
- To determine the water and glycerol permeability of rat RIN-m5F β-cells.
- To investigate the effect of acylated and desacyl ghrelin as well as GLP-1 (9-36) on insulin release, intracellular TG content and expression of AQP7 and AQP12 in rat RIN-m5F β-cells.

Universidad de Navarra

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Final Decision made for 2017IJO00417R

1 mensaje

ijo@nature.com <ijo@nature.com> 26 de mayo de 2017, 12:18
Responder a: ijo@nature.com
Para: arodmur@unav.es

Dear Dr Rodríguez:

Here is a copy of the decision letter for manuscript "Dissociation of body mass index, excess weight loss and body fat percentage trajectories after 3 years of gastric bypass: Relationship with metabolic outcomes" by Javier Gomez-Ambrosi, Patricia Andrada, Victor Valenti, Fernando Rotellar, Camilo Silva, Victoria Catalan, Amaia Rodríguez, Beatriz Ramirez, Rafael Moncada, Javier Escalada, Javier Salvador, and Gema Fruhbeck [Paper #2017IJO00417R], which you were a Contributing Author.

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Sincerely,

Editor
International Journal of Obesity
http://www.nature.com/ijo

Subject: 2017IJO00417R Decision Letter

Manuscript Number: 2017IJO00417R
Title: Dissociation of body mass index, excess weight loss and body fat percentage trajectories after 3 years of gastric bypass: Relationship with metabolic outcomes
Authors: Javier Gomez-Ambrosi, Patricia Andrada, Victor Valenti, Fernando Rotellar, Camilo Silva, Victoria Catalan, Amaia Rodríguez, Beatriz Ramirez, Rafael Moncada, Javier Escalada, Javier Salvador, and Gema Fruhbeck

Dear Dr Gomez-Ambrosi,

I am very pleased to inform you that your above mentioned manuscript has now been accepted for publication in the *International Journal of Obesity*.

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ORIGINAL ARTICLE

Role of aquaporin-7 in ghrelin- and GLP-1-induced improvement of pancreatic β-cell function after sleeve gastrectomy in obese rats

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Running title: Changes in aquaporins in β-cells after bariatric surgery.

Word count: Abstract (251); Main text (4,324); References (59)

Abbreviations: AQP, aquaporin; AUC, area under the curve; FFA, free fatty acids; GIP, gastric inhibitory polypeptide; GK, glycerol kinase; GLP-1, glucagon-like peptide-1; HFD, high-fat diet; HOMA, homeostasis model assessment; IPITT, intraperitoneal insulin tolerance test; ND, normal diet; OGTT, oral glucose tolerance test; P_f, water permeability; P_{gly}, glycerol permeability; QUICKI, quantitative insulin sensitivity check index; TG, triacylglycerol; VRAC, volume-regulated anion channel.

Disclosure statement: The authors have nothing to disclose.

Funding: This work was supported by Fondo de Investigación Sanitaria-FEDER (FIS PI12/00515, PI13/01430, PI16/00221 and PI16/01217) from the Spanish Instituto de Salud Carlos III, as well as by the Department of Health of Gobierno de Navarra (61/2014). CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN) is an initiative of the Instituto de Salud Carlos III, Spain.

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ABSTRACT

BACKGROUND/OBJECTIVES: Glycerol is a key metabolite for lipid accumulation in insulin-sensitive tissues as well as for pancreatic insulin secretion. We examined the role of aquaporin-7 (AQP7), the main glycerol channel in β-cells, and AQP12, a aquaporin related to pancreatic damage, in the improvement of pancreatic function and steatosis after sleeve gastrectomy in diet-induced obese rats.

SUBJECTS/METHODS: Male Wistar obese rats (n=125) were subjected to surgical (sham operation and sleeve gastrectomy) or dietary (pair-fed to the amount of food eaten by sleeve-gastrectomized animals) interventions. The tissue distribution and expression of AQPs in rat pancreas were analyzed by real-time PCR, Western-blot and immunohistochemistry. The effect of ghrelin isoforms and GLP-1 on insulin secretion, triacylglycerol accumulation and AQP expression was determined *in vitro* in RIN-m5F β-cells.

RESULTS: Sleeve gastrectomy reduced pancreatic β-cell apoptosis, steatosis and insulin secretion. Lower ghrelin and higher GLP-1 concentrations were also found after bariatric surgery. Acylated and desacyl ghrelin increased triacylglycerol content, whereas GLP-1 increased insulin release in RIN-m5F β-cells. Sleeve gastrectomy was associated with an upregulation of AQP7 together with a normalization of the increased AQP12 levels in rat pancreas. Interestingly, ghrelin and GLP-1 repressed AQP7 and AQP12 expression in RIN-m5F β-cells. AQP7 protein was negatively correlated with intracellular lipid accumulation in acylated ghrelin-treated cells and with insulin release in GLP-1-stimulated β-cells.

CONCLUSIONS: AQP7 upregulation in β-cells after sleeve gastrectomy contributes, in part, to the improvement of pancreatic steatosis and insulin secretion by increasing intracellular glycerol used for insulin release triggered by GLP-1, rather than for ghrelin-induced triacylglycerol biosynthesis.

Keywords: Aquaporins ● Obesity ● Pancreas steatosis ● Insulin secretion ● Bariatric surgery.

INTRODUCTION

Pancreatic β-cell function is influenced by changes in cell volume, which depend on water permeability of the plasma membrane, conferred in part by aquaporins (AQPs).[1] This integral membrane protein superfamily facilitates the transport of water and other small solutes, such as glycerol and urea, across the biological membranes.[2] Thirteen mammalian AQPs have been characterized to date, which are divided in three subfamilies based on their structural and functional properties: orthodox aquaporins (AQP0, 1, 2, 4, 5, 6 and 8), aquaglyceroporins (AQP3, 7, 9 and 10) and superaquaporins (AQP11 and 12).[3-5] AQP7 is the main glycerol channel in the pancreas and mediates the rapid entry of extracellular glycerol into β-cells.[1, 6, 7] Glycerol uptake is followed by β-cell swelling, activation of the volume-regulated anion channel (VRAC) and insulin release.[1] Glycerol kinase (GK) catalyzes the enzymatic reaction leading to the phosphorylation of glycerol to glycerol-3-phosphate, which is used as a substrate in the glycerol-3-phosphate shuttle, a process that reduces equivalents into mitochondria for use in oxidative phosphorylation and ATP production.[4, 6, 8] The increase in the cytosolic ADP:ATP ratio triggers the closure of ATP-sensitive K^+ channels, cell membrane depolarization and opening of voltage-sensitive Ca^{2+} channels, ultimately leading to the exocytosis of insulin-containing granules and insulin secretion.[1, 9, 10] Interestingly, *Aqp7*-deficient mice develop adult-onset obesity, insulin resistance and hyperinsulinemia.[11] In this regard, *Aqp7*-knockout mice show increased β-cell glycerol content and GK activity, which results in higher basal and glucose-induced insulin secretion, as well as reduced β-cell mass, indicating a more efficient insulin biosynthesis and secretion.[6, 11] On the other hand, AQP12 (originally named AQPX2) is expressed in pancreatic acinar cells and localizes on the membrane of intracellular organelles.[12, 13] The intracellular localization of this superaquaporin in pancreatic acinar cells suggests a potential role in the maturation and exocytosis of zymogen granule. However, its potential expression and function in β-cells remains unknown.

Glycerol represents an important metabolite as a substrate for *de novo* synthesis of triacylglycerols (TG) as well as glucose during fasting.[14] In this sense, the control of glycerol influx/efflux in metabolic organs, such as adipose tissue, liver and skeletal muscle, by aquaglyceroporins is important since the dysregulation of these glycerol channels is associated with metabolic diseases, such as obesity, insulin resistance and non-alcoholic fatty liver disease.[15-19] Our group has previously described that sleeve

gastrectomy restores the coordinated regulation of expression of aquaglyceroporins in adipose tissue and liver, playing a crucial role in the control of adipose and hepatic TG accumulation as well as in glucose homeostasis.[20] This bariatric surgery technique produces significant weight loss in both humans[21, 22] and rodents[20, 23] as well as a remission of β-cell dysfunction and insulin resistance.[22]

The impact of obesity and surgically-induced weight loss in pancreatic aquaporins has not yet been elucidated. Thus, our aim was to investigate the potential role of pancreatic AQP7 and AQP12 in the beneficial effect of sleeve gastrectomy on β-cell mass and steatosis in diet-induced obese rats. Since changes in gut hormones ghrelin and GLP-1 play a relevant role in for the remission of type 2 diabetes after sleeve gastrectomy,[24, 25] we analyzed the direct effect of the main isoforms of ghrelin (acylated and desacyl ghrelin) and GLP-1 on insulin release, intracellular TG accumulation and expression of AQP7 and AQP12 in rat RIN-m5F β-cells.

MATERIAL AND METHODS

Experimental animals and study design

Four-week-old male Wistar rats (n=125) were fed *ad libitum* either a normal diet (ND) (n=25) (12.1 kJ/g: 4% fat, 82% carbohydrate and 14% protein, diet 2014S, Harlan, Teklad Global Diets, Harlan Laboratories Inc., Barcelona, Spain) or a high-fat diet (HFD) (n=100) (23.0 kJ/g: 59% fat, 27% carbohydrate and 14% protein, diet F3282, Bio-Serv, Frenchtown, NJ, USA). Body weight and food intake were registered regularly to follow up the progression of diet-induced obesity. Anesthesia, sleeve gastrectomy (n=26) and sham surgery (n=27) were performed in weight-matched obese rats in a blind, randomized study according to previously described methodology.[26] After surgical interventions, animals were fed a ND. Another group of obese rats was pair-fed with ND (n=23) with the same amount of food eaten by sleeve-gastrectomized animals in order to discern the effects attributable solely to the decrease in food intake after bariatric surgery. Four weeks after the interventions, rats were sacrificed by decapitation and pancreas and blood samples were collected. A small portion of the pancreas was fixed in 4% paraformaldehyde for histological analyses. All experimental procedures conformed to the European Guidelines for the care and use of Laboratory Animals (directive 2010/63/EU) and were approved by the Ethical Committee for Animal Experimentation of the University of Navarra (049/10).

Blood and tissue analysis

Biochemical and hormonal assays were performed in sera samples as earlier described.[20, 23] Total ghrelin levels (#EZRGRT-91 K, Millipore, Billerica, MA, USA), GLP-1 (AKMGP-011, Shibayagi Co., Ltd., Japan) and GIP (#EZRMGIP-55K, Millipore) were evaluated by ELISA with inter- and intra-assay coefficients of variation being 0.8% and 2.8% (ghrelin), <5% and <5% (GLP-1) and 3.7% and 2.6% (GIP), respectively. The adipocyte insulin resistance (Adipo-IR) index, was also assessed.[18]

Oral glucose tolerance test and insulin peritoneal tolerance test

After a 12-h fasting period, oral glucose (OGTT) and insulin intraperitoneal (IPITT) tolerance tests were performed as previously described.[27] Glucose concentrations were measured before and 15, 30, 60, 90 and 120 min after the oral glucose challenge (2 g/kg of body weight) or intraperitoneal insulin administration (0.15

IU/mL) with an automatic glucose sensor (Ascensia Elite, Bayer, Barcelona, Spain) from whole blood obtained from the tail vein. The area under the curve (AUC) of blood glucose levels during OGTT and IPITT was calculated by the trapezoidal method.

RNA isolation and real-time PCR

RNA isolation and purification were performed as previously described.[20] Transcript levels of *Aqp3, Aqp7, Aqp9,* and *Aqp12* were quantified by real-time PCR (7300 Real Time PCR System, Applied Biosystems, Foster City, CA, USA). Primers and probes (**Supplemental Table 1**) were designed using the software Primer Express 2.0 (Applied Biosystems) and acquired from Genosys (Sigma, St. Louis, MO, USA). All results were normalized for the expression of *18S* rRNA (Applied Biosystems), and relative quantification was calculated as fold expression over the calibrator sample.[17]

Western-blot studies

Blots were incubated overnight at 4 ºC with rabbit polyclonal anti-AQP7 (sc-28625, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-AQP12 (CPA2913100, Acris antibodies, London, UK) antibodies or murine monoclonal anti-β-actin antibody (A5441, Sigma) diluted 1:1,000 (AQP7), 1:500 (AQP12) or 1:5,000 (β-actin) in blocking solution. The antigen-antibody complexes were detected using HRP-conjugated anti-rabbit or anti-mouse IgG antibodies and the enhanced chemiluminescence ECL Plus system (Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry of AQP7 and AQP12 in the pancreas

The immunodetection of AQP7 and AQP12 in histological sections of the pancreas was performed by an indirect immunoperoxidase method,[28] using rabbit polyclonal anti-AQP7 (sc28625, Santa Cruz Biotechnology, Inc.) and anti-AQP12 (CPA2913100, Acris) antibodies (both diluted 1:100).

Imaging and quantification of β-cell area and number

Insulin immunohistochemistry was performed to identify β-cells in the pancreas by using guinea pig polyclonal anti-insulin antibody (A0564, Dako, Golstrup, Denmark) diluted 1:100.[29] Images of insulin-positive pancreatic β-cells in Langerhans islets were captured in all fields from each animal with the 20X objective, and their area and number were assessed using the software AxioVision Release 4.6.3 (Zeiss, Göttingen, Germany).

TUNEL assay

The apoptosis of β-cells was analyzed by TUNEL in histological sections of the pancreas with the In Situ Cell Death Detection Kit, POD (11684817910, Roche, Basel, Switzerland).

Cell cultures

RIN-m5F rat insulinoma β-cells (CRL-11605, ATCC, Manassas, VA, USA) were cultured in ATCC-formulated RPMI 1640 medium with 10% fetal bovine serum (FBS) and antibiotic-antimycotic. Cells were serum-starved for 24 h and then stimulated with increasing concentrations of acylated or desacyl ghrelin (Tocris, Ellisville, MO, USA) or GLP-1 (9-36) (Bachem, Bubendorf, Switzerland) for 24 h. Insulin release was determined by ELISA (Crystal Chem, Inc., Chicago, IL, USA) and intracelullar TG content was measured as previously described.[30]

Permeability assays

Water (P_f) and glycerol (P_{gly}) permeability were measured in RIN-m5F β-cells, as previously described.[31, 32] *Pf* and *Pgly* coefficients were calculated using the model equations described by Madeira and colleagues[33] and the Berkeley Madonna software (http://www.berkeleymadonna.com/).

Statistical analysis

Data are expressed as the mean ± SEM. The PS Power and Sample Size Calculations software (edition 3.0.43) was used to determine the power of the study and sample size calculation. Statistical differences between mean values were analyzed using Student's *t* test or one-way ANOVA followed by Tukey's or Dunnett's *post-hoc* tests, where appropriate. Kruskal-Wallis followed by *U* Mann-Whiney was performed for a sample size less than 10. Pearson's correlation coefficients (r) were used to analyze the association between variables. The statistical analyses were performed using the SPSS/Windows 15.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Effect of sleeve gastrectomy on pancreatic β-cell mass and steatosis in obese rats

Obese rats exhibited higher body weight and whole-body adiposity, and sleeve gastrectomy surgery improved these parameters to a higher extent than pair-feeding, as previously reported.[20] Diet-induced obesity was associated with insulin resistance, as evidenced by higher (P<0.001) fasting glycemia, insulinemia, HOMA and adipo-IR indices (**Supplemental Table 2**) as well as increased blood glucose levels (P<0.05) and higher AUC (P<0.0001) during the OGTT and IPITT compared to control lean rats (**Supplemental Fig. 1**). Four weeks after surgical interventions, rats submitted to sleeve gastrectomy exhibited a decrease in fasting glycemia and improvement in the QUICKI and adipo-IR indices (**Supplemental Table 3**) as well as lower blood glucose levels and AUC during the OGTT and IPITT (**Supplemental Fig. 2**) compared to the sham-operated and pair-fed groups.

To gain further insight into the improved glucose metabolism after sleeve gastrectomy, we investigated the pancreatic β-cell mass and steatosis of the experimental animals. Representative images of insulin staining in the pancreas are illustrated in **Fig. 1a-b**. Quantitative analysis of β-cell mass revealed that obese rats exhibited a 40% decrease in islet density (P<0.05), (**Fig. 1d**) without changes in β-cell area and apoptosis (**Fig. 1c and 1e**). No statistically significant changes were observed in the β-cell area and number after sleeve gastrectomy (**Fig. 1f-g**). However, a significant reduction (P<0.05) in β-cell apoptosis was observed after sleeve gastrectomy (**Fig. 1h**). Obese rats showed an increase (P<0.05) in pancreatic steatosis, which was completely reversed (P<0.05) by sleeve gastrectomy (**Fig. 1i-j**). Nonetheless, no differences between the intrapancreatic TG content in sleeve gastrectomy and pair-fed groups were found (P=0.440). A positive correlation of pancreatic fat accumulation with β-cell apoptosis was found (r=0.33, P<0.05).

Obesity and weight loss achieved by sleeve gastrectomy were associated with an increased pancreatic AQP7 expression

To analyze the potential involvement of aquaglyceroporins in the changes in pancreatic β-cell mass and steatosis after sleeve gastrectomy, we first evaluated the transcript levels of *Aqp3, Aqp7* and *Aqp9* in rat pancreas. Since *Aqp10* constitutes a pseudogene in rodents,[34] its expression was not included in this study. *Aqp7* was the

most abundantly expressed aquaglyceroporin, while the expression of *Aqp3* was undetectable and *Aqp9* was only detectable in 10% of the studied samples.

We next assessed the impact of obesity and weight loss achieved by sleeve gastrectomy and pair-feeding on the mRNA and protein expression levels of pancreatic AQP7 and AQP12, the other aquaporin with known functions in the pancreas. As illustrated in **Fig. 2a-b**, tissue distribution of AQP7 and AQP12 presented a predominant immunostaining in β-cells of the Langerhans islets. Obesity was associated with an increased gene and protein expression ($P<0.05$) of pancreatic AQP7 and AQP12 (**Fig. 2c-e**). Pancreatic transcript levels of *Aqp7* were negatively correlated with total ghrelin (r=-0.37, $P=0.005$). *Aqp12* mRNA was positively associated with insulin (r=0.46, $P=0.002$), HOMA (r=0.41, $P=0.006$), serum TG (r=0.23, $P=0.043$) and intrapancreatic TG content (r=0.49, $P<0.001$) while negatively correlated with QUICKI (r=-0.36, $P<0.001$). A significant increase ($P<0.05$) in the mRNA and protein levels of AQP7 in the pancreas was observed after sleeve gastrectomy compared to sham surgery. Sleeve-gastrectomized rats exhibited a tendency towards higher AQP12 mRNA and protein in the pancreas than sham-operated animals, but fell out of statistical significance (both $P>0.05$). No changes were observed in the pair-fed group, suggesting that changes in pancreatic AQP7 and AQP12 expression are beyond caloric restriction.

Effect of ghrelin and GLP-1 on insulin release and intracellular TG content in RIN-m5F β-cells

Since ghrelin, GIP and GLP-1 are important gut hormones mediating the amelioration of glucose metabolism after bariatric surgery,[35] the fasting circulating concentrations of these factors were evaluated in our experimental animals. Obese rats showed lower ($P<0.05$) total ghrelin, without significant changes in GIP and GLP-1 levels (**Supplemental Table 2**). Sleeve gastrectomy induced a remarkable reduction ($P<0.001$) in total ghrelin levels (**Fig. 3a**), lower ($P<0.05$) GIP values (**Fig. 3b**) as well as a tendency towards higher ($P=0.155$) GLP-1 concentrations (**Fig. 3c**) compared to the other groups.

We next analyzed the effect of acylated and desacyl ghrelin as well as GLP-1 on insulin release and intracellular lipid accumulation in rat RIN-m5F β-cells, a widely used cell line based on its high insulin secretion rate. The exposition of RIN-m5F β-cells to increasing concentrations of acylated or desacyl ghrelin for 24 h did not modify

insulin release (**Fig. 3d-e**), but stimulated (P<0.05) the intracellular TG content (**Fig. 3g-h**). The treatment with increasing concentrations of GLP-1 stimulated (P<0.05) insulin secretion (**Fig. 3f**) and diminished (P<0.05) the intracytoplasmatic lipid accumulation (**Fig. 3i**) in RIN-m5F β-cells at the concentrations of 10 and 100 nmol/L.

Acylated ghrelin as well as GLP-1 downregulated AQP7 and AQP12 expression in pancreatic β-cells

AQP7 facilitates glycerol transport in β-cells.[6] Thus, we first confirmed the water and glycerol permeability of the RIN-m5F β-cell line, a widely used cell line based on its high insulin secretion rate[36] (**Fig. 4a-b**). The time course of the relative cell volume change (V/V_0) of RIN-m5F β-cells exposed to an osmotic shock with mannitol, inducing water outflow and cell shrinkage, is shown in **Fig. 4a**. The osmotic water permeability coefficient (P_f) for this cell line was $(0.8\pm0.2)\text{x}10^{-3}$cm/s. On the other hand, glycerol permeability was evaluated by computing the time course of the cell volume change of cells subjected to an osmotic shock with glycerol (**Fig. 4b**). After the first shrinkage due to water outflow, the time course of cell re-swelling (V/V_0) due to facilitated glycerol influx gave a *Pgly* of $(1.9\pm1.6)\text{x}10^{-6}$ cm/s. These permeability values are within the range of the *Pf* and *Pgly* measured in mice mature 3T3-L1 adipocytes with endogenous AQP7 expression[33] and, thus, reflect the contribution of aquaporins in RIN-m5F β-cells for water and glycerol transport.

To establish the potential mechanism of action triggered by ghrelin isoforms and GLP-1 for regulating insulin release and lipid accumulation in β-cells, the expression of AQP7 and AQP12 induced by these gut hormones was evaluated in RIN-m5F β-cells (**Fig. 4c-f**). The 24-h exposure of RIN-m5F β-cells to acylated ghrelin (**Fig. 4c-d**) and GLP-1 (**Fig. 4g-h**) downregulated AQP7 and AQP12 mRNA and protein, whereas the stimulation with desacyl ghrelin did not modify the expression of these aquaporins (**Fig. 4e-f**). The stimulation of RIN-m5F β-cells with GLP-1 did not result in a uniform concentration-dependent decrease in AQP12 mRNA expression, especially at the highest concentration (**Fig. 4g**). In this regard, it has been reported that prolonged exposure of INS-1 β-cell line to the GLP-1 receptor agonist exendin-4 induces GLP-1 receptor internalization and desensitization.[37] Interestingly, the protein expression of AQP7 was negatively associated with intracellular lipid accumulation (r=-0.43, P=0.023) in β-cells stimulated with acylated ghrelin as well as with insulin release (r=-

0.46, P=0.025) in β-cells treated with GLP-1. No correlation between AQP12 protein and these parameters was detected (all P>0.05).

DISCUSSION

Obesity-associated insulin resistance and the related compensatory hyperinsulinemia have been attributed to ectopic lipid overload.[38] The overload of FFA into the pancreas promotes β-cell hypertrophy and insulin hypersecretion, ultimately causing β-cell dysfunction and death through lipoapoptosis.[39] In line with this observation, our results showed that hyperinsulinemic, insulin-resistant obese rats showed a marked decrease in Langerhans islet number. The herein observed positive association of pancreatic fat accumulation and β-cell apoptosis highlights the relevance of lipotoxicity on β-cell dysfunction. Sleeve gastrectomy restored insulin sensitivity, as evidenced by lower basal glucose levels and AUC during the OGTT and IPITT as well as a higher QUICKI index, which is in accordance with several studies,[40, 41] including ours.[20, 23] Furthermore, this bariatric procedure improved β-cell dysfunction in obese rats, as evidenced by a decrease in β-cell apoptosis as well as improved insulin sensitivity in the fasted state. Interestingly, obesity-associated pancreatic steatosis was ameliorated after sleeve gastrectomy, which is in agreement with other studies,[42] but also after caloric restriction in the pair-fed group, suggesting that the decreased fat accumulation in the pancreas is mediated by the beneficial effects of lower energy intake. Thus, sleeve gastrectomy improves β-cell mass, apoptosis and steatosis contributing to the amelioration of insulin secretion and sensitivity after surgery.

The hormonal changes underlying the improved β-cell function after sleeve gastrectomy have not been completely unraveled. The incretin hormones GLP-1 and GIP are among the most widely studied modulators of β-cell function,[43] with the incretinic effect accounting for 70% of the insulin secretion after an OGTT.[44] GLP-1 is produced and secreted by L-cells of the small intestine in response to nutrient ingestion, whereas GIP is synthesized and released from K-cells and stimulates both insulin and glucagon secretion in the pancreas.[43] At the endocrine pancreas, GLP-1 binds its receptor GLP-1R and suppresses glucagon secretion from α-cells and potentiates insulin secretion from β-cells in a glucose-dependent manner. On the other hand, ghrelin is secreted from X/A-like cells of the oxyntic glands in the gastric fundus mucosa.[45] The clear preprandial rise and a postprandial fall of circulating ghrelin supports its role in meal initiation.[46] In the pancreas, ghrelin acts as a survival factor promoting cell survival *in vitro* in HIT-T15 pancreatic β-cells[47] and *in vivo* in streptozotocin-induced

diabetic mice.[48] In the present study, a dramatic reduction of circulating ghrelin levels, increased GLP-1 concentrations and no effect on plasma GIP was observed after sleeve gastrectomy, which is in agreement with other studies.[23, 41, 49] In RIN-m5F β-cells, GLP-1 promoted insulin secretion and reduced intracellular TG content. By contrast, we herein show, for the first time, that acylated and desacyl ghrelin induce intracellular lipid accumulation in RIN-m5F β-cells, which is in agreement with the lipogenic effect of ghrelin isoforms in other metabolic tissues, including the adipose tissue[30] and the liver.[50] Interestingly, the highest increase in intracellular TG accumulation was observed in RIN-m5F β-cells treated with the lower concentration of desacyl ghrelin. A plausible explanation for this finding may be that ghrelin downregulates its own receptor in several cell types, such as chicken and porcine pituitary somatotropes,[51] rat hypothalamic neurons[52] or human visceral adipocytes.[30] The pancreatic β-cells express the classical ghrelin receptor GHS-R 1a.[53] Nonetheless, several authors have proposed that when the biological actions induced by ghrelin are equally elicited by desacyl ghrelin, these effects cannot be mediated by GHS-R1a, because desacyl ghrelin is reportedly inactive on GHS-R1a.[54-56] Thus, further studies are warranted in order to disentangle the potential existence of an alternative ghrelin receptor in pancreatic β-cells. Our results also showed that neither desacyl nor acylated ghrelin modified insulin secretion in RIN-m5F β-cells. Similar results were found by Bando and colleagues in transgenic mice with overexpression of intraislet ghrelin and without changes in insulin secretion *in vivo*.[57] Taken together, the increased GLP-1 levels after sleeve gastrectomy might be mainly related to the improvement of insulin secretion, whereas reduced ghrelin levels appears to be responsible of the amelioration of pancreatic steatosis after surgery. The contribution of additional signals involved in β-cell function cannot be ruled out.

To gain further insight into the molecular mechanisms triggering the improvement of β-cell function, the role of ghrelin and GLP-1 in the expression of pancreatic AQP7 and AQP12 was studied. AQP7 facilitates glycerol influx into β-cells leading to insulin synthesis and exocytosis as well as TG synthesis.[6, 58] In this regard, transgenic *Aqp7*-deficient mice exhibit hyperinsulinemia and increased pancreatic insulin-1 and insulin-2 mRNA levels as well as increased intraislet glycerol and TG content.[6] Although AQP7 is the main aquaglyceroporin in the pancreas,[6, 58] our data provides evidence for the additional presence of AQP9 glycerol channel. In fact, we

have previously found[30] that acylated and desacyl ghrelin constitute negative regulators of AQP7 in adipocytes and this downregulation contributes, in part, to the lipid accumulation in fat cells. Accordingly, we found that both ghrelin isoforms diminished AQP7 expression in parallel to an increased TG content in RIN-m5F β-cells. Interestingly, GLP-1 tended to repress AQP7 expression in RIN-m5F β-cells with AQP7 protein expression being negatively associated with insulin release. Thus, it seems plausible that the reduction of AQP7 induced by ghrelin and GLP-1 might result in intracellular glycerol accumulation, which can be used for the biosynthesis of TG, as well as for insulin synthesis and secretion. Obesity and obesity-associated insulin resistance are associated with an altered transcription of AQP7 in insulin-sensitive tissues, such as adipose tissue,[15-17, 28] liver[18, 28, 59] and skeletal muscle.[19] Interestingly, sleeve gastrectomy restores the altered expression of aquaglyceroporins in epididymal and subcutaneous fat depots and liver in parallel to the improvement of whole-body adiposity and non-alcoholic fatty liver.[20] To the best of our knowledge, this is the first study describing changes in AQP7 after weight gain and weight loss in the pancreas. Importantly, both weight gain and weight loss achieved by sleeve gastrectomy were related with higher AQP7 mRNA and protein in the pancreas. The upregulation of AQP7 might constitute an adaptive response of β-cells to increase glycerol uptake and the subsequent insulin synthesis and secretion, which seems nevertheless inefficient to improve the enhanced glucose levels in the obese state, but not after bariatric surgery. This beneficial effect of sleeve gastrectomy is beyond caloric restriction, since no effects of pair-feeding on AQP7 expression in the pancreas were observed.

On the other hand, AQP12 is reportedly expressed in the rough endoplasmic reticulum (RER) and on the membranes of zymogen granules near the RER of the pancreatic acinar cells.[13] This subcellular location supports the potential role of AQP12 in the proper maturation and exocytosis of zymogen granules. Our results show that AQP12 is also expressed in β-cells of the Langerhans islets based on the immunohistochemical staining in histological sections of rat pancreas as well as by the mRNA and protein expression data in RIN-m5F β-cells and samples of rat pancreas. Interestingly, acylated ghrelin- and GLP-1-induced AQP12 downregulation in RIN-m5F cell line was neither related to insulin release nor to TG accumulation pointing to other functions of AQP12 in β-cells. In this regard, the increased pancreatic expression of AQP12 together with the positive association between this superaquaporin with markers

of insulin resistance (insulinemia and HOMA) and ectopic lipid overload (serum TG and intrapancreatic TG content) suggest that AQP12 might constitute a marker of pancreatic damage. In this sense, *Aqp12*-deficient mice are more susceptible to caerulein-induced acute pancreatitis, showing larger exocytic vesicles (vacuoles) in the pancreatic acini.[12] The normalization of pancreatic AQP12 expression after sleeve gastrectomy might reflect the restoration of pancreatic function due to the reduction of intrapancreatic steatosis and improved insulin secretion.

We herein report, for the first time, that sleeve gastrectomy restores the altered expression of pancreas-specific AQP7 and AQP12 in obese rats contributing to the prevention of steatosis and impaired insulin secretion in the pancreas. Our results identify these aquaporins as important elements in mediating part of the beneficial effects of bariatric surgery on glucose metabolism via the regulation of glycerol biodisponibility, a key metabolite for pancreatic insulin production and secretion as well as TG accumulation. In line with this observation, ghrelin and GLP-1, two important hormones involved in the resolution of insulin resistance after bariatric surgery, regulate the expression of these aquaporins in pancreatic β-cells. Further investigations are required to establish the suitability of pancreatic AQP7 and AQP12 as therapeutic targets for human obesity-associated type 2 diabetes.

ACKNOWLEDGMENTS

We thank Beatriz Ramírez (Metabolic Research Laboratory, Clínica Universidad de Navarra) for technical assistance as well as all the staff of the breeding house of the University of Navarra.

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FIGURE LEGENDS

Fig. 1. Effect of obesity and sleeve gastrectomy-induced weight loss on rat β-cell mass and steatosis. Representative images of insulin immunostaining in pancreas of control lean and obese rats (a) as well as obese animals after surgical and dietary interventions (b), magnification 200X, *scale bar*=200 μm. Bar graphs illustrate the impact of obesity and weight loss achieved by sleeve gastrectomy in β-cell area (c, f), number (d, g) and apoptosis (e, h) as well as in intrapancreatic triacylglycerol content (i, j). Statistical differences were analyzed using Student's *t* test or Kruskal-Wallis followed by *U* Mann Whitney's test, where appropriate.*P<0.05; **P<0.01; ***P<0.001 *vs* lean control rats or sham-operated group.

Fig. 2. Impact of obesity and sleeve gastrectomy-induced weight loss on AQP7 and AQP12 expression in rat pancreas. Immunohistochemical detection of AQP7 (*upper panels*) and AQP12 (*lower panels*) in rat pancreas of lean and obese rats (a) as well as four weeks after surgical and dietary interventions (b), magnification 100X, *scale bar*=200 μm. Pancreatic gene (c, d) and protein (e, f) expression of AQP7 and AQP12 in experimental animals is shown. Statistical differences were analyzed using Student's *t* test or Kruskal-Wallis followed by *U* Mann Whitney's test, where appropriate. *P<0.05 *vs* lean control rats or sham-operated group.

Fig. 3. Effect of acylated and desacyl ghrelin and GLP-1 on insulin release and intracellular lipid accumulation in rat RIN-m5F β-cells. Post-surgical serum total ghrelin (a), GIP (b) and GLP-1 (c) levels of the experimental animals after surgical and dietary interventions. RIN-m5F β-cells were treated with acylated ghrelin (d, g), desacyl ghrelin (e, h) or GLP-1 (f, i) at indicated concentrations for 24 h. Insulin release and intracellular triacylglycerol content were measured (n=9-10 per concentration). Statistical differences were analyzed using a one-way ANOVA followed by Tukey's or Dunnett's *post-hoc* test, where appropriate. *P<0.05. *P<0.01 *vs* sham-operated group or unstimulated cells.

Fig. 4. Effect of acylated and desacyl ghrelin and GLP-1 on AQP7 and AQP12 expression in rat RIN-m5F β-cells. Functional assessment of water (a) and glycerol (b) permeability in rat RIN-m5F β-cells (n=3) is shown. Bar graphs

illustrate gene and protein expression of AQP7 and AQP12 in rat RIN-m5F β-cells treated with acylated ghrelin (c, d), desacyl-ghrelin (e, f) and GLP-1 (g, h) at indicated concentrations (n=9-10 per concentration). Statistical differences were analyzed using a one-way ANOVA followed by Dunnett's *post-hoc* test. $P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *vs* unstimulated cells.

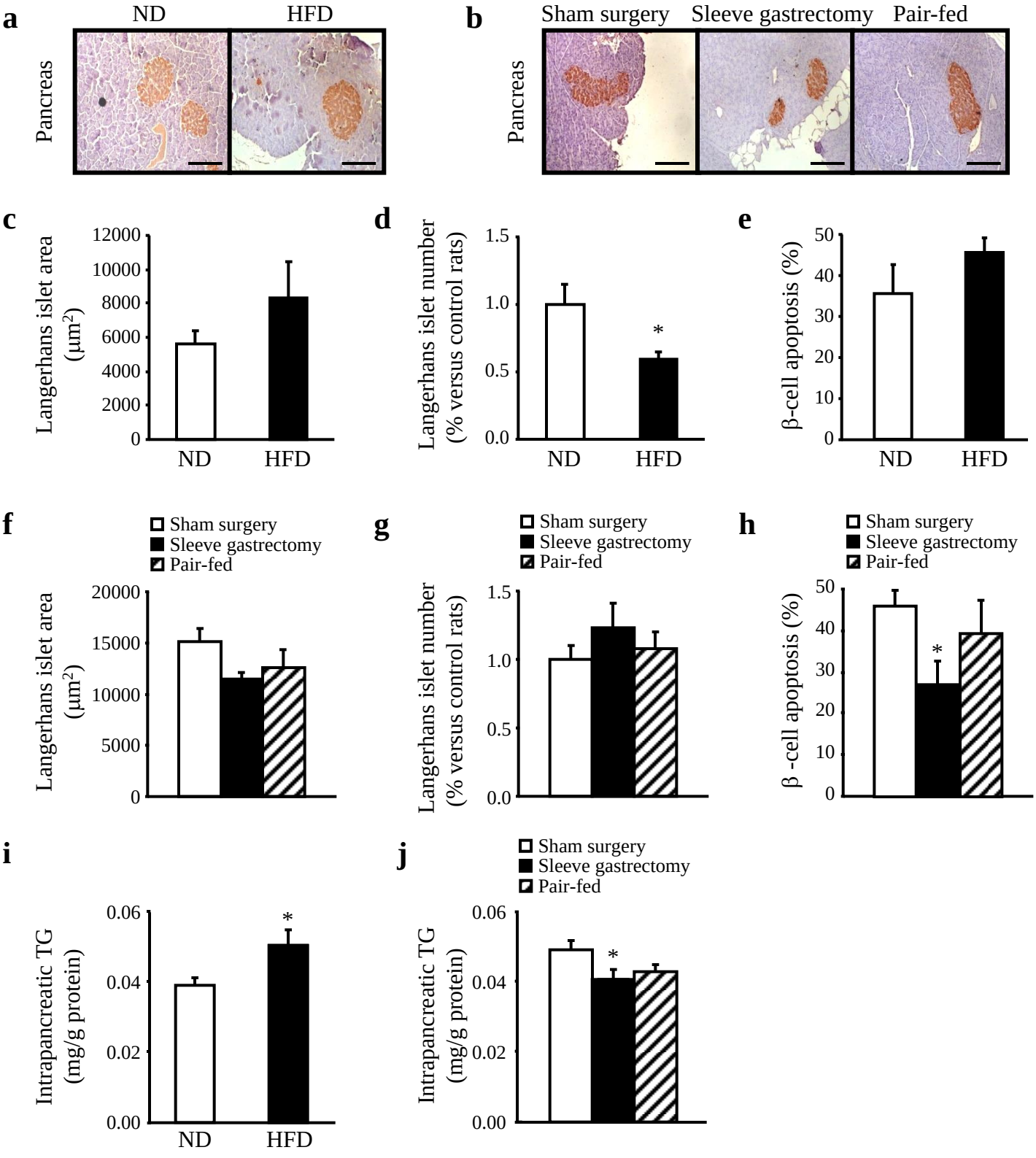

a
ND
HFD
Pancreas
b
Sham surgery
Sleeve gastrectomy
Pair-fed
Pancreas
c
Langerhans islet area (μm²)
12000
10000
8000
6000
4000
2000
0
ND
HFD
d
Langerhans islet number (% versus control rats)
1.5
1.0
0.5
0.0
*
ND
HFD
e
β-cell apoptosis (%)
50
40
30
20
10
0
ND
HFD
f
Sham surgery
Sleeve gastrectomy
Pair-fed
Langerhans islet area (μm²)
20000
15000
10000
5000
0
g
Sham surgery
Sleeve gastrectomy
Pair-fed
Langerhans islet number (% versus control rats)
1.5
1.0
0.5
0.0
h
Sham surgery
Sleeve gastrectomy
Pair-fed
β -cell apoptosis (%)
50
40
30
20
10
0
*
i
Intrapancreatic TG (mg/g protein)
0.06
0.04
0.02
0.00
*
ND
HFD
j
Sham surgery
Sleeve gastrectomy
Pair-fed
Intrapancreatic TG (mg/g protein)
0.06
0.04
0.02
0.00
*

Fig.1

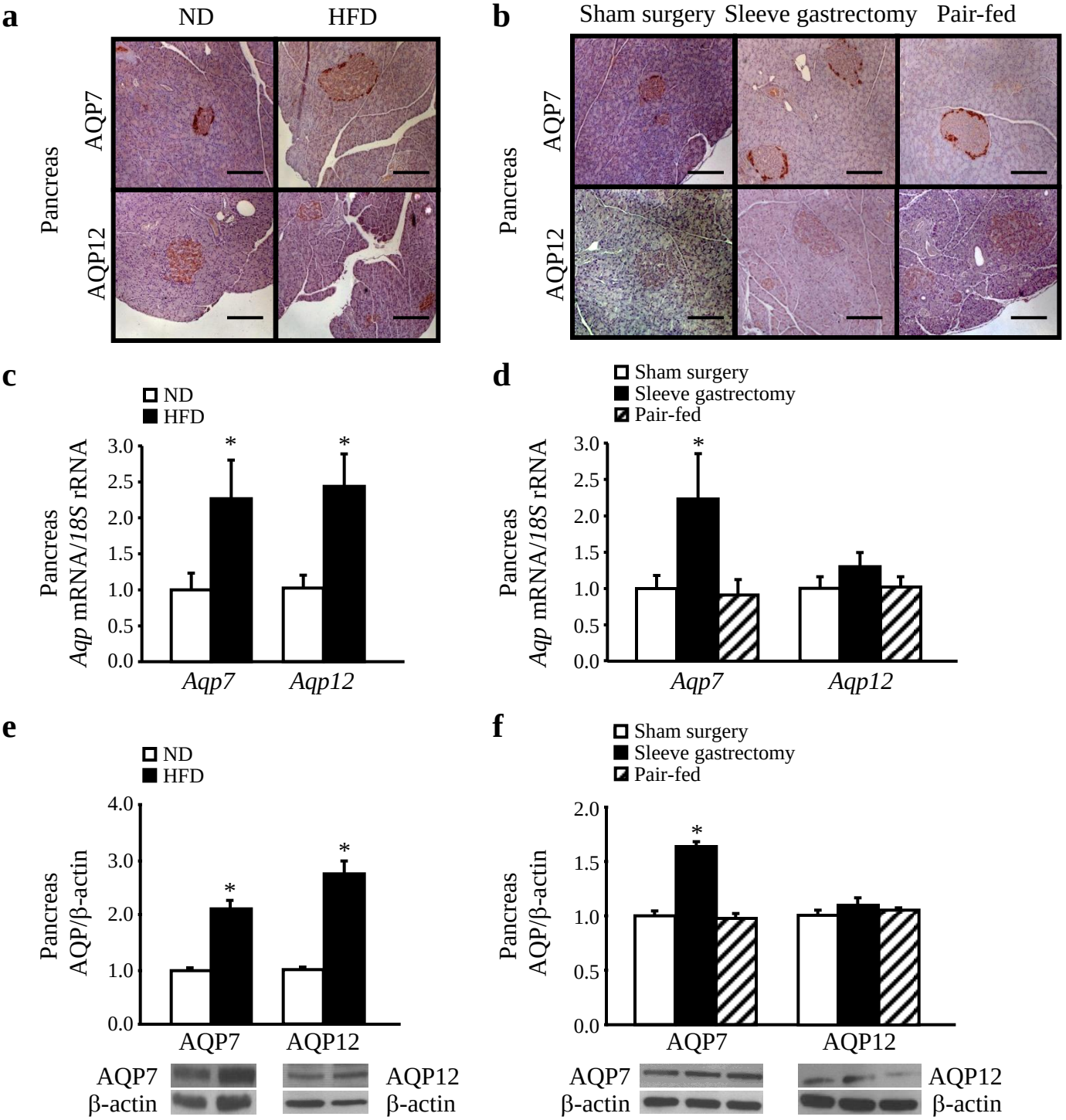

a
ND
HFD
Pancreas
AQP7
AQP12
b
Sham surgery
Sleeve gastrectomy
Pair-fed
Pancreas
AQP7
AQP12
c
ND
HFD
Pancreas
Aqp mRNA/18S rRNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Aqp7
Aqp12
d
Sham surgery
Sleeve gastrectomy
Pair-fed
Pancreas
Aqp mRNA/18S rRNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Aqp7
Aqp12
e
ND
HFD
Pancreas
AQP/β-actin
4.0
3.0
2.0
1.0
0.0
*
*
AQP7
AQP12
AQP7
β-actin
AQP12
β-actin
f
Sham surgery
Sleeve gastrectomy
Pair-fed
Pancreas
AQP/β-actin
2.0
1.5
1.0
0.5
0.0
*
AQP7
AQP12
AQP7
β-actin
AQP12
β-actin

Fig.2

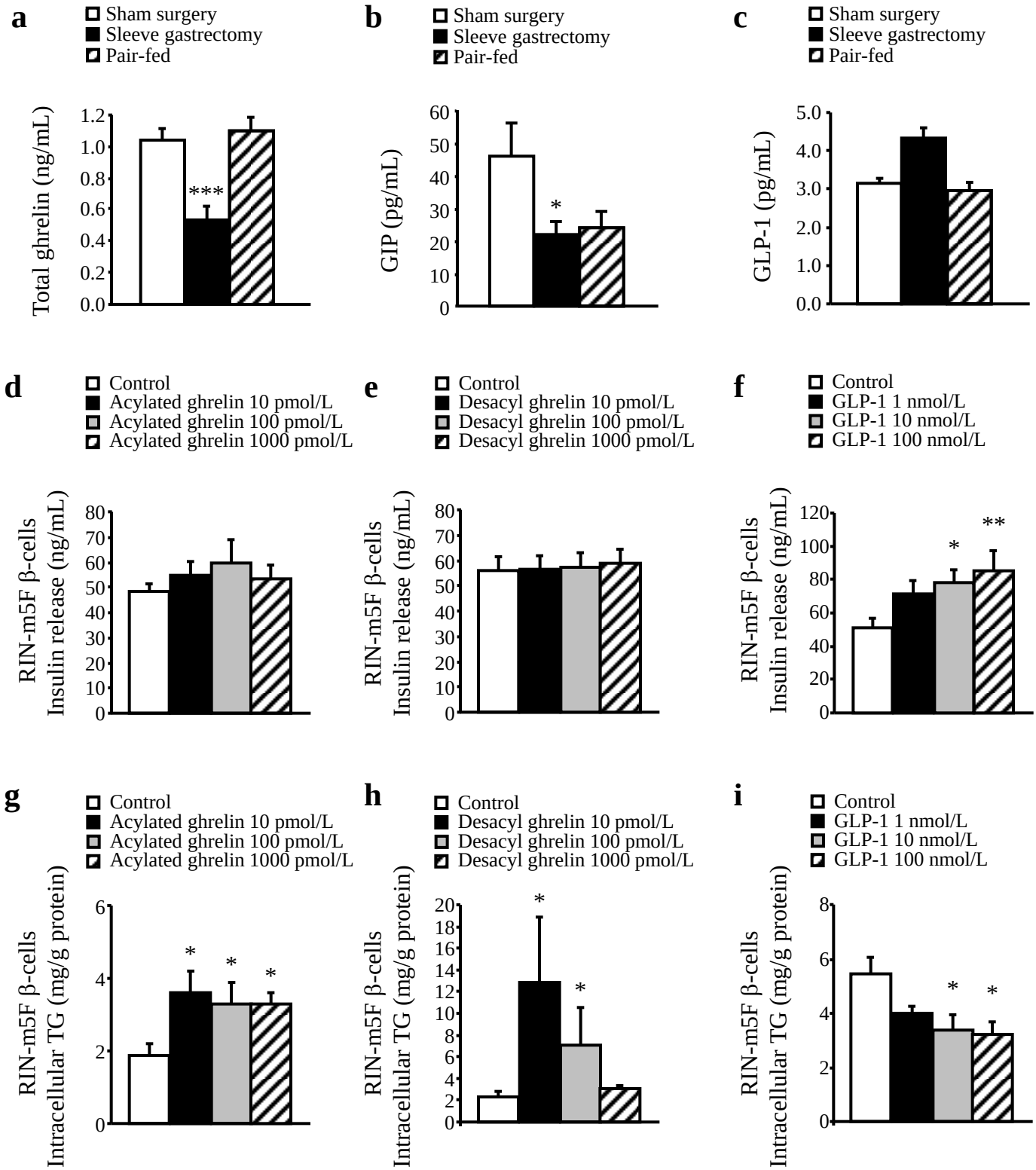

a
Sham surgery
Sleeve gastrectomy
Pair-fed
Total ghrelin (ng/mL)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
b
Sham surgery
Sleeve gastrectomy
Pair-fed
GIP (pg/mL)
60
50
40
30
20
10
0
*
c
Sham surgery
Sleeve gastrectomy
Pair-fed
GLP-1 (pg/mL)
5.0
4.0
3.0
2.0
1.0
0.0
d
Control
Acylated ghrelin 10 pmol/L
Acylated ghrelin 100 pmol/L
Acylated ghrelin 1000 pmol/L
RIN-m5F β-cells
Insulin release (ng/mL)
80
70
60
50
40
30
20
10
0
e
Control
Desacyl ghrelin 10 pmol/L
Desacyl ghrelin 100 pmol/L
Desacyl ghrelin 1000 pmol/L
RIN-m5F β-cells
Insulin release (ng/mL)
80
70
60
50
40
30
20
10
0
f
Control
GLP-1 1 nmol/L
GLP-1 10 nmol/L
GLP-1 100 nmol/L
RIN-m5F β-cells
Insulin release (ng/mL)
120
100
80
60
40
20
0
*
**
g
Control
Acylated ghrelin 10 pmol/L
Acylated ghrelin 100 pmol/L
Acylated ghrelin 1000 pmol/L
RIN-m5F β-cells
Intracellular TG (mg/g protein)
6
4
2
0
*
*
*
h
Control
Desacyl ghrelin 10 pmol/L
Desacyl ghrelin 100 pmol/L
Desacyl ghrelin 1000 pmol/L
RIN-m5F β-cells
Intracellular TG (mg/g protein)
20
18
16
14
12
10
8
6
4
2
0
*
*
i
Control
GLP-1 1 nmol/L
GLP-1 10 nmol/L
GLP-1 100 nmol/L
RIN-m5F β-cells
Intracellular TG (mg/g protein)
8
6
4
2
0
*
*

Fig.3

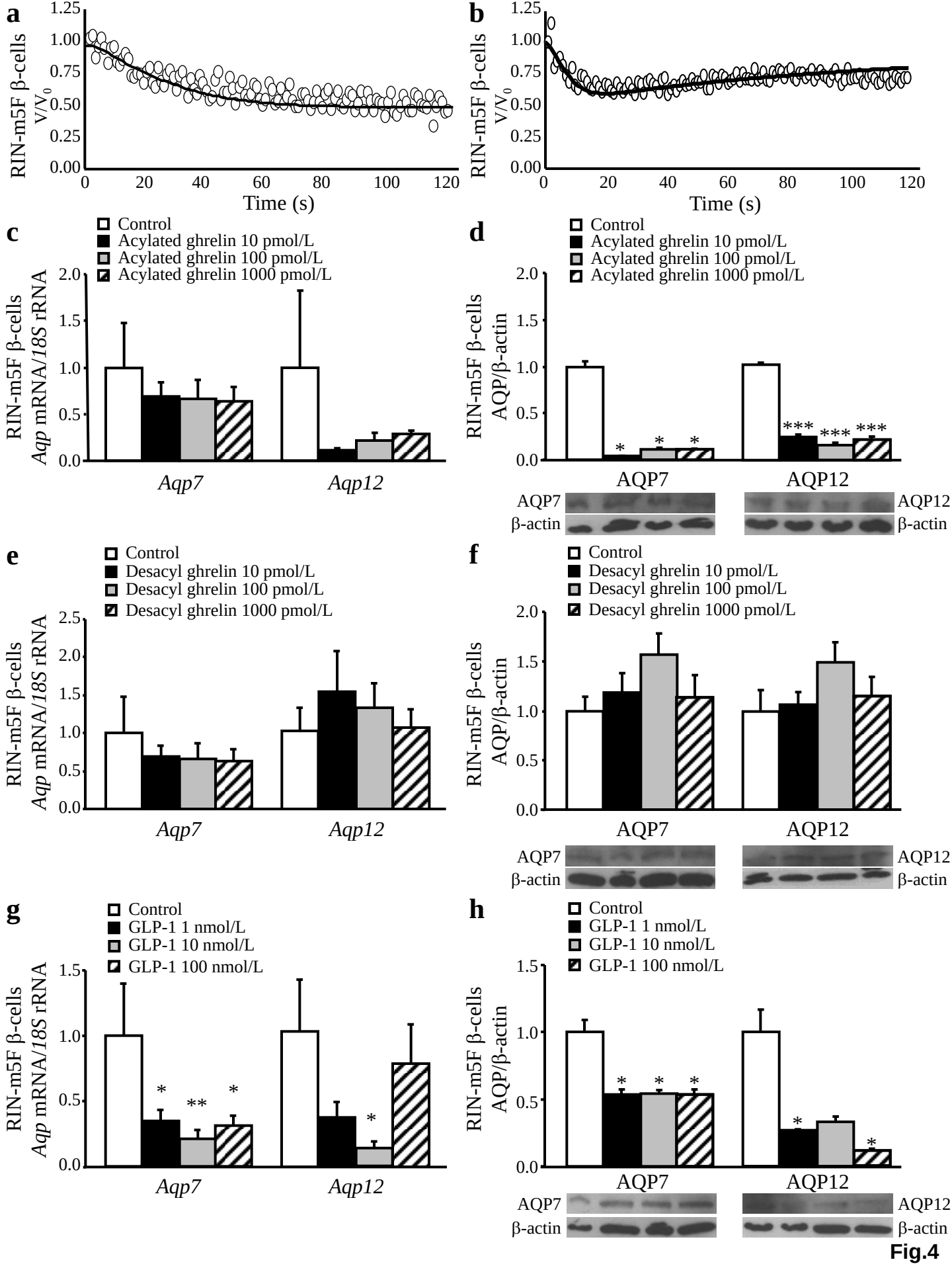

a
b
RIN-m5F β-cells
V/V0
Time (s)
c
d
e
f
g
h
Control
Acylated ghrelin 10 pmol/L
Acylated ghrelin 100 pmol/L
Acylated ghrelin 1000 pmol/L
Desacyl ghrelin 10 pmol/L
Desacyl ghrelin 100 pmol/L
Desacyl ghrelin 1000 pmol/L
GLP-1 1 nmol/L
GLP-1 10 nmol/L
GLP-1 100 nmol/L
Aqp mRNA/18S rRNA
AQP/β-actin
Aqp7
Aqp12
AQP7
AQP12
β-actin

Fig.4

Supplemental Table 1. Sequences of primers and TaqMan® probes.

Gene (GenBank accession no.)	Oligonucleotide sequence (5'-3')	Nucleotides
Aqp3 (NM_031703.1)		
Forward	CTTCTTGGGTGCTGGGATTG	412-431
Reverse	CAATGAGCTTGTTGTCTCCGG	472-492
Taqman® probe	FAM-TACTATGATGCAATCTGGG-TAMRA	443-461
Aqp7 (NM_019157.2)		
Forward	GGCTTCGTGGATGAGGTATTTG	724-745
Reverse	ACAGTCCAGCACTTCAAGGGAC	794-815
Taqman® probe	FAM-AGCTGTGTATCTTCGCCATCACG-TAMRA	761-783
Aqp9 (NM_022960.2)		
Forward	TTTGCAACATATCCAGCTCCATT	905-927
Reverse	GATCGTCTTTGCCATGTTTGACTC	986-1008
Taqman® probe	FAM-CGCCAGGTGCCTTTGTAGACCAAGTG-TAMRA	936-961
Aqp12 (NM_001109009.1)		
Forward	TCCACTGTTCTGGGAACACCTT	729-750
Reverse	TCTGCCGGTAGAACAGATTTCTCT	843-866
Taqman® probe	FAM-ATCCTGGCTGTCCTACTCCATCAGGGC-TAMRA	797-823

Aqp, aquaporin.

Supplemental Table 2. Metabolic profile of control lean and obese rats.

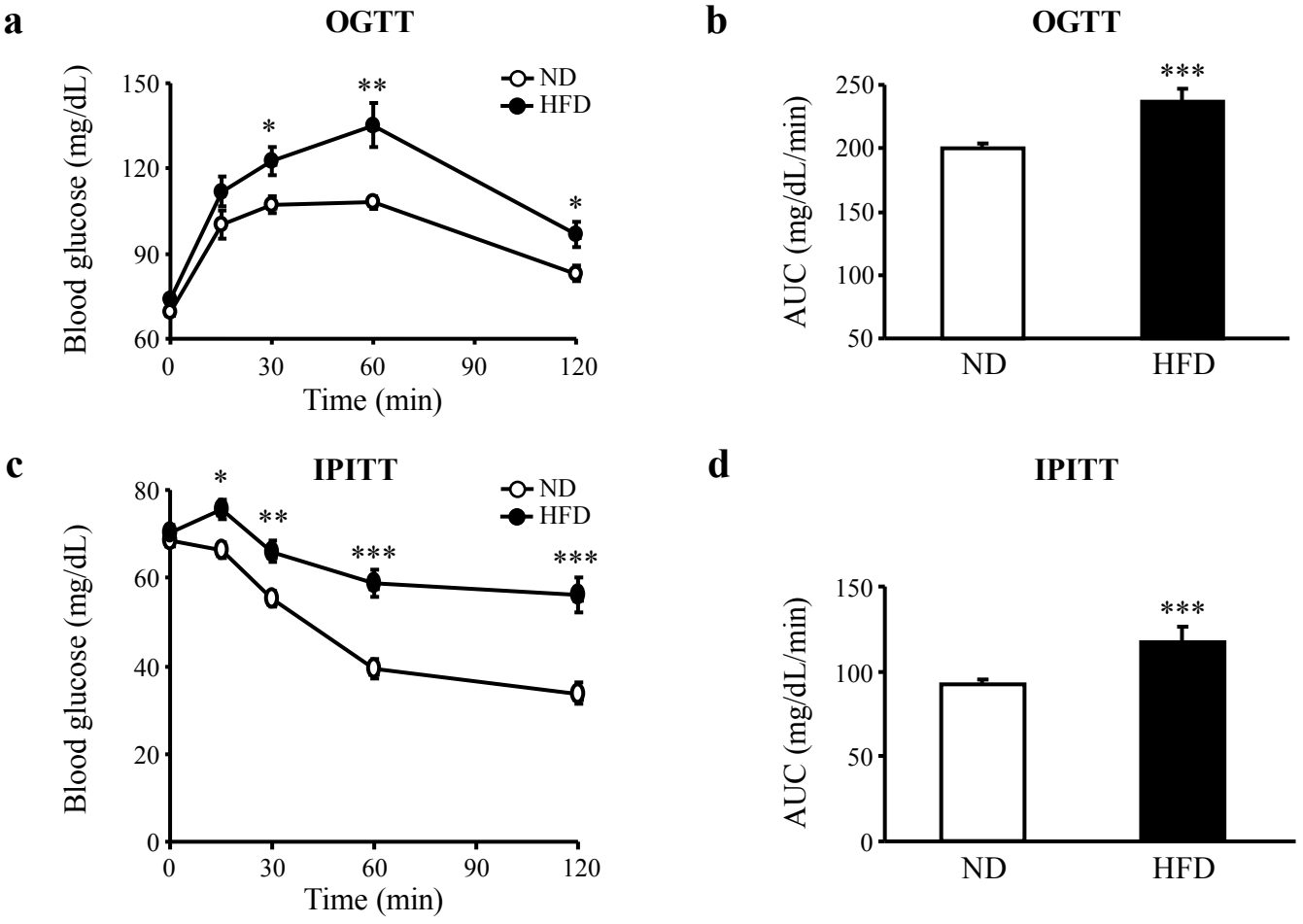
Determination	ND (n=25)	HFD (n=24)	*P*
Glucose (mg/dL)	80 ± 2	90 ± 2	**<0.001**
Insulin (ng/mL)	2.1 ± 0.4	3.6 ± 0.6	**<0.001**
HOMA	0.53 ± 0.02	0.96 ± 0.17	**<0.001**
QUICKI	0.52 ± 0.03	0.42 ± 0.02	**<0.001**
Adipo-IR	258 ± 48	400 ± 85	**<0.001**
GLP-1 (pg/mL)	3.14 ± 0.13	3.43 ± 0.17	0.208
GIP (pg/mL)	67.6 ± 7.3	49.7 ± 8.0	0.415
Total ghrelin (ng/mL)	0.97 ± 0.15	0.71 ± 0.07	**0.014**

ND, normal diet; HFD, high-fat diet; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; Adipo-IR; adipocyte insulin resistance index; GLP-1, glucagon-like peptide-1; GIP, gastric inhibitory polypeptide. Data are the mean ± S.E.M. Statistical differences were analyzed by Student's *t* test. Bold values are statistically significant *P* values.

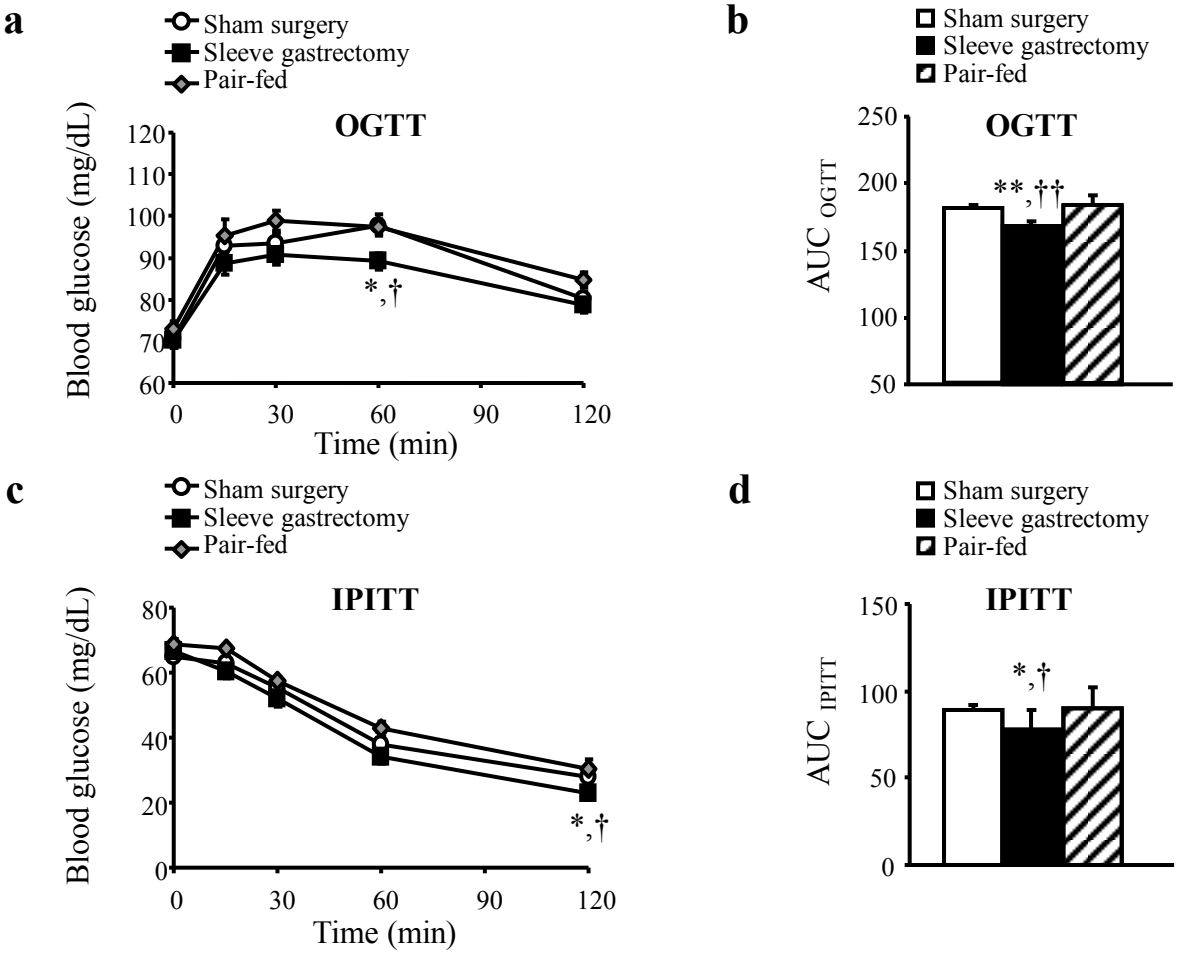
Supplemental Table 3. Metabolic profile four weeks after surgical and dietary interventions.

Determination	Sham surgery (n=27)	Sleeve gastrectomy (n=26)	Pair-fed (n=23)	*P*
Glucose (mg/dL)	79 ± 2	78 ± 2[b]	89 ± 3	**0.004**
Insulin (ng/mL)	2.2 ± 0.4	1.7 ± 0.3	1.8 ± 0.2	0.432
HOMA	0.52 ± 0.09	0.40 ± 0.07	0.47 ± 0.06	0.514
QUICKI	0.46 ± 0.01	0.56 ± 0.05[a,b]	0.44 ± 0.01	**0.011**
Adipo-IR	132 ± 15	126 ±15[a,b]	192 ± 23	**0.009**

HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index. Data are the mean ± S.E.M. Statistical differences were analyzed by one-way ANOVA followed by a Tukey's post-hoc test. Bold values are statistically significant *P* values. [a]*P*<0.05 *vs* sham surgery; [b]*P*<0.05 *vs* pair-fed group.



Supplemental Fig. 1. Impaired glucose tolerance and insulin sensitivity in diet-induced obese rats. Blood glucose levels (a, c) and AUC (b, d) during OGTT and IPITT in rats fed a normal diet (ND) or a high-fat diet (HFD). Statistical differences were analyzed by using one-way ANOVA followed by a Tukey's *post-hoc* test or a Student's *t* test, where appropriate. **P*<0.05, ***P*<0.01 *vs* control rats fed a ND.



Supplemental Fig. 2. Improved glucose tolerance and insulin sensitivity after sleeve gastrectomy in diet-induced obese rats. Blood glucose levels (a, c) and AUC (b, d) during OGTT and IPITT four weeks after surgical and dietary interventions. Statistical differences were analyzed by using one-way ANOVA followed by a Tukey *post-hoc* test. **P*<0.05, ***P*<0.01 *vs* sham-operated rats. †*P*<0.05, ††*P*<0.01 *vs* pair-fed group.

STUDY IV

4. Gastric plication improves glycaemia partly by restoring the altered expression of aquaglyceroporins in adipose tissue and liver in obese rats

Article

Méndez-Giménez L, Becerril S, Moncada R, Valentí V, Fernández S, Ramírez B, Catalán V, Gómez-Ambrosi J, Soveral G, Malagón MM, Diéguez C, Rodríguez A, Frühbeck G.

Gastric plication improves glycaemia partly by restoring the altered expression of aquaglyceroporins in adipose tissue and liver in obese rats.

Obes Surg 2017; doi: 10.1007/s11695-016-2532-2.

Hypothesis

Gastric plication improves body weight, metabolic profile and hepatic gluconeogenesis as well as steatosis in diet-induced obese rats through the regulation of aquaglyceroporins in adipose tissue and liver.

Objectives

- To confirm the effectiveness of gastric plication in the reduction of body weight, food intake and whole-body adiposity as well as in the improvement of glucose tolerance in rats fed a normal diet or a high-fat diet.
- To study the impact of gastric plication on markers of hepatic gluconeogenesis and steatosis in rats fed a normal diet or a high-fat diet.
- To analyze the impact of weight loss achieved by gastric plication and pair-feeding on the expression of aquaglyceroporins in EWAT and SCWAT (AQP3 and AQP7) as well as in the liver (AQP9).
- To evaluate the correlation of adipose and hepatic aquaglyceroporins with markers of adiposity, glucose and lipid metabolism as well as hepatic steatosis.

Méndez-Giménez L, Becerril S, Moncada R, Valentí V, Fernández S, Ramírez B, Catalán V, Gómez-Ambrosi J, Soveral G, Malagón MM, Diéguez C, Rodríguez A, Frühbeck G. Gastric Plication Improves Glycemia Partly by Restoring the Altered Expression of Aquaglyceroporins in Adipose Tissue and the Liver in Obese Rats. Obesity Surgery January 2017;27(7):1763-1774.

Supplemental table 1. Sequences of primers and TaqMan® probes.

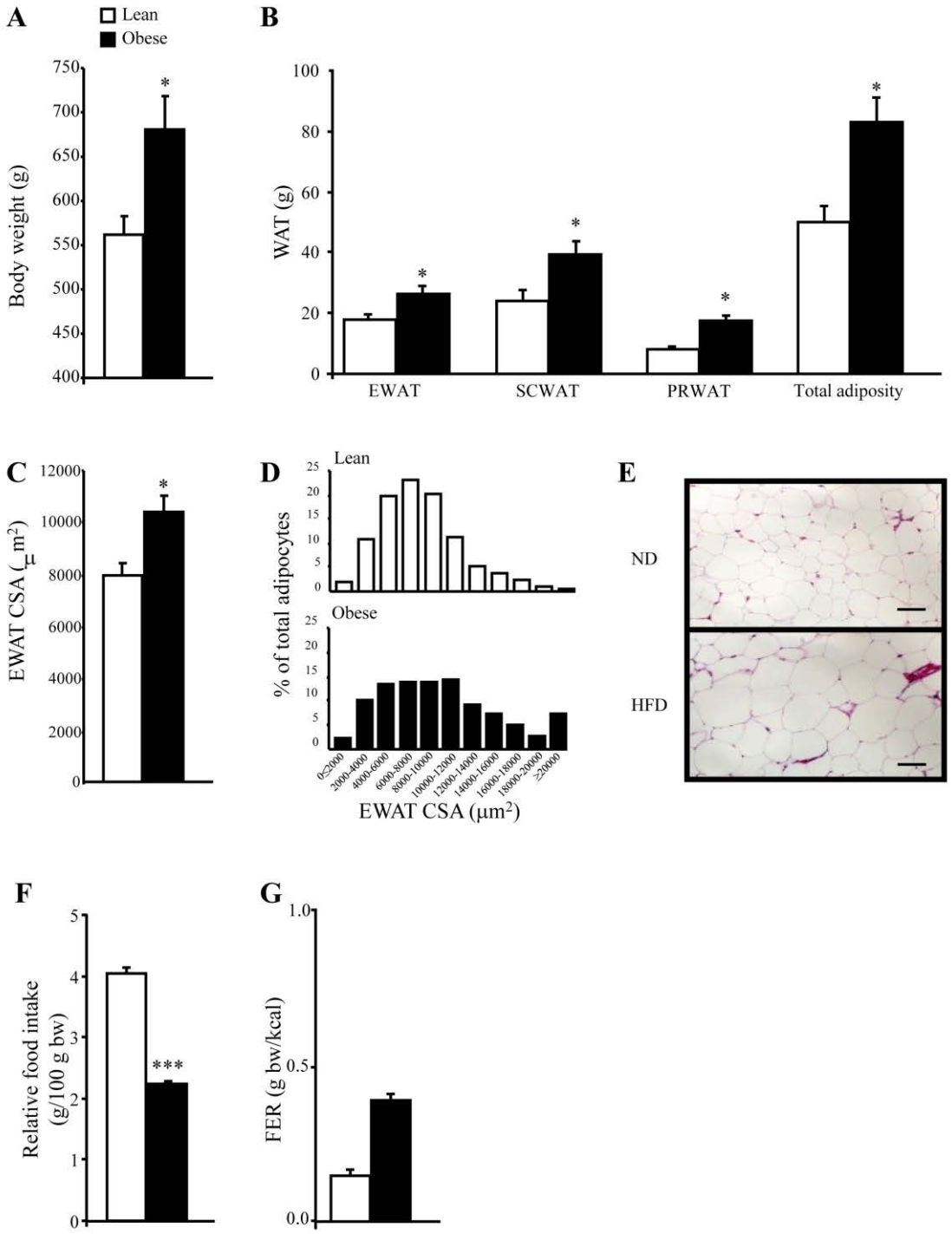
Gene (GenBank accession no.)	Oligonucleotide sequence (5'-3')	Nucleotides
Aqp3 (NM_031703.1)		
Forward	CTTCTTGGGTGCTGGGATTG	412-431
Reverse	*CAATGAGCTTGTTGTCTCCGG*	472-492
Taqman® probe	FAM-TACTATGATGCAATCTGGG-TAMRA	443-461
Aqp7 (NM_019157.2)		
Forward	GGCTTCGTGGATGAGGTATTTG	724-745
Reverse	ACAGTCCAGCACTTCAAGGGAC	794-815
Taqman® probe	FAM-AGCTGTGTATCTTCGCCATCACG-TAMRA	761-783
Aqp9 (NM_022960.2)		
Forward	TTTGCAACATATCCAGCTCCATT	905-927
Reverse	GATCGTCTTTGCCATGTTTGACTC	986-1008
Taqman® probe	FAM-CGCCAGGTGCCTTTGTAGACCAAGTG-TAMRA	936-961
Gk (NM_024381.2)		
Forward	GGGACCAGTCTGCTGCTTTG	869-888
Reverse	TGGCCCGTGTTACACAGTAAGA	947-968
Taqman® probe	FAM-ACAGGCCAAAAACACGTATGGAACAGG-TAMRA	915-941
G6pc (NM_013098.2)		
Forward	GGATCTACCTTGCGGCTCACT	575-595
Reverse	CCCGGATGTGGCTGAAAGT	646-664
Taqman® probe	FAM-CTGGAGTCTTGTCAGGCATTGCTGTGG-TAMRA	614-640
Pck1 (NM_198780.3)		
Forward	GTGATGACATTGCCTGGATGAA	1069-1090
Reverse	TAATGGCGTTCGGATTTGTCTT	1167-1188
Taqman® probe	FAM-CAAGGCAACTTAAGGGCCATCAACC-TAMRA	1101-1125
Ppara (NM_013196.1)		
Forward	AAGGCCTCAGGATACCACTATGG	699-721
Reverse	CAGCTTCGATCACACTTGTCGTA	783-805
Taqman® probe	FAM-CTGCAAGGGCTTCTTTCGGCGAAC-TAMRA	740-763
Pparg (NM_013124)		
Forward	CTGACCCAATGGTTGCTGATTAC	257-279
Reverse	CCTGTTGTAGAGTTGGGTTTTTTCA	351-375
Taqman® probe	FAM-TGAAGCTCCAAGAATACCAAAGTGCG-TAMRA	290-315
Slc2a2 (NM_012879.2)		
Forward	GTTTTTCTGTGCCGTCTTCATGT	1155-1177
Reverse	GAAGAGGAAGATGGCCGTCAT	1228-1248
Taqman® probe	FAM-TTGCTGGATAAGTTCACCTGGATG-TAMRA	1192-1215
Srebf1 (NM_001276707.1)		
Forward	ATGCGGCTGTCGTCTACCAT	2050-2069
Reverse	AGTGTGCAGGAGATGCTATATCCAT	2158-2182
Taqman® probe	FAM-CATGCCATGGGCAAGTACACAGGAGG-TAMRA	2085-2110

Aqp, aquaporin; *Gk*, glycerol kinase; *G6pc*, glucose-6-phosphatase; *Pck1*, phosphoenolpyruvate carboxykinase 1; *Ppara*, peroxisome proliferator-activator receptor α; *Pparg*, peroxisome proliferator-activator receptor γ; *Slc2a2*, solute carrier family 2 (facilitated glucose transporter), member 2; *Srebf1*, sterol regulatory element binding factor 1.

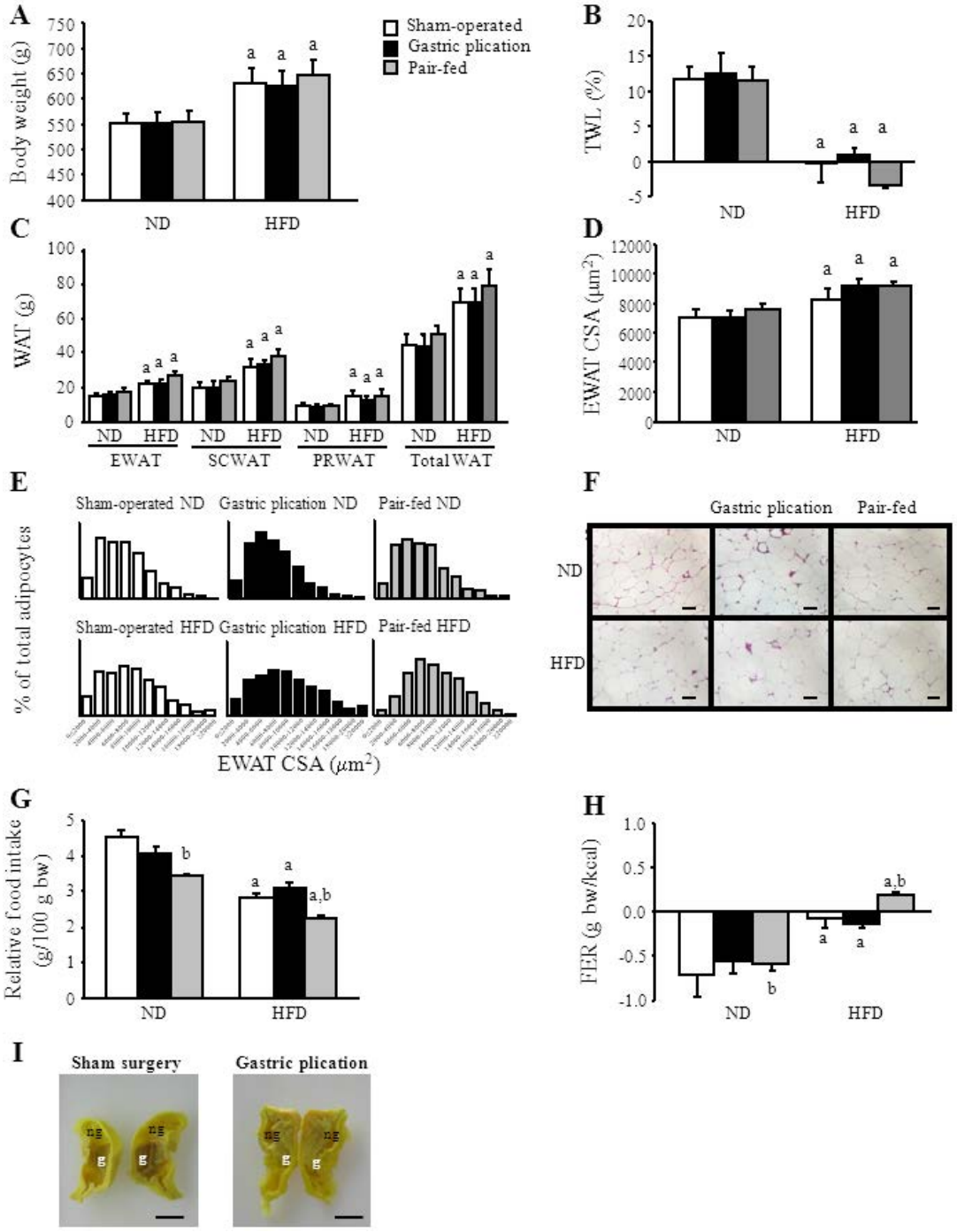
Supplemental table 2. Metabolic profile of lean and obese rats.

Determination	Lean (n=10)	DIO (n=7)	*P*
Glucose (mg/dL)	85 ± 1	93 ± 3	**0.034**
Insulin (ng/mL)	4.2 ± 0.4	5.5 ± 0.6	0.059
HOMA	1.0 ± 0.1	1.5 ± 0.2	**0.022**
QUICKI	0.40 ± 0.01	0.36 ± 0.01	**0.028**
Glycerol (mg/dL)	29 ± 3	31 ± 4	0.691
FFA (mg/dL)	17.3 ± 0.8	13.9 ± 1.0	**0.008**
Adipo-IR index	65 ± 10	84 ± 12	0.267
Triacylglycerols (mg/dL)	185 ± 34	132 ± 12	0.155
Total cholesterol (mg/dL)	101 ± 8	116 ± 13	0.310
AST (U/L)	24 ± 2	32 ± 3	**0.026**
ALT (U/L)	10 ± 2	14 ± 2	0.090
Adiponectin (μg/mL)	13.3 ± 0.5	12.1 ± 0.4	0.106
Leptin (ng/mL)	13.0 ± 1.1	23.5 ± 1.9	**0.001**
Total ghrelin (ng/mL)	1.33 ± 0.14	1.04 ± 0.07	0.089

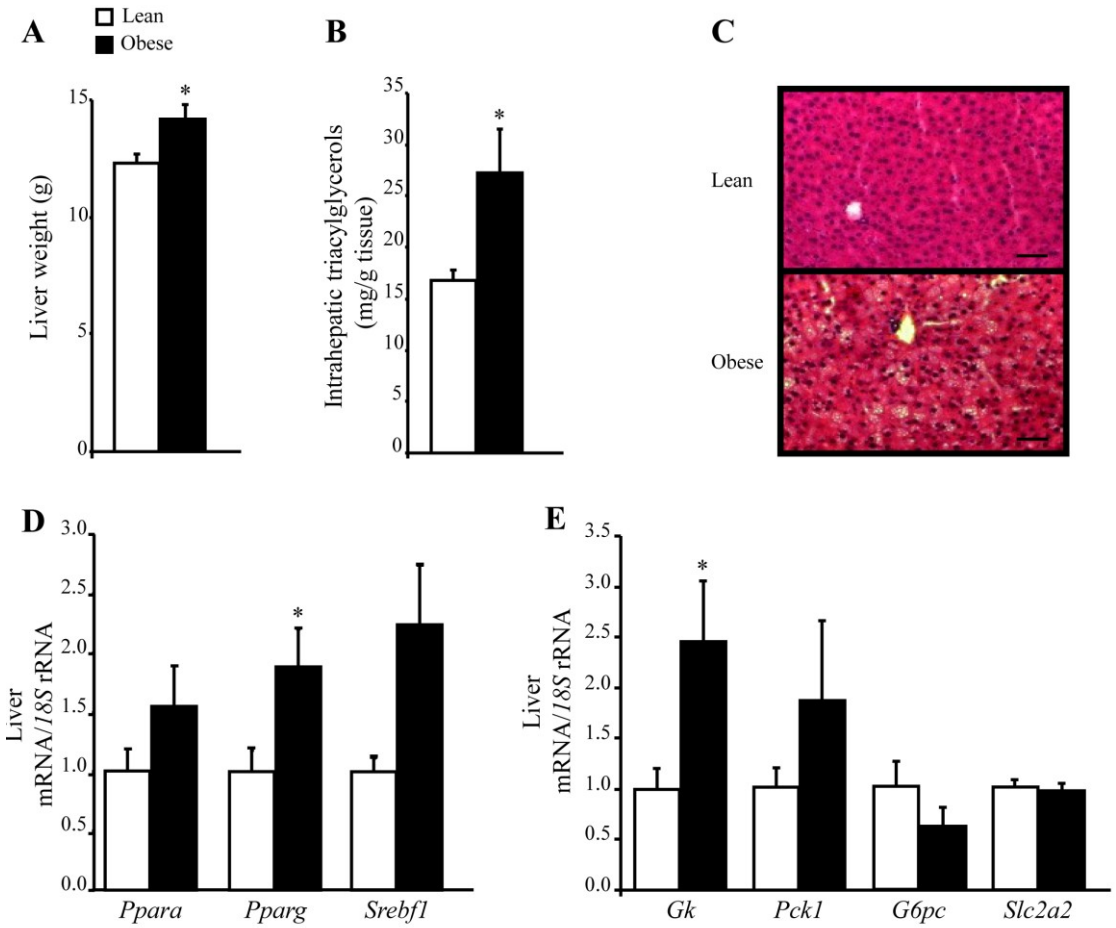
DIO, diet-induced obesity; HOMA, homeostasis model assessment; QUICKI, quantitative insulin sensitivity check index; FFA, free fatty acids; AST, aspartate transaminase; ALT, alanine transaminase. Data are the mean ± S.E.M. Statistical differences were analyzed by *t* Student's test. Bold values are statistically significant *P* values.



Supplemental Fig. 1. Body weight and whole-body adiposity in diet-induced obese rats. Bar graphs show the body weight (A), epididymal (EWAT), subcutaneous (SCWAT), perirenal (PRWAT) and total adiposity fat content (B), cell surface area (CSA) of EWAT adipocytes (C) and adipocyte size distribution (D) of lean and diet-induced obese rats. Representative histological sections of EWAT of the experimental groups (E); magnification 100X, scale bar=100 μm. Daily food intake (F) and food efficiency ratio (G) is also shown. **P*<0.05; ****P*<0.001 *vs* lean group.



Supplemental Fig. 2. Impact of gastric plication on body weight and whole-body adiposity in diet-induced obese rats. Bar graphs show the body weight (A), percentage of total weight loss (%TWL) (B), epididymal (EWAT), subcutaneous (SCWAT), perirenal (PRWAT) and whole-body white adiposity (C), cell surface area (CSA) of epididymal white adipocytes (D), as well as adipocyte size distribution (E) of experimental animals fed either a normal (ND) or a high-fat diet (HFD) after surgical interventions. Representative histological sections of EWAT of the experimental groups (F); magnification 100X, scale bar=100 μm. Daily food intake (G) and food efficiency ratio (H) is also shown. [a]$P<0.05$ effect of diet. [b]$P<0.05$ effect of surgery. (I) Representative macroscopic view of Bouin-fixed stomachs obtained from rats submitted to sham surgery (*left panel*) and gastric plication (*right panel*) (scale bar=1 cm). ng, non-glandular stomach; g, glandular stomach.



Supplemental Fig. 3. Bar graphs show the liver weight (A) and intrahepatic triacylglycerol content (B) in rats fed a normal or high-fat diet. Representative histological sections of liver of the experimental groups (C); magnification 200X, scale bar=100 μm. Hepatic expression levels of lipogenic (D) and gluconeogenic (E) genes. *P<0.05 *vs* lean group.

Discussion

1. Summary of the main findings

Glycerol constitutes an important metabolite for the control of lipid accumulation and glucose homeostasis in insulin-sensitive tissues and for pancreatic insulin secretion. The present thesis shows the role of aquaglyceroporins, which mediate glycerol transport in adipocytes (AQP3 and AQP7), hepatocytes (AQP9) and β-cells (AQP7), in the improvement of adiposity, NAFLD and β-cell function induced by two different bariatric surgery procedures, sleeve gastrectomy and gastric plication, in an experimental model of diet-induced obesity. We confirmed the alterations in the expression of aquaglyceroporins in insulin-sensitive tissues in the obese state. Obese rats exhibited excess adiposity, hepatic and pancreatic steatosis, as well as impaired glucose tolerance and insulin secretion. Obesity was associated with an increase in EWAT AQP3 and SCWAT AQP7 and a decrease in hepatic AQP9. Interestingly, *Aqp7* transcript levels in EWAT and SCWAT were positively associated with adiposity and glycemia, while hepatic *Aqp9* mRNA was negatively correlated with markers of hepatic steatosis and insulin resistance. The two studied restrictive bariatric surgery techniques exerted different impact on whole-body metabolism. On the one hand, obese rats undergoing sleeve gastrectomy showed a reduction in body weight, whole-body adiposity and hepatic steatosis as well as improved glucose tolerance four weeks after surgery. Sleeve gastrectomy down-regulated AQP7 in both EWAT and SCWAT, without changing hepatic AQP9. Thus, sleeve gastrectomy restores the coordinated regulation of fat-specific AQP7 and liver-specific AQP9, thereby improving whole-body adiposity and hepatic steatosis (**Table 3**). By contrast, gastric plication did not change body weight and induced a modest reduction in whole-body adiposity and hepatosteatosis. However, gastric plication improved basal glycemia by downregulating AQP3, which entails lower efflux of glycerol from fat, lower plasma glycerol availability, and a reduced use of glycerol as a substrate for hepatic gluconeogenesis.

Our results show, for the first time, that obesity is associated with higher AQP7 levels in the pancreas, which is involved in insulin secretion and TG accumulation in β-cells. Sleeve gastrectomy improved glucose tolerance and reduced pancreatic β-cell mass, apoptosis, steatosis and insulin secretion in obese rats. Circulating levels of ghrelin and GLP-1, two important gut hormones mediating the resolution of insulin resistance after bariatric surgery, were decreased and increased, respectively, after

sleeve gastrectomy. Interestingly, ghrelin and GLP-1 not only increased intracellular TG content and insulin secretion, respectively, but also downregulated AQP7 expression *in vitro* in RIN-m5F β-cells. AQP7 protein was negatively correlated with intracellular lipid accumulation in acylated ghrelin-treated cells and with insulin release in GLP-1-stimulated β-cells. Together, sleeve gastrectomy improves excess lipid accumulation and impaired insulin secretion in obese rats with AQP7 contributing to this beneficial effect through the regulation of glycerol availability in β-cells (**Table 3**).

Table 3. Summary of the functional role of the changes in AQP expression after sleeve gastrectomy and gastric plication.

AQP	Tissue (subcellular location)	Changes in obesity	Changes after surgery		Functional changes associated with AQPs after surgery
			Sleeve gastrectomy	Gastric plication	
AQP3	WAT (lipid droplets)	▲	▲	▼	Improvement in lipolysis
AQP7	WAT (membrane)	▲	▼	=	Reduction in adipocyte size
	Pancreas (membrane)	▲	▲	No data available	Improved insulin synthesis and secretion
AQP9	Liver (membrane)	=▼	▲	▼	Decrease in hepatic gluconeogenesis and lipogenesis
AQP12	Pancreas (cytoplasm)	=▼	-	No data available	Reduction of pancreatic damage

▲Increase; ▼ Decrease.

2. Effect of sleeve gastrectomy and gastric plication on body weight, whole-body adiposity and metabolic profile in obese rats

The prevalence of obesity and obesity-associated comorbidities, such as T2D and NAFLD, has markedly increased during the past three decades, becoming a major health problem worldwide. Bariatric surgery has emerged as the most effective treatment to induce sustainable weight loss and improve metabolic profile in obesity (Frühbeck, 2015). In this thesis, we have characterized the changes in body composition as well as metabolic profile after two different bariatric surgical procedures, namely sleeve gastrectomy and gastric plication, in diet-induced obese rats. The survival rate of both surgical techniques was 100% with no animals being excluded due to

complications, confirming the safety of both sleeve gastrectomy (Rubino *et al*, 2016) and gastric plication (Kourkoulos *et al*, 2012, Talebpour *et al*, 2012). In study II, our results showed that four weeks after surgery, rats undergoing sleeve gastrectomy exhibited a decrease in body weight and in all WAT depots (EWAT, SCWAT and PRWAT) as well as lower adipocyte hypertrophy compared to the other animal groups of the study. By contrast, animals submitted to gastric plication presented similar body weight, whole-body adiposity and adipocyte cell surface area (CSA) compared to sham-operated rats 1 month after surgery. One plausible explanation for the different results obtained for weight loss consists in the distinct gastric capacity limitation approach. By using the technique of sleeve gastrectomy, around the 60-80% of the stomach is cut along the greater curvature leaving a narrow tube and excluding the gastric fundus, while in the procedure of gastric plication, the stomach is invaginated inside the gastric lumen without resection. In this regard, the reduction of circulating ghrelin, a hormone mainly produced in the fundus of the stomach with orexigenic and adipogenic properties (Rodríguez *et al*, 2009), appears to be involved in the weight-loss effects of sleeve gastrectomy. Rats submitted to sleeve gastrectomy exhibit a dramatic reduction in circulating concentrations of ghrelin, which may contribute to the higher weight loss when compared to pair-fed rats. By contrast, in line with reports of other authors (Ivano *et al*, 2013, Darido *et al*, 2014), animals that underwent the gastric plication procedure exhibited higher serum total ghrelin levels than sham-operated rats. The elevated concentration of ghrelin may promote the continued eating of the rats even if the stomach is full, due to the reduction of the mechanosensitivity of the gastric vagus nerve (Page *et al*, 2007). Taken together, the increase in circulating total ghrelin after gastric plication might explain, in part, the lack of effect of this surgical technique in food intake, total adiposity, and body weight compared to sleeve gastrectomy.

The observed changes in the metabolic profile after sleeve gastrectomy and gastric plication are summarized in **Table 4**. The results obtained in the present thesis evidence that sleeve gastrectomy was associated with an improvement in insulin sensitivity and lipid profile compared to sham-operated group, which is in agreement with previous results of our group (Rodríguez *et al*, 2012b, Rodríguez *et al*, 2012a, Moncada *et al*, 2016a, Moncada *et al*, 2016c) and others (Patrikakos *et al*, 2009, Stefater *et al*, 2010, Wilson-Pérez *et al*, 2013b). Moreover, our data showed that sleeve gastrectomy reduced intrahepatic TG accumulation and macrovesicular steatosis of

obese rats, which is in accordance with previous reports showing the beneficial effects of this bariatric procedure on the fatty liver of experimental models of genetic and diet-induced obesity (Wang *et al*, 2009, Kawano *et al*, 2013). On the other hand, gastric plication improved basal glycemia and glucose tolerance in obese rats, which is in accordance with data reported by other authors (Guimarães *et al*, 2013). In contrast with results of other authors (Talebpour *et al*, 2015), we did not find an improvement in serum TG and cholesterol levels in obese rats submitted to gastric plication. Moreover, our data support the notion that hepatic steatosis was not improved after gastric plication despite the improvement in hyperglycemia. Taken together, sleeve gastrectomy constitutes a more effective technique to improve obesity-associated metabolic derangements than gastric plication.

Table 4. Metabolic effects of sleeve gastrectomy and gastric plication in obese rats.

Characteristic	Sleeve gastrectomy	Gastric plication
Excess body weight	Decreased	Very modest reduction
Excess adiposity	Decreased	No effect
Hyperglycemia	Improved	Improved
Insulin resistance	Improved	Improved
Dyslipidemia	Improved	No effect
NAFLD	Improved	No effect

3. Role of aquaglyceroporins in the improvement of adiposity after bariatric surgery

Obesity is associated with increased lipolysis due to higher lipolytic activity of β_3-adrenergic receptors and reduced anti-lipolytic action of insulin, leading to elevated circulating concentrations of FFA and glycerol (Méndez-Giménez *et al*, 2014). Glycerol is released to the bloodstream through AQP7 and, to a lesser extent, via AQP3 in adipocytes to compensate body energy demands (Hara-Chikuma *et al*, 2005, Rodríguez *et al*, 2011b, Madeira *et al*, 2013). AQP3 and AQP7 facilitate glycerol efflux from adipocytes in response to lipolysis induced by β-adrenergic agonists via its translocation from the cytosolic fraction (AQP3) or lipid droplets (AQP7) (Kishida *et al*, 2000, Yasui

H. *et al*, 2008, Rodríguez *et al*, 2011b). Our findings provide evidence that obese rats present an increase of AQP3 in EWAT and AQP7 in SCWAT suggesting a higher lipolytic response and glycerol release in both fat depots, which is in accordance with previous results of our group and others (Marrades *et al*, 2006, Prudente *et al*, 2007, Catalán *et al*, 2008, Rodríguez *et al*, 2011b). Plausible explanations for the upregulation of WAT aquaglyceroporins in obese animals include: i) the decrease in circulating ghrelin, a negative regulator of AQP7 expression in adipocytes (Rodríguez *et al*, 2009); ii) the activation of the transcription factors PPARα and PPARγ since rat genes encoding *Aqp3, Aqp7* and *Aqp9* present PPAR-response elements (PPRE) in their gene promoters (Kishida *et al*, 2001a, Méndez-Giménez *et al*, 2014); and iii) the altered expression of adipokines, including leptin, adiponectin, lipocalin-14 or apelin-13 (Rodríguez *et al*, 2011b, Guo *et al*, 2014, Rodríguez *et al*, 2015a, Lee J. T. *et al*, 2016, Tardelli *et al*, 2017), which regulate the expression of aquaglyceroporins in adipose tissue (**Figure 15**).

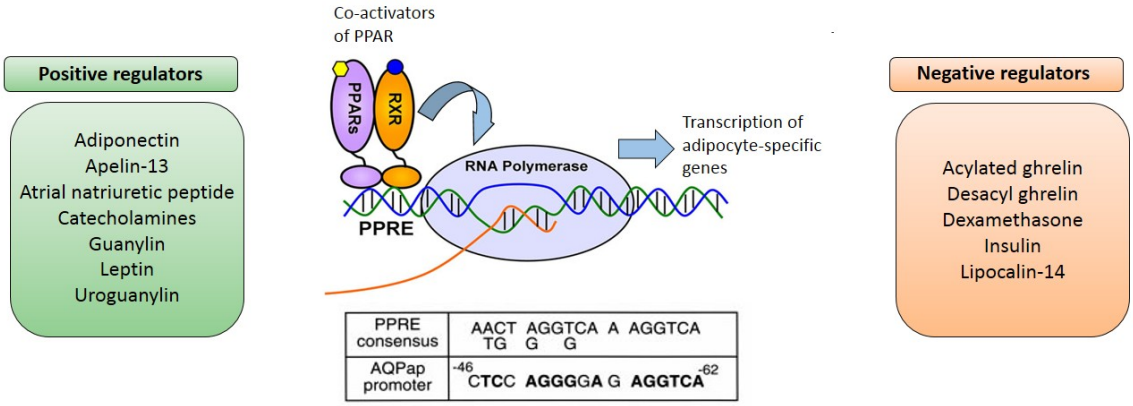


Figure 15. Factors involved in the regulation of the expression of aquaglyceroporins in adipocytes [modified from (Rosen *et al*, 2000)].

Sleeve gastrectomy, but not gastric plication, reduced adipocyte hypertrophy of obese rats. In order to unravel the molecular mechanisms involved in adiposity changes after both bariatric surgery techniques, the expression of aquaglyceroporins was evaluated in different fat depots of our experimental animals. AQP7 is considered a marker of mature adipocytes, since the expression of this aquaglyceroporin is almost negligible in undifferentiated preadipocytes, while AQP7 becomes markedly expressed during the late adipogenesis in a process mediated by PPARγ, the master adipogenic transcription factor (**Figure 15**) (Kishida *et al*, 2001a). Interestingly, sleeve gastrectomy was associated with a downregulation of AQP7 and PPARγ in EWAT and SCWAT, and a positive association of *Aqp7* and *Pparg* transcript levels was observed in both fat

depots. However, gastric plication did not modify the expression of AQP7 in adipose tissue. Together, the lower expression of AQP7 in both fat depots after sleeve gastrectomy appears to be related to the reduction in adipocyte cell size after sleeve gastrectomy. In line with this observation, the remodeling of EWAT after exercise training in obese rats is also related with a decrease in AQP7 content (Rocha-Rodrigues *et al*, 2016).

In 3T3-L1 adipocytes, AQP3 is translocated in response to the lipolytic action of leptin from the plasma membrane to lipid droplets, a step that might facilitate glycerol mobilization after lipolysis (Rodríguez *et al*, 2015a). Sleeve gastrectomy induced an increase in EWAT AQP3, which may reflect an improvement in the lipolytic rate in this fat depot following this surgical intervention. By contrast, animals submitted to gastric plication exhibited a decrease in the expression of AQP3 in EWAT and SCWAT suggesting a reduction in the release of glycerol from both fat depots to the bloodstream. In conclusion, changes in the expression of adipose AQP3 after sleeve gastrectomy and gastric plication might reflect modifications in adipocyte lipolysis.

4. Impact of sleeve gastrectomy and gastric plication on hepatosteatosis in diet-induced obese rats

NAFLD is commonly associated with obesity, dyslipidemia, insulin resistance and T2D (European Association for the Study of the Liver *et al*, 2016). The mechanisms underlying intrahepatic TG accumulation, as the hallmark of NAFLD, include increased delivery of FFA to the liver, inadequate FFA oxidation, and increased *de novo* lipogenesis (Utzschneider *et al*, 2006, Ezquerro *et al*, 2016). In the studies II, III and IV of the present thesis, obese rats exhibited insulin resistance, evidenced by higher glucose levels during the oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT), hyperinsulinemia, higher HOMA index and adipose tissue insulin resistance index (adipo-IR), hypoadiponectinemia, as well as worse lipid profile, supported by higher total cholesterol levels compared to their lean counterparts. Moreover, obese animals developed hepatosteatosis, evidenced by an increase in liver weight and intrahepatic TG content as well as macrovesicular steatosis in the liver histological sections compared to lean control rats.

The transcription factors PPARα, PPARγ and sterol regulatory element-binding transcription factor 1 (SREBF1) represent key elements in the control of hepatic lipid

metabolism (Tontonoz *et al*, 1993, Costet *et al*, 1998, Kersten *et al*, 1999, Musso *et al*, 2009, Rodríguez *et al*, 2009, Gong *et al*, 2016). PPARs belong to the nuclear receptor superfamily and form a heterodimer with retinoid X receptor (RXR) that bind to DNA at the PPRE in the gene promoters, resulting in gene transcription (Evans *et al*, 2004). PPARα was the first PPAR to be identified and is predominantly expressed in the liver, where it is a major activator of FFA β-oxidation (Evans *et al*, 2004, Poulsen *et al*, 2012). In this regard, it is well established that impaired PPARα function is associated with hepatic lipid accumulation (Reddy, 2001). PPARγ is highly expressed in WAT and BAT and, to a lesser extent, in hepatocytes (Braissant *et al*, 1996, Escher *et al*, 2001). PPARγ promotes lipid storage in the adipose tissue, thereby reducing FFA delivery and lipotoxicity in non-adipose organs, such as the liver or pancreas (Chawla *et al*, 1994, Fajas *et al*, 1997). Furthermore, PPARγ plays an important role in increasing insulin sensitivity with PPARγ agonists being currently used to treat diabetes. SREBF1 (formerly known as adipocyte determination and differentiation factor 1, ADD1) belongs to the basic helix-loop-helix family of transcription factors and induces the transcription of lipogenic genes (including ACC, FAS and LPL) with sterol response elements (SRE) in their gene promoters (Tontonoz *et al*, 1993, Kim *et al*, 2004). In studies II and IV, obese rats exhibited increased transcript levels of *Ppara*, *Pparg* and *Srebf1*, confirming the important role of these lipogenic transcription factors in the onset of NAFLD in obesity.

Certain studies investigating the effect of bariatric surgery on NAFLD have shown an improvement in serum transaminases and hepatic histologic features after surgery (Dixon *et al*, 2006, Burza *et al*, 2013). Our data showed that sleeve gastrectomy induced the highest reduction of liver weight and intrahepatic TG levels compared to sham surgery and pair-feeding, which is in accordance with other reports (Wang *et al*, 2009, Kawano *et al*, 2013). Study II also demonstrated that sleeve gastrectomy was associated with a downregulation of *Pparg* and *Srebf1* in obese rats, suggesting the implication of these lipogenic transcription factors in the amelioration of the hepatosteatosis after this bariatric surgery technique. In another study of our group (Ezquerro *et al*, 2016), an upregulation of *Ppara* and *Cpt1a* together with an increased mitochondrial DNA content was observed in the liver after sleeve gastrectomy, suggesting an increased flux of FFA towards mitochondrial β-oxidation and higher mitochondrial copy number after this bariatric surgery procedure. Conversely, gastric

plication surgery did not improve the fatty liver of obese rats as evidenced by similar liver weight, intrahepatic TG accumulation and hepatic *Ppara, Pparg* and *Srebf1* expression.

Several plausible explanations of the different impact of sleeve gastrectomy and gastric plication on the amelioration of NAFLD are summarized in **Figure 16**. Firstly, fasting serum FFA constitute the majority of fat delivered to the liver and contribute to TG synthesis and accumulation in NAFLD (Zhang J. *et al*, 2014). Sleeve gastrectomy tended to decrease circulating FFA, whereas gastric plication did not change serum FFA levels and, hence, the increased FFA delivery remained unchanged. Secondly, while sleeve gastrectomy reduced hepatic steatosis through the down-regulation of transcription factors involved in lipogenesis, gastric plication induced a modest, but not significant, reduction in intrahepatic TG accumulation and lipogenic factors *Ppara*, *Pparg* and *Srebf1*. Thirdly, in the context of obesity and diabetes, insulin no longer suppressed hepatic gluconeogenesis, while continuing to activate lipogenesis, a state referred to as "selective insulin resistance" (Kubota *et al*, 2016). This state of "selective insulin resistance" has been related to defective insulin receptor substrates 1 and 2 (IRS1/2) in the periportal zone (primary site of gluconeogenesis), which otherwise is enhanced in the perivenous zone (primary site of lipogenesis) of the liver. Both sleeve gastrectomy (studies II and III) and gastric plication (study IV) improved glycemia and glucose tolerance, which is in accordance with previous reports (Guimarães *et al*, 2013). However, it can be speculated that gastric plication might cause a differential hepatic distribution of IRS1 and 2 that restores the altered gluconeogenesis, but not lipogenesis. Finally, we have recently demonstrated that both acylated and desacyl ghrelin regulate hepatic lipogenesis, mitochondrial FFA β-oxidation and autophagy in rat hepatocytes (Ezquerro *et al*, 2016). In study II, total ghrelin levels after sleeve gastrectomy were diminished due to the resection of the gastric fundus, which is consistent with the literature (Frühbeck *et al*, 2004a, Peterli *et al*, 2012). In this sense, the decrease in the most abundant isoform, desacyl ghrelin after sleeve gastrectomy contributes to the reduction of lipogenesis, whereas the increased relative acylated ghrelin levels activate factors involved in mitochondrial FFA β-oxidation and autophagy in obese rats, thereby ameliorating NAFLD (Ezquerro *et al*, 2016). Conversely, gastric plication surgery exhibits higher ghrelin levels due to the exposition of the greater curvature to the gastric lumen, which might enhance the ability of both isoforms to promote hepatic

lipogenesis. In summary, although other bariatric procedures such as sleeve gastrectomy or RYGB ameliorate NAFLD (Froylich *et al*, 2016), gastric plication cannot be considered an effective method to reduce hepatosteatosis in obesity.

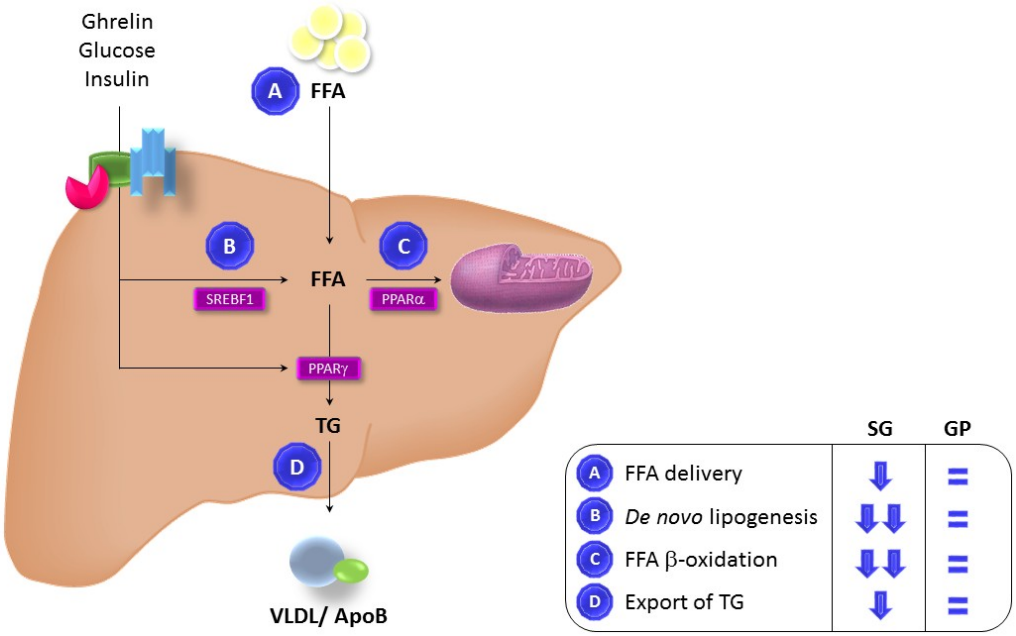


Figure 16. Schematic view of the impact of sleeve gastrectomy (SG) and gastric plication (GP) on the mechanisms involved in the onset of NAFLD in the context of obesity. FFA derived from adipocyte lipolysis (A) or through hepatic *de novo* lipogenesis (B). FFA once in the liver, can be used as a substrate for mitochondrial FFA β-oxidation (C) or converted to TG for storage and secretion in the form of VLDL assembled with apolipoprotein B [modified from (Gong *et al*, 2016)]. Sleeve gastrectomy, but not gastric plication, improves hepatosteatosis ameliorating several pathways of hepatic lipid metabolism.

5. Role of aquaglyceroporins in the amelioration of non-alcoholic fatty liver disease after bariatric surgery

AQP9 is the most abundant glycerol channel in rodents and human liver, and it is mainly localized at the sinusoidal plasma membrane that faces the portal vein (Jelen *et al*, 2011, Calamita *et al*, 2012, Gena *et al*, 2013, Rodríguez *et al*, 2014). This aquaglyceroporin allows the influx of glycerol and urea into the hepatocytes, and its expression is markedly increased during fasting (Carbrey *et al*, 2003, Rojek A. M. *et al*, 2007, Jelen *et al*, 2012). Plasma glycerol is introduced into the hepatocytes via AQP9 (Rojek A. M. *et al*, 2007, Calamita *et al*, 2012, Gena *et al*, 2017), where it is converted to glycerol-3-phosphate by GK and is used as a substrate for *de novo* synthesis of glucose (gluconeogenesis) and TG (lipogenesis) (Jelen *et al*, 2011, Lebeck, 2014). Regarding the participation of AQP9 in the onset of hepatosteatosis, our group has previously reported a downregulation of hepatic AQP9 and reduced glycerol

permeability in parallel to the degree of steatosis in both genetically obese *ob/ob* mice (Gena *et al*, 2013) as well as obese patients with NAFLD (Rodríguez *et al*, 2014). In the present study, we found a downregulation of hepatic AQP9 in diet-induced obese rats. The hepatic *Aqp9* expression was negatively associated with markers of fatty liver, such as intrahepatic TG and with insulin resistance, including insulinemia and HOMA index. In this sense, it is well known that insulin resistance constitutes a major feature of NAFLD (Kalhan *et al*, 2001, Reshef *et al*, 2003, Chalasani *et al*, 2012, Méndez-Giménez *et al*, 2014). The diet-induced obese rats used in studies II, III and IV developed insulin resistance, as evidenced by higher glycemia, insulinemia, HOMA, and adipo-IR indices. Taken together, the decreased hepatic AQP9 expression in both genetic and diet-induced obesity appears to be a compensatory mechanism whereby the liver counteracts further TG accumulation within its parenchyma as well as further aggravation of the hyperglycemia by reducing glycerol permeability.

The molecular mechanisms whereby bariatric surgery ameliorates NAFLD is poorly understood. In the present thesis, we investigated whether the reduction of hepatosteatosis after bariatric surgery is also related to changes in hepatic AQP9 expression. In study II, our data revealed a slight increase of hepatic AQP9 expression after sleeve gastrectomy. Several plausible mechanisms might explain the effect of sleeve gastrectomy on hepatic AQP9 expression. Firstly, AQP9 is positively regulated by the activation of PPARα and PPARγ (Kishida *et al*, 2001a, Lebeck *et al*, 2015). Interestingly, we also found a positive association of hepatic of *Aqp9* expression with *Pparg* suggesting a positive modulation of this aquaporin by this transcription factor in the liver. Secondly, our group recently reported that leptin replacement improved the fatty liver of leptin-deficient *ob/ob* mice in parallel to an increase in hepatic AQP9 content (Rodríguez *et al*, 2015a). Owing to its ability to induce weight loss and decrease adiposity, it can be speculated that sleeve gastrectomy, but not gastric plication, improves leptin sensitivity in the liver, and hence, contributes to increase hepatic AQP9 expression. Thirdly, in rodents, the promoter of *Aqp9* gene presents a negative IRE with insulin repressing the expression of this aquaglyceroporin in the liver (Kuriyama *et al*, 2002). In study II, AQP9 was negatively correlated with insulin and HOMA, both markers of insulin resistance. Sleeve gastrectomy improved glycemia and insulin sensitivity, which is in agreement with several studies (Wilson-Pérez *et al*, 2013b, Basso *et al*, 2016), including ours (Rodríguez *et al*, 2012b). Thus, the increase in

hepatic AQP9 might also reflect the decrease in insulinemia observed in sleeve gastrectomy. Altogether, sleeve gastrectomy restores the coordination of AQP7 in the adipose tissue and AQP9 in the liver, leading to normal circulating glycerol levels (**Figure 17**).

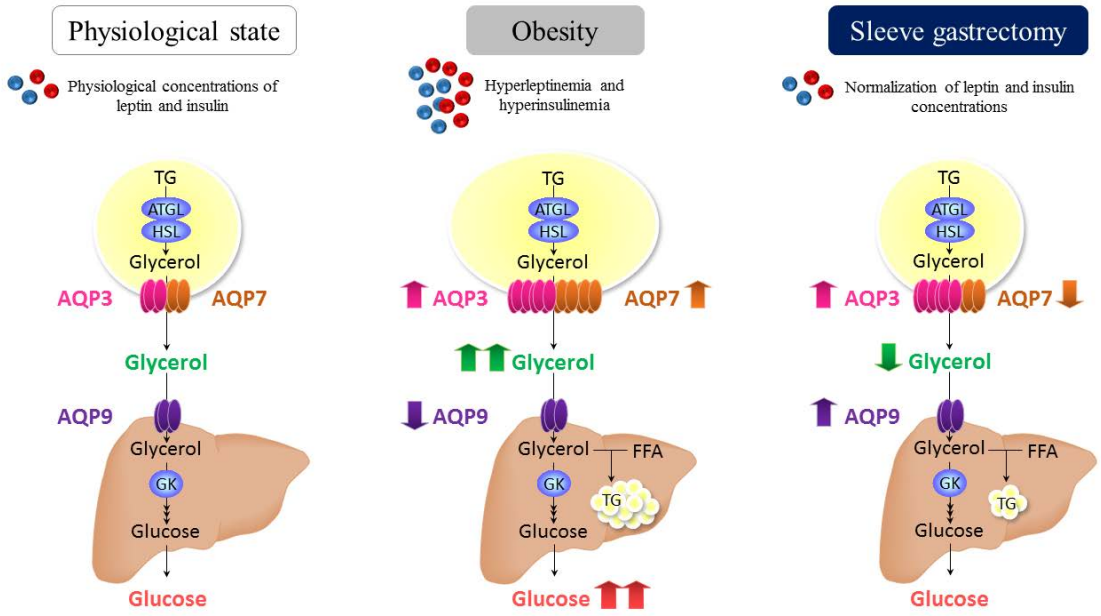


Figure 17. Proposed working model for the role of aquaglyceroporins in the improvement of hepatic steatosis and gluconeogenesis in the physiological state, obesity and after sleeve gastrectomy. Obesity is associated with an increased expression of aquaglyceroporins AQP3 and AQP7 in the adipose tissue leading to an increased glycerol output from fat cells and glycerol use for hepatic gluconeogenesis and lipogenesis increase. Sleeve gastrectomy restores the coordinated regulation of fat-specific AQP7 and liver-specific AQP9, contributing to a reduction in circulating glycerol that reduced the excessive lipid accumulation in liver parenchyma as well as decreasing whole-body glucose levels.

In study IV, gastric plication did not change the expression of AQP9 or factors involved in lipogenesis (*Ppara, Pparg* and *Srebf1*) or gluconeogenesis (*Gk, Pck1, G6pc* and *Slc2a2*) in the liver. Despite the lack of effect of gastric plication on hepatosteatosis, this restrictive bariatric surgery technique improved basal glycemia and glucose tolerance, which is in accordance with data reported by other authors (Guimarães *et al*, 2013). It seems plausible that the decrease in adipose AQP3 after gastric plication might contribute to the prevention of excessive plasma glycerol availability used for hepatic gluconeogenesis, thereby preventing high circulating glucose levels (**Figure 18**). However, the contribution of other mechanisms in insulin-sensitive organs cannot be discarded.

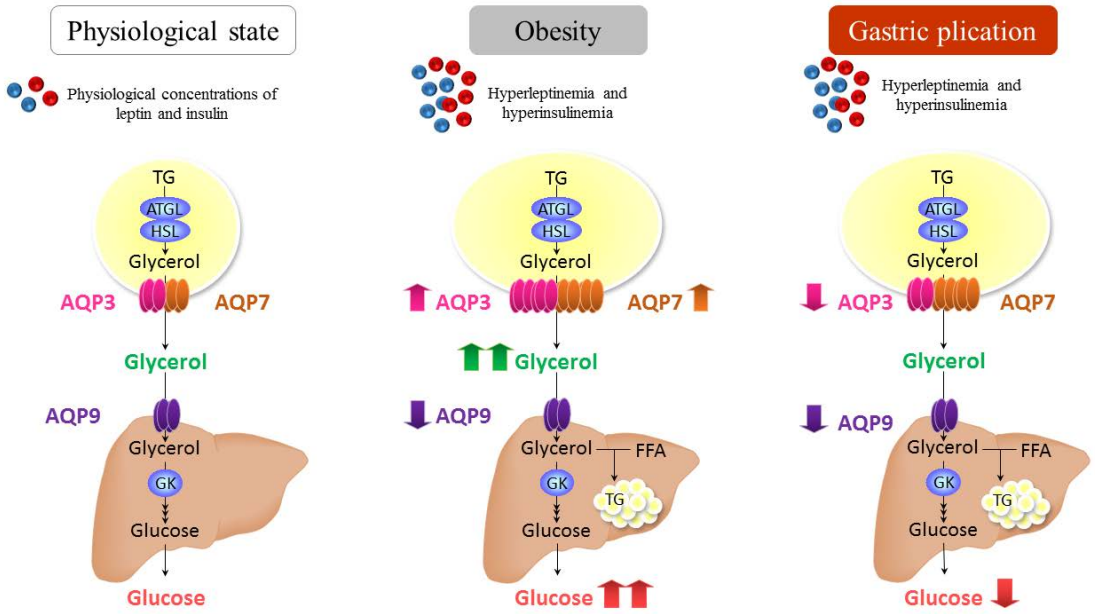


Figure 18. Impact of gastric plication in hepatic steatosis and gluconeogenesis. Gastric plication induced a decrease in the expression of AQP3 in the adipose tissue, which might contribute to the prevention of excessive plasma glycerol availability used for hepatic gluconeogenesis, thereby preventing high glucose levels.

6. Influence of sleeve gastrectomy on β-pancreatic function in diet-induced obese rats

Obesity is commonly associated with insulin resistance (Kahn *et al*, 2006). Under normal conditions, pancreatic islet β-cells increase insulin secretion sufficiently to overcome the reduced efficiency of insulin action, thereby maintaining normal glucose tolerance. In order to maintain an appropriate long-term glycemic control in insulin-resistant states, the number of pancreatic islet β-cells or β-cell mass, is expanded (de Koning *et al*, 2008). T2D occurs when pancreatic β-cell dysfunction leads to impaired insulin secretion in the context of insulin resistance (Turner *et al*, 1999, Heine *et al*, 2006). The β-cell dysfunction is characterized by a decreased insulin gene expression, blunted glucose-stimulated insulin secretion as well as increased β-cell apoptosis rates (Wajchenberg, 2007). Accordingly, in study III, we found that hyperinsulinemic and insulin-resistant obese rats exhibited adaptive changes in β-cell mass, evidenced by a 40% decrease in islet density as well as a slight, but not significant, increase in β-cell apoptosis. Sleeve gastrectomy restored insulin sensitivity, as evidenced by improved glucose levels during the OGTT and IPITT as well as a higher quantitative insulin sensitivity check index (QUICKI), which is in agreement with several studies (Rodríguez *et al*, 2012b, Wilson-Pérez *et al*, 2013b, Basso *et al*,

2016). Furthermore, this bariatric procedure improved insulin sensitivity in the fasted state.

Obesity-associated insulin resistance and hyperinsulinemia have been attributed to ectopic lipid overload, with lipotoxicity being a major contributor of β-cell dysfunction (Lee Y. *et al*, 2010, van Raalte *et al*, 2010, Ou *et al*, 2013). In this regard, obesity is strongly associated with pancreatic steatosis (Mathur *et al*, 2007, Lee J. S. *et al*, 2009, Lee Y. *et al*, 2010), which has been proposed as a link for the development of obesity-associated metabolic derangements including the metabolic syndrome (Lee J. S. *et al*, 2009), NAFLD (van Geenen *et al*, 2010) and T2D (Smits *et al*, 2011). The overload of FFA in β-cells promotes endoplasmic reticulum stress, oxidative stress, mitochondrial uncoupling and dysfunction, islet inflammation and β-cell apoptosis (van Raalte *et al*, 2010). In line with these observations, in the present thesis, obese rats showed pancreatic steatosis with pancreatic fat accumulation being positively associated with β-cell apoptosis. Interestingly, sleeve gastrectomy ameliorated obesity-associated pancreatic steatosis and reduced β-cell apoptosis, which is in agreement with other studies (Honka *et al*, 2015). Thus, sleeve gastrectomy improves β-cell apoptosis and steatosis contributing to the improvement of insulin secretion and sensitivity after surgery.

Bariatric surgery improves insulin sensitivity 2-fold to 3-fold within days after this procedure, which implicates mechanisms independent of weight loss that involve the modulation of intrinsic gut hormones via the gastro-entero-insular axis (Frühbeck, 2015). The incretin hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are among the most widely studied modulators of β-cell function, with the incretin effect accounting for 70% of the insulin secretion after an OGTT (Hussain A. *et al*, 2010, Romero *et al*, 2012). At the endocrine pancreas, GLP-1 binds its receptor GLP-1R and suppresses glucagon secretion from α-cells and potentiates insulin secretion from β-cells in a glucose-dependent manner. GIP is synthesized and secreted from K-cells and stimulates both insulin and glucagon secretion in the pancreas (Hussain M. A. *et al*, 2016). On the other hand, ghrelin acts as a survival factor promoting cell survival *in vitro* in HIT-T15 pancreatic β-cells (Granata *et al*, 2006) and *in vivo* in streptozotocin-induced diabetic mice (Bando *et al*, 2013). The ability of ghrelin to modulate insulin secretion in pancreatic islets remains controversial, with

some authors- pointing to an inhibitory effect of acylated ghrelin on insulin release *in vitro* (Qader *et al*, 2008) and others reporting that *in vivo* insulin secretion and islet architecture are not significantly different in transgenic overexpression of intraislet ghrelin (Bando *et al*, 2012). In study III, obese rats submitted to sleeve gastrectomy showed a dramatic reduction of circulating ghrelin levels, increased GLP-1 concentrations and no effect on plasma GIP compared to the pair-fed group, which is in accordance with other studies (Rodríguez *et al*, 2012b, Al-Sabah *et al*, 2014, Basso *et al*, 2016). In the *in vitro* experiments in RIN-m5F β-cells, we found that GLP-1 (9-36) promoted insulin secretion and reduced intracellular TG content. By contrast, acylated and desacyl ghrelin induced intracellular lipid accumulation in RIN-m5F β-cells, which is in agreement with the lipogenic effect of ghrelin isoforms in other metabolic tissues, including adipose tissue (Rodríguez *et al*, 2009) and liver (Porteiro *et al*, 2013, Ezquerro *et al*, 2016). However, contrary to other reports (Qader *et al*, 2008), neither desacyl nor acylated ghrelin modified insulin secretion in RIN-m5F β-cells. Taken together, the increased GLP-1 levels after sleeve gastrectomy might be mainly related to the improvement of insulin secretion, whereas reduced ghrelin levels appears to be responsible of the amelioration of pancreatic steatosis after surgery. Nonetheless, the contribution of additional hormones involved in β-cell function cannot be discarded.

7. Role of aquaglyceroporins in the restoration of β-pancreatic function after bariatric surgery

Insulin release can be induced not only by the activation of metabolically-regulated K_{ATP} channels, but also by VRAC that are activated by D-glucose concentrations within the range effective in modulating electrical activity in β-cells (Best *et al*, 2010). More precisely, the intracellular accumulation of lactate and $HCO3^-$ anions generated by the catabolism of D-glucose leads to β-cell swelling and, hence, the activation of the volume-sensitive anion channels, which induces plasma membrane depolarization and the subsequent gating of voltage-dependent Ca^{2+} channels and insulin secretion (Malaisse, 2008, Louchami *et al*, 2012). In line with this observation, AQP7 allows a rapid influx of glycerol to β-cells leading to β-cell swelling (Matsumura *et al*, 2007, Best *et al*, 2009). The activation of VRAC in response to β-cell swelling results in Cl^- efflux thereby generating an inward (depolarizing) current, leading to activation of voltage-sensitive Ca^{2+} channels, Ca^{2+} influx and hence, insulin exocytosis

(Muoio *et al*, 2008, Best *et al*, 2009, Virreira *et al*, 2011, Louchami *et al*, 2012). AQP7 not only induces insulin synthesis and exocytosis in β-cells, but also TG synthesis (Matsumura *et al*, 2007, Louchami *et al*, 2012) (**Figure 19**). In this regard, *Aqp7*-deficient mice exhibit hyperinsulinemia and increased pancreatic insulin-1 and insulin-2 transcript levels as well as increased intraislet glycerol and TG content (Matsumura *et al*, 2007). In study III, we first confirmed the water (*Pf*) and glycerol (*Pgly*) permeability of the rat RIN-m5F β-cells, a widely used cell line based on its high insulin secretion rate (Hohmeier *et al*, 2004). We found that the permeability values were within the range of the *Pf* and *Pgly* measured in mature murine 3T3-L1 adipocytes with endogenous AQP7 expression (Madeira *et al*, 2013) and, thus, reflect the contribution of aquaporins in RIN-m5F β-cells for water and glycerol transport. In line with previous studies (Matsumura *et al*, 2007, Louchami *et al*, 2012), we found that AQP7 was mainly localized in the β-cells of the Langerhans islets of our experimental animals.

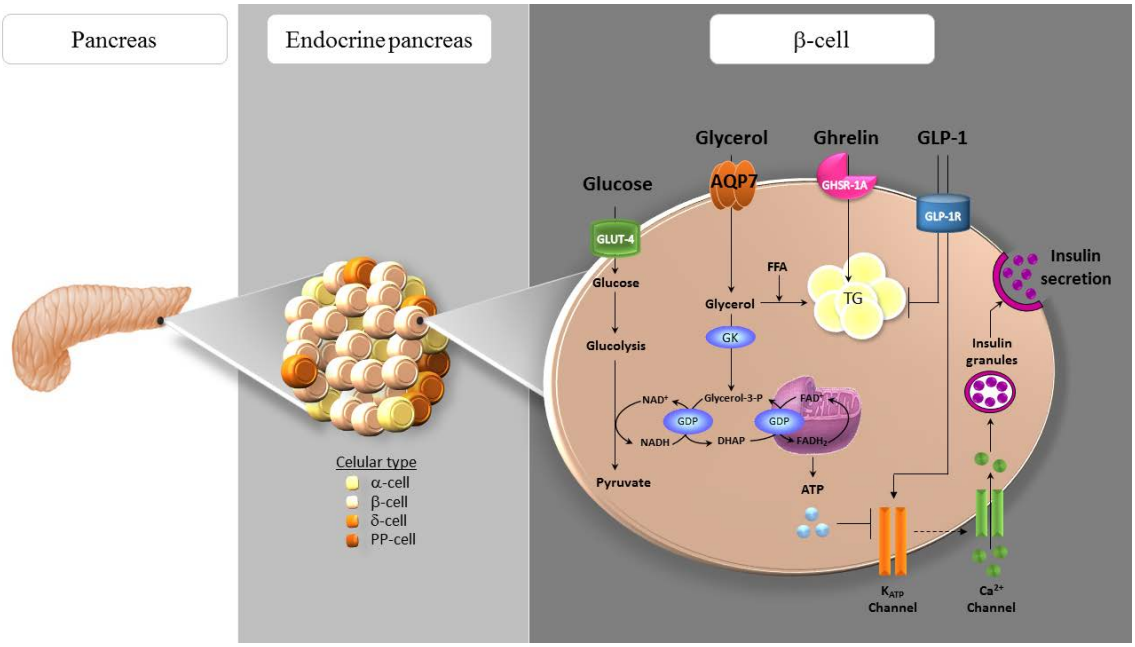


Figure 19. Role of ghrelin and GLP-1 in AQP7-induced insulin secretion and TG accumulation in β-cells. AQP7 facilitates glycerol influx to β-cells. The increase in intracellular glycerol and the consequent activation of GK activity, in turn, stimulate the pro-insulin mRNA and insulin secretion, probably through their participation in the glycolysis and glycerol-phosphate shuttle activities in β-cells. Glycerol can be also used as a substrate for *de novo* synthesis of TG. Both ghrelin and GLP-1 down-regulated AQP7 expression in β-cells. The subsequent increase in intracellular glycerol might be used for the biosynthesis of TG

induced by ghrelin as well as for insulin synthesis and secretion triggered by GLP-1. PP: pancreatic polypeptide.

To gain further insight into the molecular mechanisms triggering the improvement of β-cell function, the role of ghrelin and GLP-1 in the expression of pancreatic AQP7 was studied. Acylated and desacyl ghrelin constitute negative regulators of AQP7 in adipocytes and this downregulation contributes, in part, to the lipid accumulation in fat cells (Rodríguez *et al*, 2009). Accordingly, in study III, we found that acylated and desacyl ghrelin diminished AQP7 expression in parallel to an increased TG content in RIN-m5F β-cells. However, we could not replicate the ability of ghrelin to stimulate insulin secretion reported by other authors (Yada *et al*, 2014). Interestingly, GLP-1 (9-36) showed a tendency towards a downregulation of AQP7 in RIN-m5F β-cells with AQP7 protein expression being negatively associated with insulin release. Thus, it seems plausible that the reduction of AQP7 induced by ghrelin and GLP-1 might result in intracellular glycerol accumulation, which can be used for the biosynthesis of TG as well as for insulin synthesis and secretion (**Figure 19**).

Obesity and obesity-associated insulin resistance are associated with an altered gene expression profile of AQP7 in insulin-sensitive tissues, such as adipose tissue (Marrades *et al*, 2006, Prudente *et al*, 2007, Catalán *et al*, 2008, Rodríguez *et al*, 2011b), liver (Rodríguez *et al*, 2011b, Rodríguez *et al*, 2014, Rodríguez *et al*, 2015a) and skeletal muscle (Wakayama *et al*, 2014). To the best of our knowledge, we report, for the first time, that both weight gain and weight loss achieved by sleeve gastrectomy were related with higher AQP7 mRNA and protein levels in rat pancreas. AQP7 upregulation might constitute an adaptive response of β-cells to increase glycerol uptake and the subsequent insulin synthesis and secretion, which seems nevertheless inefficient to reduce the hyperglycemia in the obese state, but not after bariatric surgery. This beneficial effect of sleeve gastrectomy is beyond food intake reduction, since no effects of pair-feeding on AQP7 expression in the pancreas were observed.

In the present thesis, we also studied the potential role of AQP12, the other pancreatic aquaporin, in the improvement of β-cell function after bariatric surgery, AQP12 is reportedly expressed in the acinar cells of the pancreas and a potential role of this superaquaporin in the maturation and exocytosis of zymogen granules due to its intracellular location has been proposed (Itoh *et al*, 2005, Ohta *et al*, 2009). In study III, we show that AQP12 is also expressed in β-cells of the Langerhans islets based on the

immunohistochemical staining in histological sections of rat pancreas as well as by the gene and protein expression data in RIN-m5F β-cells and rat pancreas. Interestingly, acylated ghrelin- and GLP-1-induced AQP12 downregulation in the RIN-m5F cell line was neither related to insulin release nor to TG accumulation pointing to other functions of AQP12 in β-cells. In this regard, the increased pancreatic expression of AQP12 together with the positive association between this superaquaporin with markers of insulin resistance (insulinemia and HOMA) and ectopic lipid overload (serum TG and intrapancreatic TG content) suggest that AQP12 might constitute a marker of pancreatic damage. In line with this observation, *Aqp12*-knockout mice present an increased susceptibility to caerulein-induced acute pancreatitis, showing larger exocytic vesicles (vacuoles) in the pancreatic acini (Ohta *et al*, 2009). The normalization of pancreatic AQP12 expression after sleeve gastrectomy might reflect the restoration of pancreatic function due to the reduction of intrapancreatic steatosis and improved insulin secretion.

In conclusion, sleeve gastrectomy restores the altered expression of pancreas-specific AQP7 and AQP12 in obese rats contributing to the prevention of excess lipid accumulation and impaired insulin secretion in β-cells. Our results identify these aquaporins as key elements in mediating part of the beneficial effects of bariatric surgery on glucose metabolism via the regulation of glycerol availability, a key metabolite for pancreatic insulin synthesis and secretion as well as TG accumulation. In line with this observation, ghrelin and GLP-1, two important hormones involved in the resolution of insulin resistance after bariatric surgery, regulate the expression of these aquaporins in pancreatic β-cells.

Conclusions

1. Sleeve gastrectomy decreases body weight and adiposity and improves insulin sensitivity, β-cell function, lipid profile and hepatosteatosis of obese rats. By contrast, gastric plication improves basal glycemia and glucose tolerance, with a modest reduction in whole-body adiposity and intrahepatic triacylglycerol accumulation.

2. Sleeve gastrectomy and gastric plication downregulate the expression of AQP7 and AQP3, respectively, in epididymal and subcutaneous white adipose tissue. These changes are associated with improvement of adipocyte metabolism after bariatric surgery, with the reduction of AQP7 being related to lower adipocyte hypertrophy whereas the decrease in AQP3 reflects a reduction in adipocyte glycerol release.

3. Sleeve gastrectomy, but not gastric plication, induces a slight increase in hepatic AQP9 expression, which might reflect the recovery of glycerol uptake due to the improvement of hepatic steatosis and gluconeogenesis following weight loss achieved with this bariatric surgery technique.

4. Sleeve gastrectomy is associated with an increase in pancreatic AQP7 expression and a normalization of the increased AQP12 levels in the pancreas of obese rats. The upregulation of AQP7 appears to be an adaptive response of β-cells to increase glycerol uptake and the subsequent insulin synthesis and secretion. The normalization of AQP12 levels might reflect the reduction of β-cell injury induced by bariatric surgery, since this superaquaporin is positively associated with markers of insulin resistance and pancreatic steatosis.

5. Ghrelin and GLP-1 constitute negative regulators of AQP7 in RIN-m5F β-cells. The subsequent increase in intracellular glycerol might be used for the biosynthesis of triacylglycerols induced by ghrelin as well as for insulin synthesis and secretion triggered by GLP-1.

Concluding remarks

Sleeve gastrectomy constitutes a more effective technique to improve obesity-associated metabolic derangements than gastric plication. Our results identify aquaglyceroporins as key elements in mediating part of the beneficial effects of bariatric surgery via the regulation of glycerol availability, a key metabolite for the control of fat accumulation control, whole-body glucose homeostasis and insulin secretion. The altered expression of aquaglyceroporins in adipose tissue (AQP3 and AQP7), liver (AQP9) and pancreas (AQP7) in diet-induced obese rats is restored after sleeve gastrectomy. These changes contribute, in part, to the prevention of adipocyte hypertrophy and excessive lipid accumulation in the liver and pancreas as well as with an amelioration of β-cell function and insulin sensitivity after sleeve gastrectomy. By contrast, gastric plication restores glycemia by AQP3 downregulation, which entails lower efflux of glycerol from fat, lower plasma glycerol availability, and a reduced use of glycerol as a substrate for hepatic gluconeogenesis.

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Zhu J, Gupta R, Safwa M. The Mechanism of metabolic surgery: gastric center hypothesis. *Obes Surg* 2016;26:1639-41.

Other related publications

1. Regulation of adipocyte lipolysis

Article

Frühbeck G, **Méndez-Giménez L**, Fernández-Formoso JA, Fernández S, Rodríguez A. Regulation of adipocyte lipolysis.
Nutr Res Rev 2014;27(1):63-93.

Main objective

Overview of the control of adipocyte lipolysis by classic and novel factors together with analysis of the molecular mechanisms underlying this catabolic process as well as its involvement in the onset of obesity-associated diseases.

Specific objectives

- To review the control of lipolysis by classic factors, such as catecholamines, insulin, cytokines and other hormones, including ghrelin or the endocannabinoid system.
- To identify the influence of the subcellular compartmentalization of lipases.
- To outline the relevance of lipid droplet proteins and lipid-binding proteins.
- To characterize the changes in adipocyte lipolysis in human obesity and their metabolic impact.

Nutrition Research Reviews (2014), **27**, 63–93
doi:10.1017/S095442241400002X

Regulation of adipocyte lipolysis

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Abstract

In adipocytes the hydrolysis of TAG to produce fatty acids and glycerol under fasting conditions or times of elevated energy demands is tightly regulated by neuroendocrine signals, resulting in the activation of lipolytic enzymes. Among the classic regulators of lipolysis, adrenergic stimulation and the insulin-mediated control of lipid mobilisation are the best known. Initially, hormone-sensitive lipase (HSL) was thought to be the rate-limiting enzyme of the first lipolytic step, while we now know that adipocyte TAG lipase is the key enzyme for lipolysis initiation. Pivotal, previously unsuspected components have also been identified at the protective interface of the lipid droplet surface and in the signalling pathways that control lipolysis. Perilipin, comparative gene identification-58 (CGI-58) and other proteins of the lipid droplet surface are currently known to be key regulators of the lipolytic machinery, protecting or exposing the TAG core of the droplet to lipases. The neuroendocrine control of lipolysis is prototypically exerted by catecholaminergic stimulation and insulin-induced suppression, both of which affect cyclic AMP levels and hence the protein kinase A-mediated phosphorylation of HSL and perilipin. Interestingly, in recent decades adipose tissue has been shown to secrete a large number of adipokines, which exert direct effects on lipolysis, while adipocytes reportedly express a wide range of receptors for signals involved in lipid mobilisation. Recently recognised mediators of lipolysis include some adipokines, structural membrane proteins, atrial natriuretic peptides, AMP-activated protein kinase and mitogen-activated protein kinase. Lipolysis needs to be reanalysed from the broader perspective of its specific physiological or pathological context since basal or stimulated lipolytic rates occur under diverse conditions and by different mechanisms.

Key words: Catecholamines: Insulin: Hormone-sensitive lipase: Adipocyte TAG lipase: Perilipin: Adipokines: Lipid mobilisation

Introduction

Under normal conditions, the adipose tissue is able to fine-tune a series of neuroendocrine signals to precisely adapt the balance between TAG synthesis (lipogenesis) and breakdown (lipolysis) to meet physiological needs. In higher eukaryotes adipocyte TAG depots represent the major energy reserve of the organism as a result of the constant flux between lipolysis and re-esterification[1–5]. During energy surplus adipocytes accomodate the excess fuel as TAG for retrieval during periods of negative energy balance such as fasting, starvation or long-term exercise. The hydrolysis of TAG produces NEFA and glycerol that are released into the vasculature for use as energy substrates by other organs. Since TAG are not able to pass through biological membranes they need to be cleaved by TAG hydrolases, also termed lipases, before entering or exiting cells[6,7]. The ability to rapidly mobilise lipid reserves as NEFA to subvene energy demands represents a highly adapted metabolic response. In addition, the balance between the lipogenic drive and the lipolytic rate prevents an exaggerated elevation of plasma NEFA, which is considered a key aetiological factor in the development of insulin resistance[8,9]. Thus, the fat-storing ability of adipocytes prevents the appearance of lipotoxicity (lipid-induced dysfunction) and lipoapoptosis (lipid-induced programmed cell death) in other

Abbreviations: ACSL1, long-chain acyl-CoA synthetase 1; AMPK, AMP-activated protein kinase; AQP, aquaporin; ATGL, adipocyte TAG lipase; cAMP, cyclic AMP; CB, cannabinoid receptor; CD36, fatty acid translocase; CGI-58, comparative gene identification-58; Cide, cell death-inducing DFFA (DNA fragmentation factor-α)-like effector; COPI, coat protein complex I; DAG, diacylglyerol; ERK, extracellular signal-related kinase; FABP4, fatty acid-binding protein 4; FATP, fatty acid transport protein; *G0S2*, G0/G1 switch gene 2; GH, growth hormone; G_i, G-inhibitory protein; GLP-1, glucagon-like peptide-1; HSL, hormone-sensitive lipase; IRS, insulin receptor substrate; LC3, light chain 3; LPL, lipoprotein lipase; MAP, mitogen-activated protein; MGL, monoacylglycerol lipase; mTOR, mammalian target of rapamycin; PDE-3B, phosphodiesterase-3B; PI3K, phosphatidyl inositol 3-kinase; PKA, protein kinase A; PTH, parathyroid hormone; RNAi, RNA interference; ZAG, Zn-α_2-glycoprotein.

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tissues (especially skeletal muscle and liver)[10–12]. While the metabolic importance of lipolysis remains unchanged, established models of adipose tissue lipolysis have undergone substantial revision lately. Notably, adipocyte lipid droplets are now considered dynamic organelles critical for the handling of lipid stores, containing specific structural proteins and lipid-metabolising enzymes involved in the modulation of both basal and hormone-regulated lipolysis[13–17]. Current knowledge in this field is reviewed from the broader perspective of providing an overview of the classic lipolytic factors as well as by focusing on the recently identified influence of the subcellular compartmentalisation of lipases, the relevance of lipid droplet proteins and lipid-binding proteins, as well as the activation of the different signalling pathways together with their regulation.

Control of lipolysis

Lipolysis constitutes the catabolic process leading to the breakdown of TAG into glycerol and NEFA in the adipose tissue[2]. Basal lipolytic activity of adipocytes is conditioned by sex, age, physical activity, fat depot location, species and genetic variance, whereas stimulated adipocyte lipolysis is regulated by multiple factors, which are depicted in Fig. 1 [18,19]. Interestingly, fat cell lipolysis exhibits species-unique characteristics based on the predominance of specific receptors and their relative density and expression[20,21]. A decreased lipolytic rate is observed both in the early years of life and the elderly in relation to the action of catecholamines and insulin[22–25]. For the same BMI, women exhibit higher NEFA circulating concentrations than men due to their constitutively larger fat depots and subcutaneous adipocytes[26]. Regional differences in the sensitivity to catecholamine-stimulated and insulin-inhibited lipolysis further underlie these sex-specific characteristics, which will be described more extensively below. An increased basal lipolysis together with an enhanced lipolytic sensitivity to catecholamines take place during situations of negative energy balance such as fasting, starvation or semi-starvation, contributing to the increased mobilisation of NEFA from adipocytes and the subsequent fat mass loss when maintained over time[2]. As in situations of energy deprivation, during prolonged exercise plasma NEFA increase in response to the elevated release of catecholamines and decreased production of insulin[27]. Both short- and long-term endurance training make adipocytes more sensitive to catecholamine stimulation via adrenoceptor signal transduction changes[28–31].

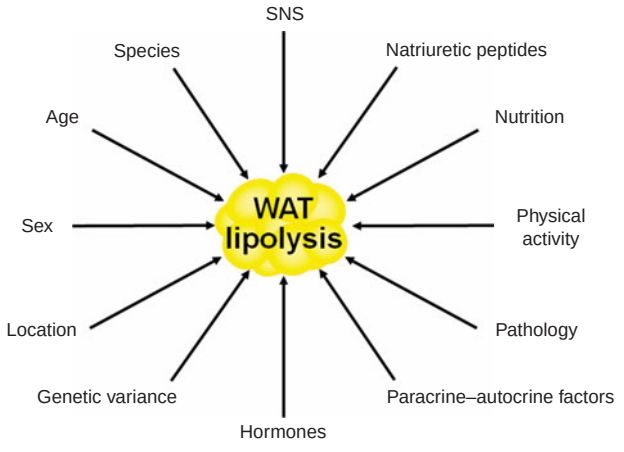

Fig. 1. Main factors influencing adipocyte lipolysis. SNS, sympathetic nervous system; WAT, white adipose tissue. (A colour version of this figure can be found online at http://www.journals.cambridge.org/nrr)

Some dietary compounds also have the capacity to exert a direct impact on lipolysis regulation. The well-known lipolytic effect of caffeine and other methylxanthines occurs by elevating the cyclic AMP (cAMP) intracellular levels by two mechanisms. On the one hand, this is through A_1-adenosine receptor antagonism, leading to a reduction of adenylyl cyclase activity and subsequent increased lipolysis. On the other hand, methylxanthines further prevent the breakdown of cAMP by inhibiting phosphodiesterase activity[3]. Thus, coffee consumption increases lipid turnover and raises plasma NEFA, while a high intake of methylxanthines may also contribute to weight loss and maintenance through an enhanced fat oxidation and thermogenesis[32,33]. Another dietary compound influencing adipocyte lipolysis is Ca, with high intakes being associated with decreased adiposity and a reduced risk of obesity in diverse epidemiological studies[3]. Ca supplementation reportedly favours weight loss in both obese mice and human subjects undergoing energy-restricted diets, stimulating lipolysis via inhibition of the secretion of parathyroid hormone (PTH)[34] and the subsequent activation of 25-hydroxycholecalciferol to 1,25-dihydroxycalciferol[35–38]. While acute ethanol intake exerts an anti-lipolytic effect, chronic ethanol consumption suppresses the β-adrenergic receptor-mediated lipolytic action via an increased activation of phosphodiesterase, resulting in a decreased protein kinase A (PKA) stimulation and a diminished activating phosphorylation of perilipin-1 and hormone-sensitive lipase (HSL)[39].

Genetic variance also plays a role in determining lipolytic rate[5,18,40]. Variations in adrenoceptors have been intensely analysed for their putative functional effects on lipolysis and association with the development of obesity. The most studied are the polymorphisms in codon 64 of the β_3-adrenergic receptor and in codons 16, 27 and 164 of the β_2-adrenoceptor. The Trp64Arg missense mutation of the β_3-adrenergic receptor gene was reportedly associated with decreased lipolysis induced by β_3-adrenoceptor agonists[41]. However, other studies have failed to show any phenotypic effect of this polymorphism, so its true pathophysiological contribution to fat metabolism and energy homeostasis in humans remains controversial[18]. Noteworthy, variations in non-coding regions of calpain 10 lead to a decreased β_3-adrenergic receptor

function. In the β_2-adrenergic receptor gene the Arg16Gly mutation has been shown to be associated with altered β_2-adrenergic receptor function, with carriers of this mutation showing a five-fold increased agonist sensitivity[18]. The Gln27Glu substitution was found to be twice as common in obese than in non-obese subjects in some populations, with homozygotes exhibiting an average excess fat mass of 20 kg and about 50 % larger fat cells[42]. On the contrary, the rare Thr164Ile substitution in the β_2-adrenergic receptor gene has not been consistently observed in obese individuals. Polymorphisms in the G-β_3 gene, encoding for a specific G-coupling protein that links α- as well as β-adrenergic receptors to adenylate cyclase, alter catecholamine-induced lipolysis in human fat cells, improving the lipolytic function of β-adrenoceptors at the same time as enhancing the anti-lipolytic activity of α_2-adrenoceptors. Furthermore, variations in intronic dinucleotide repeats of the HSL gene are accompanied by a decreased function of the lipase with a reduced lipolytic effect of catecholamines[43,44].

Classic factors

In humans the main elements controlling lipolysis are the activity of the autonomic nervous system and the endocrine influence derived from the release of insulin[2,18,45]. Adipose tissue is richly innervated by both the sympathetic and parasympathetic nervous systems with nerve terminals running along blood vessels and a certain number of adipocytes in direct contact with nerve varicosities. Thus, electrical stimulation of sympathetic nervous system nerve endings results in an increase in lipolytic activity, while surgical sympathectomy reportedly reduces lipolysis in the denervated adipose depot[46–49]. Although the parasympathetic nervous system has been shown to also innervate white adipose tissue and decrease lipolysis, stimulating an increase in insulin sensitivity[50,51], its true functional role has been subsequently questioned[52].

Catecholamine-induced regulation. Catecholamines, adrenaline and noradrenaline, exert their impact on lipolysis upon binding to the diverse adrenergic receptor subtypes located on the plasma membrane of adipocytes[2,45,53]. These receptors are linked to G-proteins, with G-protein receptor complexes regulating adenylate cyclase in the cell membrane. In mammals at least four adrenoceptors exert their action with marked species characteristics[4]. In humans β_1- and β_2-adrenoceptors are the most active lipolytic elements, while the contribution of β_3-adrenergic receptors remains to be better established. The presence of β_3-adrenoceptors in human white adipocytes has been clearly proven with tissue and subcellular distribution as well as response to stimulators being consistent with participation in lipolysis[54]. However, the failure of β_3-adrenoceptor agonists to elicit clear-cut lipolytic and weight-loss effects in obese patients casted doubts on the true physiological relevance of this β-adrenoceptor subtype in humans[55,56]. Contrarily, β_3-adrenoceptors are abundantly expressed in adipocytes of rodents[57]. Upon binding to their ligand, β-adrenergic receptors initiate the activation of the lipolytic cascade through the stimulation of cAMP production and subsequent activation of the cAMP-dependent PKA, which is followed by the phosphorylation of perilipin and HSL, ultimately leading to lipolysis stimulation (Fig. 2). Another peculiarity of human adipocytes resides in the presence of abundant α_2-adrenoceptors, which are coupled to G-inhibitory proteins (G_i), thereby inhibiting cAMP production and, thus, lipolysis[58,59]. Therefore, the balance between the lipolytic effect of β-adrenergic receptors and the opposing anti-lipolytic activity of α_2-adrenoceptors also determines the net outcome of catecholamine-induced fat mobilisation in humans. The identification of brown adipose tissue in human adults beyond the vestigial amounts originally acknowledged and its association with BMI and adiposity has triggered a re-focusing of attention to the true relevance of β_3-adrenoceptors in lipid metabolism and energy homeostasis[60,61].

Hormone-mediated control. A number of hormones are known to participate in the regulation of lipolysis. Among all endocrine factors, insulin is quantitatively and qualitatively the most relevant one. The impact of growth hormone (GH), adrenocorticotropic hormone, cortisol, thyroid hormones, PTH and glucagon is comparatively much more reduced than that of insulin. The mechanisms of action of all are briefly discussed below.

Hormone-mediated control: insulin. Insulin is a key regulator of white adipose tissue biology, controlling not only lipogenesis but also the rate of lipolysis and NEFA efflux. Insulin regulates glucose uptake by adipocytes and triggers fatty acid transport protein translocation as well as fatty acid uptake by fat cells[62]. Binding of insulin to its specific cell-surface receptor produces tyrosine phosphorylation and activation of the insulin receptor, which leads to the interaction with the insulin receptor substrates (IRS-1 and IRS-2), in turn activating the phosphatidyl inositol 3-kinase (PI3K) complex[2]. Insulin powerfully inhibits basal and catecholamine-induced lipolysis through phosphorylation (via a PKB/Akt-dependent action) and activation of phosphodiesterase-3B (PDE-3B). The phosphodiesterase catalyses the breakdown of cAMP to its inactive form, thereby decreasing cAMP levels, which in turn reduces PKA activation and, therefore, also translates into preventing HSL stimulation. Insulin may also suppress lipolysis through phosphorylation of the regulatory subunit of protein phosphatase-1 (PP-1), which once activated rapidly dephosphorylates and deactivates HSL, thus decreasing the lipolytic rate[63]. The anti-lipolytic effect of insulin is observed already minutes upon binding of the hormone to its receptors.

Hormone-mediated control: growth hormone. While insulin repesents the primary anabolic hormone exerting the main influence periprandially, GH operates directly and through stimulation of insulin growth factor-1, insulin

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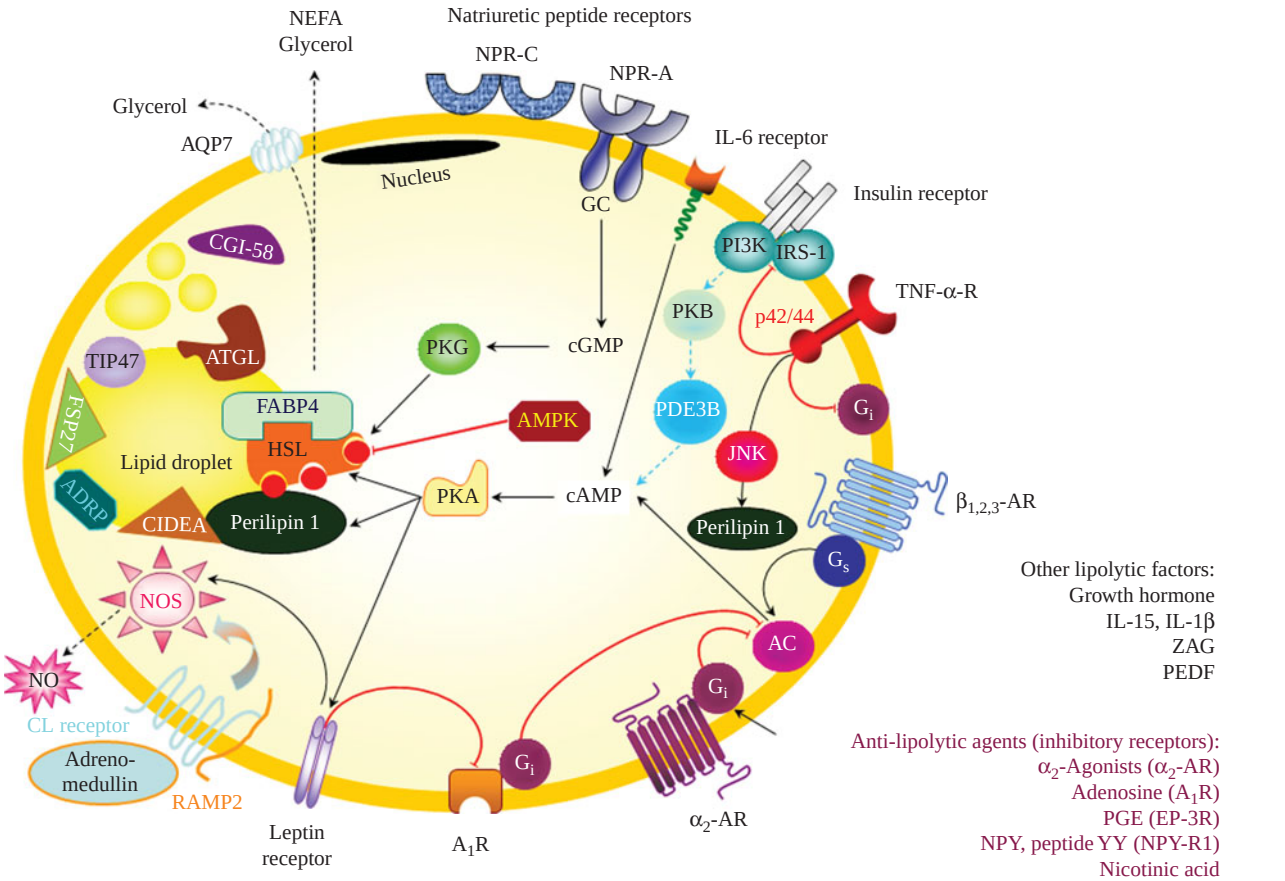

Fig. 2. Principal regulators and major pathways involved in adipocyte lipolysis. A_1R, A_1 adenosine receptor; AC, adenylyl cyclase; ADRP, adipophilin/adipocyte differentiation-related protein; AMPK, AMP-activated protein kinase; AQP7, aquaporin 7; AR, adrenoreceptor; ATGL, adipocyte TAG lipase; cAMP, cyclic AMP; CGI-58, comparative gene identification-58; cGMP, cyclic GMP; CIDEA, cell death-inducing DFFA (DNA fragmentation factor-α)-like effector A; CL, calcitonin receptor-like; EP-3R, PGE receptor 3; FABP4, fatty acid binding protein 4; FSP27, fat-specific protein 27; GC, guanylyl cyclase; G_i, inhibitory GTP-binding proteins; G_s, stimulatory GTP-binding proteins; HSL, hormone-sensitive lipase; IRS-1, insulin receptor substrate-1; JNK, Jun kinase; NOS, NO synthase; NPR, natriuretic peptide receptor; NPY, neuropeptide Y; NPY-R1, neuropeptide Y receptor 1; PDE3B, phosphodiesterase 3B; PEDF, pigment epithelium-derived factor; PI3K, phospatidylinositol-3 kinase; PKA, protein kinase A; PKB, protein kinase B; PKG, protein kinase G; RAMP2, receptor activity modifying protein-2; TIP47, tail-interacting protein of 47 kDa; TNF-α-R, TNF-α receptor; ZAG, zinc-α_2-glycoprotein. (A colour version of this figure can be found online at http://www.journals.cambridge.org/nrr)

and NEFA during stress and fasting[64]. Thus, GH represents a less potent though critically important regulator of lipolysis, which influences body composition, stimulating muscle mass accretion at the same time as reducing adiposity by a direct lipolytic effect using cAMP- and PKA-dependent pathways. GH-deficient individuals can experience up to a 40 % reduction in plasma NEFA and lipolysis that are returned to normal values by GH replacement therapy. Interestingly, GH activates adenylyl cyclase by selectively shifting the $G_i\alpha_2$ subunit and removing cAMP production inhibition[65]. Exogenous GH administration produces an increase in NEFA after 2–3 h, thus reflecting a delayed lipolytic effect when compared with that of catecholamines. In this context, small physiological GH pulses reportedly increase interstitial glycerol levels in abdominal and femoral fat[66]. In addition, suppression of the normal nocturnal rise in GH is followed by a reduction in subsequent lipolysis in subcutaneous adipose tissue[67]. Endogenous GH has been shown to play a limited metabolic role during the daily fed–fast cycle, whereas it is essential for the increased lipolytic rate observed with more prolonged fasting[68]. Recently, adipocyte-specific disruption of JAK2 (JAK2A) in mice has been shown to result in GH resistance in adipocytes, with reduced lipolysis and increased body fat, thereby offering complementary mechanistic insights into the well-recognised effects of GH on lipid flux[69].

Hormone-mediated control: other hormones. Cortisol also exerts a lipolytic effect, which is less potent than that of catecholamines at the same time as being delayed (minutes in the case of adrenaline *v.* hours for cortisol)[62,70]. Importantly, the *in vivo* lipolysis stimulation is counteracted by the corticoid-induced insulin release[71,72], whereby the net outcome of a short-term treatment with a standard dose of corticosteroids is an increase in abdominal adipose tissue lipolysis, without changes in GH concentrations, hyperglucagonaemia and insulin resistance. While a stimulation of lipolysis in human adipose tissue has been also ascribed to PTH[20,73], it has also been suggested that a PTH excess

may promote weight gain by impeding catecholamine-induced lipolysis[34]. Whereas in rodents testosterone up-regulates catecholamine-induced lipolysis[74], in humans testosterone in physiological concentrations causes a depot-specific reduction of catecholamine-stimulated lipolysis in subcutaneous fat cells, probably due to reduced protein expression of β_2-adrenoceptors and HSL[75–77]. The relevance of androgen signalling in lipolysis regulation became evident from the observation that late-onset obesity development in androgen receptor-null male mice was caused in part by a decreased lipolytic activity[78]. The direct molecular mechanism accounting for the hypertrophic adipocytes and expanded white adipose tissue of these mice depends on an altered lipid homeostasis characterised by a decreased lipolysis but not an increased lipogenesis. Interestingly, transcripts for HSL were strikingly decreased, whereas those for lipogenic genes were unchanged or decreased. Androgens slightly decrease lipoprotein lipase (LPL) activity in human adipose tissue organ cultures, but markedly inhibit adipogenesis in primary preadipocyte cultures obtained from subcutaneous and omental depots of both sexes[79]. Thus, the androgenic effects on adipose tissue in men as opposed to women may differ more in terms of the magnitude of their negative impact on adipogenesis and lipid synthesis rather than in the direction of the lipolytic action.

Although commonly acting in rodent fat cells as lipolytic agents via stimulatory GTP-binding protein (G_s protein)-coupled receptors, thyrotropin-stimulating hormone, adrenocorticotropic hormone and α-melanocyte-stimulating hormone are either ineffective or very weak stimulators of lipolysis in human adipocytes[62]. Neither glucagon nor glucagon-like peptide-1 (GLP-1) has been clearly shown to stimulate lipolysis *in vitro*. Likewise, no significant effects of glucagon or GLP-1 on lipolytic rate or adipose tissue blood flow following local or experimental intravenous normo- and hyperglucagonaemia have been observed[80,81]. However, during the present decade the role of the GLP-1/GLP-1 receptor system in lipolysis has experienced renewed interest[82]. A dose-dependent lipolytic effect of GLP-1 in 3T3-L1 adipocytes in a receptor-dependent manner involving downstream adenylate cyclase/cAMP signalling has been shown[83].

Cytokines and other 'newcomers'

Over the past years adipose tissue has been recognised as an extraordinarily active endocrine organ with the ability to secrete numerous products of diverse nature such as hormones, cytokines, enzymes, complement factors, vasoactive peptides and growth factors, among others[84–87]. All these adipose-derived factors, collectively termed adipokines, are involved in a pleiad of physiological functions ranging from energy homeostasis to reproduction, including inflammation and immunity as well as angiogenesis and bone metabolism, among others[88–94]. The dynamic cross-talk of adipokines with other non-metabolic biological processes extends to the cardiovascular[95–99], gastrointestinal[100–103], respiratory[104–106] and muscular[107–110] systems. In addition to their participation in plentiful diverse physiological functions, many of the recently identified hormones and adipokines have also been shown to be able to directly affect lipolysis.

Cytokine regulation of lipolysis. Cytokine release by both adipocytes and stromovascular cells underlies the participation of adipose tissue in a dynamic cross-talk and potent feedback signalling with key neuroendocrine organs involved in the regulation of food intake, lipid metabolism, glucose disposal, energy expenditure and the stress response[111,112]. The complex secretory activities of adipose tissue also contribute to the development of insulin resistance and atherogenic processes[113–115]. The release of cytokines further exerts important local autocrine and paracrine effects, mainly involved in adipose tissue remodelling, adipogenesis, angiogenesis, inflammation and immunity. Noteworthy, cytokines, like TNF-α, as well as some interleukins and adipokines, are important regulators of spontaneous lipolysis.

Cytokine regulation of lipolysis: TNF-α. TNF-α is produced in large amounts by adipocytes and other cell types within adipose tissue[84,116]. In humans, contrarily to rodents, TNF-α is not released from adipose tissue into the circulation but rather acts predominantly as a local factor[117–119]. As with other lipolytic agents, important species differences have also been observed as regards TNF-α action. TNF-α is able to stimulate lipolysis by at least three separate mechanisms[117,120,121]. First, it inhibits insulin receptor signalling, thereby counteracting the anti-lipolytic effect of the hormone. In this respect, TNF-α operates via the inactivation of IRS-1. This can be brought about by the inhibition of tyrosine phosphorylation and by a reduction in the amount of IRS-1 in adipocytes. In fact, TNF-α counteracts tyrosine phosphorylation by promoting serine phosphorylation of IRS-1. The most important TNF-α effect on adipocyte IRS-1 is mediated through the p42/44 mitogen-activated protein (MAP) kinase (Fig. 2). Second, TNF-α is able to stimulate lipolysis by inhibiting the G_i-protein-coupled adenosine receptor signalling to counteract the anti-lipolytic effect of adenosine. TNF-α markedly decreases the protein content of all three $G_i\alpha$ subtypes in rodent fat cells, without changing the amount of G_s protein or β-subunit of the G-protein complex. This decrease in G_i protein mitigates the anti-lipolytic effect of adenosine. Interestingly, TNF-α decreases G_i-protein content through an induction of protein degradation by the proteasomal pathway[122]. However, the TNF-α–G_i interaction appears to be specific for rodents because it has not been observed in human fat cells. The third way by which TNF-α induces lipolysis is via direct stimulation of basal lipolysis through interactions with the lipid-binding protein perilipin. Only TNF-α receptor 1 and MAP kinases promote lipolytic effects in fat cells leading to phosphorylation and

decreased production of perilipin, the adipose lipid droplet coating protein that protects it from being hydrolysed by HSL[117,123,124]. Three MAP kinases, namely p44/42, Jun kinase (JNK) and p38, are activated by TNF-α in fat cells but only the first two have been linked to lipolysis so far.

Mechanistically, TNF-α can stimulate lipolysis in the absence of insulin, thus providing evidence that it does not simply antagonise the anti-lipolytic effects of insulin. Moreover, extracellular glucose is required for the TNF-α-induced lipolytic effect, suggesting that a certain nutritional state or substrate availability is required[119]. The downstream signals of the TNF-α receptor 1-dependent pathway involve the activation of extracellular signal-related kinases (ERK1/2), JNK, AMP-activated protein kinase (AMPK), inhibitor of κB kinase (IKK) and PKA[119,125,126]. However, in fat cells the TNF-α-induced activation of ERK1/2, JNK and IKK is rapid and transient, while TNF-α-induced lipolysis takes more than 6 h, suggesting the existence of more distant events that are likely to be controlled by transcriptional regulation[119,127].

Cytokine regulation of lipolysis: IL-6 and IL-15. The IL-6 receptor and glycoprotein 130, key elements of the cytokine pathway, are expressed in human adipocytes, pointing to a direct autocrine/paracrine action of IL-6 on fat cells[62]. Infusions of recombinant human IL-6 have been reported to increase plasma NEFA and glycerol concentrations, leading the authors to conclude that IL-6 represents a novel lipolytic factor that operates as a potent stimulator of lipolysis[128,129]. Interestingly, IL-6 infusions were accompanied by parallel increases in plasma cortisol and adrenaline levels, whereas the potential effect on GH concentrations was not analysed. In this regard, it is difficult to establish whether the increased lipolysis depends on the direct action of IL-6 or rather reflects the effects of other lipolytic factors such as GH, cortisol and noradrenaline[130]. A more recent study has shown that higher circulating IL-6 concentrations are associated with an increased isoproterenol-stimulated lipolysis especially in omental adipocytes in women[131]. In any case, the reported effect on lipolysis of IL-6 is relatively modest compared with that elicited by catecholamines and insulin. The potential involvement of IL-6 during the practice of exercise or other situations related to severe illness, where a clear need for an elevated lipid fuel takes place, has been set forward[132,133].

Another member of the interleukin family has been proposed to participate in the modulation of lipolysis. The administration of IL-15 has been shown to produce a significant reduction in white adipose tissue via both a decreased rate of lipogenesis and a reduction in LPL activity, without a concomitant decrease in food intake[134]. Comparative studies with other cytokines revealed that IL-15 is apparently more potent in its acute stimulation of lipolysis than IL-6 and TNF-α[135]. Noteworthy, when specific inhibitors of PKA or Janus kinase were present an attenuation of the lipolytic effect of IL-15 was observed. IL-15 is known to be highly expressed in skeletal muscle, exerting a potent anabolic effect on muscle protein accretion while decreasing fat depots in adipose tissue[136]. Taking these observations together, it can be speculated that IL-15 may operate as a homeorhectic factor that mobilises and directs energy away from the adipocyte to other cells during the acute phase of the inflammatory response.

Interestingly, IL-1β and TNF-α have been shown to activate MAP3K8, also called Tpl2, which is expressed in adipocytes and is implicated in cytokine-induced lipolysis[127]. Pharmacological inhibition or silencing of Tpl2 was able to prevent MAP kinase kinas/ERK1/2 activation by these cytokines but not by insulin, thereby providing evidence of its involvement in ERK1/2 activation particularly in response to inflammatory stimuli[127].

Cytokine regulation of lipolysis: leptin. More than a decade ago the identification of functional leptin receptors (OB-R) in white adipose tissue suggested the involvement of leptin in the direct peripheral regulation of adipocyte metabolism[137–139]. In fact, leptin was shown to directly participate in lipid metabolism control through the inhibition of lipogenesis and the stimulation of lipolysis. Leptin reportedly exerts an autocrine–paracrine lipolytic effect on isolated white adipocytes both *in vitro* and *ex vivo*[140–143].

Adenosine A_1 receptors have been shown to be markedly expressed in adipocytes and influence fat cell metabolism via the regulation of adenylyl cyclase and, therefore, participate in lipolysis control via the inhibitory guanosine 5′-triphosphate (GTP) binding proteins, G_i[144,145]. The adenosinergic system increases leptin secretion by directly activating adenosine A_1 in white adipose tissue[146]. In this respect, a defective leptin-induced stimulation of lipolysis that opposes the adenosine-mediated tonic inhibition was identified[143]. Interestingly, the lipolytic effect of leptin is located at the adenylyl cyclase-inhibitory G protein step (Fig. 2), providing an explanation for the defective stimulation of adipocyte adenylate cyclase and the blunted lipolysis observed in leptin-deficient and OB-R-lacking rodents as well as in morbidly obese humans[147–149]. Moreover, storage of surplus energy in white adipose tissue and the development of diet-induced obesity require the blockade of a latent leptin-stimulated energy sump in white adipocytes[150]. In this regard, the pleiotropic effects of leptin in other metabolically relevant organs like brown adipose tissue, skeletal muscle, pancreas, liver and heart need to be considered[108,151–157].

Cytokine regulation of lipolysis: adiponectin. Adiponectin, also known as Acrp30, AdipoQ, apM1 or GBP28, is a hydrophilic 30-kDa protein highly expressed and secreted by adipocytes[88,90]. The three-dimensional structure of the C-terminal globular domain of adiponectin shows a high structural homology with TNF-α, another well-known lipolytic cytokine[158]. Interestingly, HSL activity has been shown to be positively correlated to adiponectin expression, with percentage body fat and adiponectin

mRNA arising as the only independent predictors of adipose tissue HSL activity explaining 26 % of its variability[159]. Increased adipose tissue mass has been suggested to explain the association between low adiponectin and reduced NEFA tolerance[160]. Adiponectin has been shown to inhibit spontaneous and catecholamine-induced lipolysis in human adipocytes of non-obese subjects through AMPK-dependent mechanisms[161]. In contrast to most adipokines, which are markedly up-regulated in obesity, adipose tissue expression and circulating concentrations of adiponectin are decreased in both overweight and obesity, thereby implying a plausibly decreased impact on overall lipolysis. Adiponectin gene knockout mice and primary adipocytes obtained from these mice exhibit an increased lipolysis[162]. Moreover, adiponectin was shown to suppress HSL activation without modifying adipocyte TAG lipase (ATGL) and comparative gene identification-58 (CGI-58) expression in adipocytes. In addition, adiponectin reportedly reduced the type 2 regulatory subunit RIIα protein levels of PKA by reducing its protein stability, with ectopic expression of RIIα abolishing the inhibitory effects of adiponectin on lipolysis in adipocytes[162]. The proportion of secreted high-molecular-weight *v.* total adiponectin has been shown to be higher in visceral than in subcutaneous adipose tissue explants in non-obese individuals, while no differences were observed in obese individuals[163]. More recently, full-length adiponectin was shown to exert an anti-lipolytic effect in non-obese subcutaneous adipose tissue, while the globular and trimeric isoforms exhibited anti-lipolytic activity in obese subcutaneous and visceral adipose tissue, respectively[164].

Other elements involved in lipolysis. Analysis of the involvement of other factors in the control of lipolytic pathways is unravelling a huge number of potential modulators, which vary greatly not only in their biochemical structure but also in their main physiological effect and the signalling cascade activated.

Other elements involved in lipolysis: nitric oxide. NO or related redox species have been described to act as regulators of lipolysis both in rodent and human adipocytes[165–170]. Inhibition of NO release increased lipolysis independently of local blood flow changes. While chemical NO donors stimulate basal lipolysis, they block the characteristic isoproterenol-induced lipolytic activity via the inhibition of adenylyl cyclase and PKA. Inducible NO synthase has emerged as a negative modulator of lipolysis via an oxidative signalling pathway upstream of cAMP production[169].

A functional relationship between leptin and NO has been established in several physiological processes[139,171–175]. Given the co-localisation of both factors in fat cells and their involvement in lipolysis, a potential role of NO in the leptin-induced lipolytic effect seemed plausible. In fact, 1 h after exogenous leptin administration a dose-dependent increase in both serum NO concentrations and basal adipose tissue lipolytic rate was observed[143]. Up to 27 % of the variability taking place in lipolysis was attributable to the changes in NO concentrations. The leptin-induced NO production in white adipocytes was shown to be mediated through PKA and MAP kinase activation[176]. Inhibition of NO synthesis by N^{ω}-nitro-L-arginine methyl ester (L-NAME) pretreatment was followed by a reduction in the leptin-mediated lipolysis stimulation compared with leptin-treated control animals. Contrarily, in adipocytes obtained from rats under acute ganglionic blockade, the leptin-induced lipolytic effect did not show differences with the lipolytic rate achieved by leptin in control rats. The NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) was able to exert a significant inhibitory effect on isoproterenol-stimulated lipolysis. Thus, NO has emerged as a potentially relevant autocrine–paracrine physiological signal to fine-tune lipolysis by facilitating leptin-induced lipolysis and, at the same time, being able to inhibit catecholamine-induced lipolysis[173].

Other elements involved in lipolysis: natriuretic peptides. Until recently, human fat cell lipolysis was thought to be mediated essentially by a cAMP-dependent PKA-regulated pathway under the control of catecholamines and insulin. However, Lafontan *et al.*[177] provided evidence that natriuretic peptides also have the ability to potently stimulate lipolysis in human adipocytes to the same degree as a non-selective β-adrenoceptor agonist. This lipolytic effect is mediated mainly by natriuretic peptide receptor type A through a cyclic GMP-dependent PKG (cGK-I) signalling pathway (Fig. 2) that does not involve PDE-3B inhibition or cAMP production and PKA activity[178–182]. Noteworthy, *in vitro* studies have shown that HSL can also be phosphorylated by the cyclic GMP-dependent signalling cascade. In fact, cGK-I phosphorylates perilipin and HSL. Increases in plasma atrial natriuretic peptide levels by physiological (exercise) or pharmacological stimuli are followed by an enhanced lipid mobilisation[183,184]. In humans atrial natriuretic peptide also reportedly induces postprandial lipid oxidation, energy expenditure, and concomitantly arterial blood pressure[185,186]. Taken together, this pathway that participates in lipid mobilisation and energy homeostasis becomes especially important during chronic treatment with β-adrenoceptor antagonists, which inhibit catecholamine-induced lipolysis but enhance cardiac atrial natriuretic peptide release.

Other elements involved in lipolysis: endocannabinoid system. Our understanding of the participation of the endocannabinoid system in energy homeostasis has progressed enormously over the past years[187–189]. In particular, the observation of the presence of G protein-coupled cannabinoid receptor (CB) CB1 receptors in adipocytes provided a clue for the involvement of endocannabinoids in the peripheral control of lipid metabolism[190–193]. Selective CB1 antagonism was shown to coordinately induce key genes of the fatty acid catabolic pathway, thereby favouring lipolysis and reducing fat storage in adipose tissue[191]. Interestingly, the selective

antagonism of CB1 receptors reportedly induced β_3-adrenoceptors and GH receptors at the same time as repressing the expression of catechol-*O*-methyltransferase, an enzyme involved in the degradation of catecholamines. The reduced expression of this methyltransferase along with the induction of the receptors of two well-known hormones with lipolytic effects further supports the molecular basis for the participation of endocannabinoids in the modulation of lipolysis.

Amides of fatty acids with ethanolamine (FAE) are biologically active lipids participating in a variety of physiological effects, including appetite regulation. While the polyunsaturated FAE anandamide (arachidonoylethanolamide) increases food intake by activating G protein-coupled cannabinoid receptors, the monounsaturated FAE oleoylethanolamide (OEA) reduces feeding as well as body-weight gain and stimulates lipolysis by activating the nuclear receptor PPAR-α[194,195].

Other elements involved in lipolysis: ghrelin. Beyond its strong orexigenic effect, the gastrointestinal twenty-eight-amino acid octanoylated peptide ghrelin exerts a wide spectrum of actions including the inhibition of isoproterenol-induced lipolysis in rodent adipocytes[196]. Both ghrelin and des-acyl ghrelin have been shown to antagonise the catecholamine-stimulated lipolysis via a non-type 1A GH secretagogue receptor. Moreover, acylated and unacylated ghrelin have been also shown to attenuate isoproterenol-induced lipolysis in isolated rat visceral adipocytes through activation of phosphoinositide 3-kinase γ and PDE-3B[197]. However, ghrelin infusion in human subjects was observed to induce acute insulin resistance and lipolysis independent of GH signalling[198]. All of the elements of the ghrelin system have been identified in human adipocytes, including receptors and isoforms as well as the ghrelin-*O*-acyltransferase or GOAT enzyme[199,200]. Interestingly, in differentiating omental adipocytes, incubation with both acylated and desacyl ghrelin increased PPAR-γ and sterol regulatory element-binding protein-1 mRNA levels, as well as fat storage-related proteins, like acetyl-CoA carboxylase, fatty acid synthase, LPL and perilipin[199]. Consequently, both ghrelin forms stimulate intracytoplasmatic lipid accumulation at the same time as exhibiting an anti-lipolytic effect.

Other elements involved in lipolysis: other miscellaneous agents. The potent anti-lipolytic effect of nicotinic acid together with its specific binding to adipose tissue was firmly established more than half a century ago[201,202]. However, the mechanistic basis for this action on lipolysis control has been provided only more recently[203]. Activation of the nicotinic acid receptor triggers an inhibitory G-protein signal, which decreases cAMP concentrations in adipocytes, thereby inhibiting lipolysis. Continuous 24 h nicotinic acid infusion in rats reportedly alters gene expression and basal lipolysis in adipose tissue, producing a NEFA rebound and insulin resistance[204] that are consistent with clinical observations following treatment with this compound.

Other agents originating from either adipocytes or surrounding cells are known to negatively control adenylyl cyclase activity and inhibit lipolysis via their interaction with plasma membrane receptors belonging to the seven-transmembrane domain receptor family. Autacoid agents, as already mentioned including adenosine, prostaglandins and their metabolites, exert a clear anti-lipolytic effect. Whereas adenosine and neuropeptide Y reportedly inhibit lipolysis, for PGE_2 a biphasic effect has been put forward with nanomolar concentrations suppressing lipolysis, but micromolar levels resulting in lipolysis stimulation[63]. On the contrary, PGI_2 showed no effect or exerted also a biphasic effect, whereby nanomolar concentrations stimulated lipolysis, whereas at micromolar levels lipolysis was suppressed.

Cachexia-inducing tumours produce a lipid-mobilising factor (LMF) that causes an immediate glycerol release when incubated with murine adipocytes, with the stimulation of lipolysis by LMF being associated with an elevation in intracellular cAMP concentrations[205–207]. Zn-α_2-glycoprotein (ZAG), a tumour-related LMF of 43 kDa, has been found to be expressed in 3T3-L1 cells as well as in the major fat depots of mice, being up-regulated in rodents with cancer cachexia[208]. Both ZAG expression and protein have been also detected in human adipocytes of visceral and subcutaneous origin. Remodelling of adipose tissue together with decreased lipid storage constitute a hallmark of cancer patients with cachexia. In addition to ATGL- and HSL-enhanced lipolysis, in cancer other factors such as ZAG have been shown to participate in TAG degradation leading to white adipose tissue atrophy. ZAG expression and release by adipose tissue are up-regulated in weight-losing cancer patients, suggesting that ZAG operates both locally and systemically to stimulate lipid mobilisation[206]. However, ZAG did not display the thermogenic effects of the β-adrenoceptor agonist, nor did it increase β_3-adrenoceptor or *UCP1* (uncoupling protein 1) gene expression in brown adipose tissue, thereby implying that it does not behave as a typical $\beta_{3/2}$-adrenoceptor agonist[209]. Thus, ZAG has emerged as a novel adipokine, being identified as an additional adipose tissue factor closely related to body weight loss not only via modulation of lipolysis in fat cells but also by activating AMPK in skeletal muscle cells[208,210].

The octapeptide angiotensin II (Ang II) is the active component of the renin–angiotensin system (RAS). A local RAS is present in adipose tissue, with all the elements of the system, including angiotensinogen, renin and angiotensin-converting enzyme, having been identified in adipocytes[211]. Noteworthy, Ang II has been shown to decrease local blood flow in a dose-dependent manner and to inhibit lipolysis in adipose tissue with the effects being similar in both normal-weight and obese individuals[212]. In the last decade evidence has been provided that adipose tissue is a source of vasoactive peptides that further exert metabolic actions[213]. Thus, endothelin-1 is

a powerful vasoconstrictor primarily produced and secreted by endothelial cells to operate on the underlying vascular smooth muscle cell layer that can also act on adipocytes inducing lipolysis via the ERK pathway[214,215]. In human subjects endothelin-1 has been shown to selectively counteract insulin inhibition of visceral adipocyte lipolysis, decreasing the expression of insulin receptor, IRS-1 and PDE-3B and increasing the expression of the endothelin receptor-B (ET_BR) in visceral but not subcutaneous adipocytes[216]. The ET_BR-mediated effects were signalled via the PKC and calmodulin pathways. Subsequently, it was further observed that long-term incubation of human adipocytes with endothelin-1 increases lipolysis via the activation of ET_AR[217]. Likewise, the fifty-two-amino acid vasoactive peptide adrenomedullin together with its receptor components (calcitonin receptor-like receptor and receptor activity modifying protein-2 (CRLR/RAMP2)) have been identified to be concomitantly expressed in adipose tissue (Fig. 2), exhibiting a tissue-specific up-regulation during the development of obesity[218,219]. Interestingly, in adipose tissue adrenomedullin acts as an autocrine–paracrine factor to regulate lipid mobilisation, inhibiting lipolysis through NO-mediated β-adrenergic agonist oxidation[220]. In this context, it has been proposed that adrenomedullin alone is devoid of lipolytic function and inhibits β-adrenergic-stimulated lipolysis by shifting the concentration–response curve for isoproterenol by a NO-dependent mechanism; specifically, adrenomedullin-induced NO modifies isoproterenol through an extracellular oxidative reaction to yield its aminochrome, isoprenochrome. However, other studies have provided evidence for adrenomedullin dose-dependently elevating cAMP levels and the lipolytic rate[221]. In this case, adrenomedullin was shown to increase the phosphorylation of PKA, ERK and Akt and would reportedly exhibit additive effects on isoproterenol-induced lipolysis.

Apelin represents a further peptide with vasoactive characteristics that has been subsequently shown to be secreted by adipocytes of both humans and rodents, being up-regulated in states of obesity[222]. The identification in adipocytes of apelin and the apelin receptor (APJ), a G-protein-coupled receptor, supported a plausible autocrine participation of this peptide in adipobiology. In this line, apelin was shown to dose-dependently stimulate AMPK phosphorylation in human adipose tissue, which was associated with increased glucose uptake[223]. Apelin reportedly decreased isoproterenol-induced NEFA and glycerol release in 3T3-L1 cells and isolated adipocytes abrogating the catecholamine-induced HSL phosphorylation via G-protein q polypeptide (G_q), G_i pathways and AMPK activation[224]. The apelin-induced inhibition of basal lipolysis was exerted through AMPK-dependent enhancement of perilipin expression by preventing lipid droplet fragmentation and hormone-stimulated acute lipolysis inhibition mediated by decreasing perilipin phosphorylation[225]. Moreover, apelin also suppressed adipogenesis through MAP kinase kinase/ERK signalling.

Pigment epithelium-derived factor (PEDF) is a 50-kDa protein of the non-inhibitory serpin family of serine protease inhibitors originally identified as a regulator of hepatic TAG metabolism involved in the development of insulin resistance in obesity[6,226,227]. Subsequently it was tested whether this adipocyte-secreted factor also exhibits autocrine–paracrine lipolytic effects. PEDF was shown to stimulate TAG hydrolysis in adipose tissue, muscle and liver via ATGL[228]. The exact mechanisms underlying the participation of PEDF in insulin resistance, obesity and non-alcoholic fatty liver disease still need to be fully elucidated[229–231]. The potential role of other recently identified adipose-related factors on lipolysis such as serum amyloid A, osteopontin, osteocalcin, osteoprotegerin, obestatin, lipocalin 2, visfatin, nerve growth factor-inducible derived peptides, omentin, mammalian chitinase-like protein YKL40, chemerin, vitamin D and tenascin C, among others, beyond their originally reported effects merits to be specifically investigated[111,227,232–245].

Influence of subcellular compartmentalisation of lipases

Multicellular organisms ranging from insects to mammals have evolved specialised systems to store surplus lipid energy for subsequent mobilisation in times of need. In mammals the storage and mobilisation of lipids are fundamental functions of adipocytes. About 80 % of the total adipose tissue weight is due to the fat content, with over 90 % of lipids being stored as TAG[246]. The major secretory products of adipose tissue are NEFA[247], which are derived from the lipolysis of stored TAG in a process involving three main steps and requiring, at least, three different lipases, which are regulated by both adipocyte and non-adipocyte factors[7]. Thus, the classic lipolytic pathway encompasses the three following consecutive steps: (i) TAG hydrolysation by ATGL to generate fatty acids and diacylglyerol (DAG)[248]; (ii) subsequently, HSL catalyses the hydrolysis of DAG to monoacylglycerol (MAG) and fatty acids[249,250]; (iii) monoacylglycerol lipase (MGL) is required to complete the hydrolysis of MAG into one fatty acid and glycerol[251]. HSL and ATGL are quantitatively the most important lipases based on the blunted isoprenaline-induced lipolysis observed in adipocytes of *Atgl*- and *Hsl*-knockout mice[248,252].

TAG hydrolysis. Only a decade ago the initiation of TAG hydrolysis was thought to be exclusively controlled by HSL[2–7,253–255]. However, the generation of *Hsl*-knockout mice revealed the existence of residual HSL-independent TAG lipase activity, pointing to the existence of previously unidentified adipose tissue lipases. Currently, ATGL is well recognised to be the lipase responsible for initiating TAG breakdown to yield DAG[5,6]. ATGL is a 54-kDa TAG hydrolase, also named phospholipase A2ξ or desnutrin, belonging to the family of patatin-like phospholipase domain-containing proteins (PNPLA) with specificity for TAG as a substrate[6,248,256,257]. *Atgl*-knockout mice and

knockdown studies in adipocytes provided evidence for the involvement of ATGL in TAG but not DAG hydrolysis. *Atgl*-null mice exhibited a blunted lipolysis, producing a more than 75 % reduction in NEFA release and a significant TAG accumulation in adipocytes leading to obesity[248,258]. The co-activator of ATGL, CGI-58, also known as α/β-hydrolase domain-containing protein 5 (ABHD5), was shown to stimulate TAG hydrolase activity in wild-type and *Hsl*-deficient but not *Atgl*-deficient mice. ATGL and HSL are responsible for 95 % of TAG lipase activity, thereby suggesting a complementary relationship between the two lipases[257–259].

ATGL is highly expressed in adipose tissue, with its expression being profoundly elevated during adipocyte differentiation. Two phosphorylation sites (Ser404 and Ser428) have been identified within the C-terminal region of ATGL. Furthermore, the enzymic activity and its interaction with CGI-58 are dependent on the C-terminal region[260]. Overexpression of *Atgl* elevates TAG hydrolysis as well as basal and catecholamine-stimulated lipolysis, while *Atgl* silencing decreases TAG hydrolase activity, TAG storage and lipid droplet size[257]. Alterations of *Atgl* expression resulted in dramatic changes in whole-cell lipolysis. Conversely, silencing of *Atgl* or CGI-58 significantly reduced basal lipolysis and essentially abolished forskolin-stimulated lipolysis. Taken together, these findings suggest that in humans the ATGL–CGI-58 complex acts independently of HSL and precedes its action in the sequential hydrolysis of TAG.

Fasting, glucocorticoids and PPAR agonists increase *Atgl* mRNA expression, whereas food intake and insulin decrease it[261,262]. Cellular TAG lipolysis by ATGL produces essential mediators involved in lipid ligand generation for PPAR activation, with *Atgl* deficiency in mice reducing mRNA levels of PPAR-α and PPAR-δ target genes[263]. While mammalian target of rapamycin (mTOR)-dependent signalling has been observed to decrease *Atgl* mRNA expression, FoxO1 activation by SIRT1-mediated deacetylation elevated it[262,264–266]. However, the role of AMPK in lipolysis control remains controversial[267–271]. In this sense, the precise mechanisms of ATGL regulation need to be fully established. Recently, a protein encoded by the G0/G1 switch gene 2 (*G0S2*) has been identified as a selective regulator of ATGL by attenuating its action both *in vitro* and *in vivo*[272,273]. *G0S2* is highly expressed in adipose tissue and differentiated adipocytes interacting specifically with ATGL to inhibit its TAG hydrolase activity. While knockdown of endogenous *G0S2* enhances both basal and stimulated lipolysis in adipocytes, overexpression of *G0S2* decreases the lipolytic rate of adipocytes and adipose tissue explants. *G0S2* has been further shown to regulate human lipolysis influencing ATGL activity and intracellular localisation by anchoring the lipase to lipid droplets (Fig. 3) independently of the C-terminal lipid-binding domain of ATGL[273]. Moreover, *G0S2* expression has been observed to be diminished in poorly controlled type 2 diabetes, thereby establishing a potential link between adipose tissue *G0S2* down-regulation and insulin resistance. Given that the above-mentioned characteristics reveal ATGL as an attractive therapeutic target, the development and characterisation of a selective small-molecule inhibitor of ATGL, atglistatin, may prove of interest for the pharmacological treatment of dyslipidaemic and metabolic disorders[274].

Diacylglycerol hydrolysis. HSL, an 84-kDa cytoplasmic protein with demonstrated activity for a wide range of substrates including TAG, DAG, cholesteryl esters and retinyl esters, was presumed to be the rate-limiting enzyme in the initial steps of the lipolytic process. However, several important findings challenged this view of the unique regulatory and rate-limiting role of HSL on lipolysis, pointing to the existence of alternative lipases targeting TAG molecules to counterbalance the strong affinity of HSL for DAG[4,5,7,250,257,275]: (i) PKA-dependent HSL phosphorylation led only to a 2- to 3-fold increase in TAG hydrolase activity, while whole-cell lipolysis resulted in a 100-fold increase; (ii) *Hsl*-null mice exhibited a normal body weight with decreased adiposity; (iii) these mutants further showed DAG adipocyte accumulation; (iv) the existence of residual TAG hydrolase activity and lipolysis despite HSL silencing or specific pharmacological inhibition; and (v) failure of HSL overexpression to promote whole-cell lipolysis. As mentioned previously, the identification of ATGL provided explanations for these findings[250,254,276].

Fig. 3 illustrates ATGL and HSL regulation in basal and stimulated conditions. ATGL and HSL have the capacity to hydrolyse *in vitro* the first ester bond of TAG. ATGL exhibits 10-fold higher substrate specificity for TAG than DAG, selectively enabling the first step in TAG hydrolysis, leading to the formation of DAG and fatty acid. An important step in lipolysis activation comprises the translocation of HSL from a cytosolic compartment to the surface of the lipid droplet. Upon lipolytic stimulation, HSL moves from the cytosol to the surface of lipid droplets where it interacts with perilipin-1 and neutral lipids. Noteworthy, adipocytes lacking perilipin-1 are incapable of translocating HSL to the lipid droplet after increases in cAMP[277,278]. Perilipin-1 operates as a dynamic scaffold to coordinate the access of enzymes to the lipid droplet in a way that is responsive to the metabolic state of the adipocyte[279,280]. Thus, in basal conditions (Fig. 3(a)) perilipin-1 limits lipase access to the lipid droplet[281]. Lipolysis stimulation is followed by HSL translocation from the cytosol to lipid droplets and redistribution of ATGL, resulting in enriched colocalisation of the two lipases. Interestingly, the ATGL–CGI-58 complex acts independently of HSL and precedes its action in the sequential hydrolysis of TAG in humans. The increased number of ATGL–CGI-58 complexes formed following perilipin-1 phosphorylation (which releases CCI-58) and docked on small lipid droplets govern PKA-stimulated lipolysis (Fig. 3(b)). The association

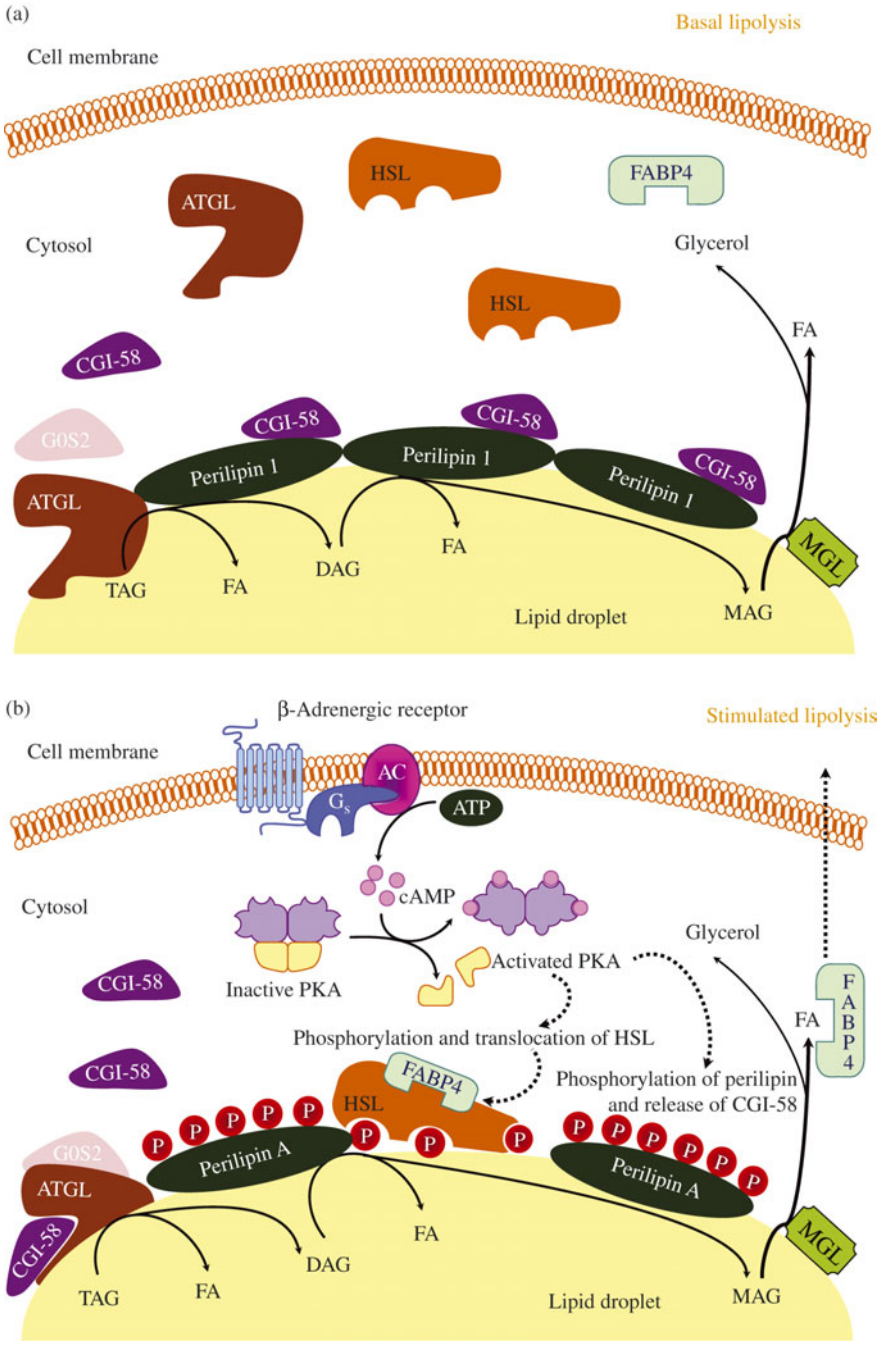

Fig. 3. Schematic representation of basal (a) and stimulated (b) lipolysis, the catabolic pathway by which TAG are hydrolysed into fatty acids (FA). AC, adenylyl cyclase; ATGL, adipocyte TAG lipase; cAMP, cyclic AMP; CGI-58, comparative gene identification-58; DAG, diacylglycerol; FABP4, fatty acid binding protein 4; G0S2, G0/G1 switch gene 2; G_s, stimulatory GTP-binding proteins; HSL, hormone-sensitive lipase; MAG, monoacylglycerol; MGL, monoacylglycerol lipase; P, phosphate; PKA, protein kinase A. (A colour version of this figure can be found online at http://www.journals.cambridge.org/nrr)

between fatty acid binding protein 4 (FABP4) and HSL represents a further regulatory step. Fatty acid binding to FABP4 and HSL phosphorylation precede the association of FABP4 and HSL. FABP4 also participates in the trafficking of fatty acids from the site of hydrolysis (i.e. the lipid droplet) to the plasma membrane. In addition to supporting fatty acid trafficking to the plasma membrane in a reaction that is independent of its physical association with HSL, FABP4 bound to fatty acids associates with activated, phosphorylated HSL on the surface of lipid droplets. The sequential effect of ATGL-accentuated TAG hydrolysis, phosphorylated HSL and MGL activity yields massive increases in NEFA release in response to PKA activation.

The expression profile of HSL basically mirrors that of ATGL, given that both enzymes coordinatedly hydrolyse TAG and, therefore, share some regulatory characteristics but differ in the mechanisms of enzyme control[6]. Whereas

β-adrenergic stimulation exerts ATGL regulation mainly via CGI-58 recruitment, HSL constitutes the main target for PKA-catalysed phosphorylation[282]. Adipocyte HSL encompasses an N-terminal domain (that interacts with FABP4) and a C-terminal catalytic domain (that contains the active site as well as a regulatory module with all the known phosphorylation sites of HSL)[4,255,283]. Phosphorylation of HSL at Ser563, Ser659 and Ser660 by PKA and at Ser660 via the ERK pathway activate lipolysis[284]. The PKA-dependent lipolytic effect is exerted increasing HSL's intrinsic activity and promoting its access to TAG molecules within the adipocyte. Conversely, AMPK exerts an anti-lipolytic effect, blocking the translocation of HSL to the lipid droplets by its phosphorylation at Ser565[261]. Deactivation of lipolysis mediated by insulin is associated with down-regulation of HSL and ATGL expression[285,286]. Moreover, insulin signalling phosphorylates and activates PDE isoforms via PKB, cAMP hydrolysis and PKA inhibition, resulting in the prevention of HSL and perilipin-1 phosphorylation, HSL activation and translocation as well as CGI-58-mediated ATGL activation. The peripheral control of insulin is accompanied by a central mechanism via the sympathetic nervous system that reduces the activitiy of both HSL and ATGL[287].

Monoacylglycerol hydrolysis. The final step of lipolysis is catalysed by MGL, which is constitutively expressed in adipose tissue and has no affinity for DAG, TAG or cholesteryl esters[255]. The enzymic activity of MGL is required in the final hydrolysis of the 2-monoacylglycerols produced by HSL activation. Site-directed mutagenesis has shown the relevance of Ser122, Asp239 and His269 in the lipase and esterase activities of MGL[255,288].

Other lipases. The contribution of alternative lipases to ATGL and HSL to the overall lipolytic capacity and maintenance of the highly dynamic TAG turnover has yet to be completely discerned. Potential TAG hydrolases have been identified within members of the carboxylesterase/lipase and the patatin homology domain families[6]. Carboxylesterase-3/TAG hydrolase-1 is supposedly involved in HSL-independent lipolysis in adipocytes and participates in the assembly and secretion of VLDL in the liver [289,290]. Among the patatin homology domain family, PNPLA4 and PNPLA5 have been observed to exhibit TAG hydrolase, DAG transacylase and retinylester hydrolase activity *in vitro*, which needs to be confirmed *in vivo* [291]. Noteworthy, the member with the highest ATGL homology is PNPLA3 or adiponutrin[292–295].

Lipid droplet proteins. Cytoplasmic lipid droplets are organelles in which cells store neutral lipids for use as an energy source in times of need, but they also play important roles in the regulation of key metabolic processes, with excess accumulation of intracellular lipids being associated with obesity, type 2 diabetes and atherosclerosis. Fat droplets may constitute up to 95 % of the total adipocyte volume, being mainly composed by TAG. Intracellular TAG storage droplets have emerged as extraordinarily dynamic organelles, with signalling events underlying lipid mobilisation by shuttling protein trafficking to a specialised subset of these droplets[15]. Thus, lipid droplet scaffold proteins are key elements in organising and directing the lipolytic signalling cascade[15,246].

The function of lipid droplets is regulated by their coating proteins, collectively termed PAT proteins after perilipin, adipophilin/adipocyte differentiation-related protein (ADRP), and tail-interacting protein of 47 kDa (TIP47)[4,296,297]. Further members of the family are S3-12, oxidative tissue-enriched PAT protein (OXPAT), myocardial lipid droplet protein (MLDP) and lipid storage droplet protein 5 (LSDP5)[298,299]. The members of this family share varying levels of sequence similarity, lipid droplet association and functions in stabilising lipid droplets.

Lipid droplet proteins: perilipin. Lipid droplets in most tissues are coated by two or more members of the perilipin family, which are now numbered according to the order of discovery[291]. Expression of perilipin-1 is mainly restricted to white and brown adipocytes and, to a lesser extent, steroidogenic cells of adrenal cortex, testes and ovaries. Perilipin-2 (formerly adipophilin or ADRP) and perilipin-3 (formerly TIP47) are ubiquitously expressed and, therefore, lipid droplet components of most tissues. While perilipin-4 (formerly S3-12) is primarily expressed in white adipocytes, perilipin-5 (formerly OXPAT, MLDP, or LSDP5) is expressed in brown adipocytes as well as myocytes of skeletal muscle and heart, all of which rely on lipolysis to provide fatty acids to mitochondria for β-oxidation to drive either ATP production or heat generation. Thus, the perilipin composition of lipid droplets within a specific tissue constitutes an important component of lipolysis regulation.

Perilipin is the best-known member of the PAT family, with perilipin-1 being the predominant isoform found in mature adipocytes, the most abundant protein on the lipid droplet surface and the major substrate for cAMP-dependent PKA in lipolytically stimulated adipocytes[297,300–308]. Perilipin limits the access of cytosolic lipases to lipid droplets, thereby facilitating TAG storage under basal conditions (Fig. 3(a)). When energy is needed, perilipin is phosphorylated by PKA, facilitating maximal lipolysis by ATGL and HSL (Fig. 3(b)). Thus, perilipin expression and its phosphorylation state are key in lipolysis control, with phosphorylation of Ser492 producing a lipid droplet remodelling, widely increasing the surface area for lipase binding, while Ser517 is essential for ATGL-dependent lipolysis in stimulated conditions[4]. Perilipin-1 is also phosphorylated by the cyclic GMP-dependent PKG.

Perilipin ablation confers resistance to genetic or diet-induced obesity, producing a lean phenotype with smaller adipocytes, increased basal lipolysis and attenuated stimulated lipolysis[301]. Recently, perilipin-1 has been shown to move between the fat droplet and the endoplasmic reticulum[309], which is physiologically reasonable given that

lipid droplets are largely derived from the endoplasmic reticulum. In this regard, perilipin-mediated lipid droplet formation in adipocytes was demonstrated to promote sterol regulatory element-binding protein-1 (SREBP-1) processing and TAG accumulation, suggesting an interplay between lipid droplet formation and SREBP-1 activation via a positive feedback loop[310]. Therefore, the lysosomal protein degradation machinery of perilipin may constitute a target mechanism for enhancing adipocyte lipolysis. Interestingly, a genome-wide RNA interference (RNAi) screen in *Drosophila* S2 cells highlighted the relevance of elements of the vesicle-transport systems in lipolysis regulation through the identification of the vesicle-mediated coat protein complex I (COPI) as an evolutionary-conserved regulator of PAT protein composition at the lipid droplet surface[311,312]. In addition to regulating PAT protein composition, COPI promotes the association of ATGL with the lipid droplet surface to mediate lipolysis. These genes are conserved in mammalian cells, thus suggesting that a similar complex might be operative in adipocytes. Although COPI-mediated transport reportedly participates in delivery of ATGL to the lipid droplet surface, depletion of β-COP (a subunit of the COPI coat complex) does not affect association of ATGL with lipid droplets or ATGL-mediated lipolysis, pointing to the possibility of alternative transport mechanisms implicated in the regulation of lipid homeostasis[313].

Lipid droplet proteins: coactivator comparative gene identification-58 (CGI-58) or α/β-hydrolase domain-containing protein 5 (ABHD5). CGI-58 lacks lipase activity in itself but potently and selectively stimulates lipolysis by activating ATGL. As mentioned above, in basal unstimulated conditions CGI-58 binds tightly to lipid droplets by interacting with perilipin-1 and is unable to activate ATGL[4]. However, following β-adrenoceptor stimulation CGI-58 is quickly dispersed to the cytosol, favouring ATGL co-localisation and migration to small lipid droplets. Thus, under stimulated conditions, the intracellular cAMP elevation and PKA activation promote perilipin-1 phosphorylation, which is followed by the dissociation from perilipin of CGI-58, which subsequently interacts with ATGL and activates TAG hydrolysis (Fig. 3(b)). In addition to ATGL activation, a further physiological function for CGI-58 in phospholipid synthesis with lysophosphatidic acid acyltransferase activity has been observed[4].

Lipid droplet proteins: Cide domain-containing proteins. A further family of lipid droplet-associated proteins encompasses the cell death-inducing DFFA (DNA fragmentation factor-α)-like effectors (Cide), which includes three members (Cidea, Cideb and Cidec/Fsp27) with tissue-specific expression[5]. In spite of Cidea and Cideb not being expressed in white adipose tissue, their deletion yielded rodents with lower body weight and improved insulin sensitivity as well as resistant to diet-induced obesity[314,315]. In the *Cidea* knockout model the elevated energy expenditure was attributable to brown adipose tissue via enhanced AMPK activity leading to increased fatty acid oxidation[316]. The *Cideb* mutants exhibited a decreased hepatic VLDL secretion and *de novo* fatty acid oxidation related to enhanced hepatic oxidative activity[317,318]. Cidea is also involved in human adipocyte lipolysis, TAG deposition and fatty acid oxidation via cross-talk with TNF-α, which inhibits the transcription of the gene[319–321]. Cidea co-localises with perilipin around lipid droplets in fat cells. An increased lipolysis is observed in Cidea-depleted human adipocytes. Contrarily, ectopical expression of Cidea in preadipocytes markedly enhances lipid droplet size, promoting lipid accumulation[322]. Noteworthy, Cidea expression is elevated in human cancer cachexia, exhibiting a correlation with elevated NEFA concentrations and weight loss[323]. In humans Cidec, also referred to as fat-specific protein 27, FSP27, is predominantly expressed in subcutaneous adipocytes, being down-regulated in response to a reduced energy intake[324]. Small interfering RNA-mediated knockdown of Cidec translated into an increased basal release of NEFA, and decreased responsiveness to adrenergic lipolysis stimulation[4,325]. The interaction between the diverse lipases is also starting to be unfolded. FSP27 and perilipin-1 interaction promotes the formation of large lipid droplets in human adipocytes[326–329]. Recently, the unilocular to multilocular transformation that takes place during 'browning' of white adipose tissue has been related to Cide-triggered dynamic changes in lipid droplet-associated proteins[330].

Lipid droplet proteins: other proteins (GPIHBP1 and Rab). Glycosylphosphatidylinositol-anchored HDL-binding protein (GPIHBP1) is a 28-kDa glycosylphosphatidylinositol-anchored glycoprotein located on the luminal surface of endothelial cells in tissues where lipolysis takes place such as adipose tissue, skeletal muscle and heart[7,331]. The expression of GPIHBP1 in mice is modulated by fasting and refeeding as well as by PPAR-γ agonists. GPIHBP1 knockout mice exhibit chylomicronaemia, even on a low-fat diet, with highly elevated plasma TAG concentrations[332–334]. GPIHBP1 is highly expressed in the same tissues that express high levels of LPL, namely, heart, adipose tissue, and skeletal muscle where it binds both LPL and chylomicrons, suggesting that GPIHBP1 functions as a platform for LPL-dependent lipolytic processing of TAG-rich lipoproteins, stabilising LPL without activating it.

Rab GTPases, which are key regulators of membrane trafficking, have emerged as particularly relevant molecules in the highly dynamic cellular interactions involved in lipid mobilisation. In this sense, proteomic analyses have consistently identified the small GTPase Rab18 as a component of the lipid droplet coat[335]. Thus, Rab18 provides an excellent marker to follow the dynamics of lipid droplets in living cells as well as to gain insight into the complex regulatory mechanisms involved in lipid storage and release[336–338]. In 3T3-L1 adipocytes, stimulation of lipolysis increases the association of Rab18 with lipid droplets,

suggesting that Rab18 recruitment is regulated by the metabolic state of individual lipid droplets. Furthermore, Rab1a and its effector protein are reportedly involved in the CD36 trafficking signalling pathway[259].

Integral membrane proteins and transporters. While the main signalling cascades and regulators of lipolysis have been identified, the cellular interactions involved in lipid mobilisation and release still remain to be completely disentangled. Except in adipocytes, lipid droplets are normally small, mobile and interact with other cellular compartments in cells. On the contrary, fat cells are composed mainly of very large, immotile lipid droplets. The striking morphological differences between lipid droplets in adipocytes and non-adipocytes suggest that key differences must exist in the way in which lipid droplets in different cell types interact with other organelles to facilitate lipid transfer. A plethora of molecules involved in these interactions are now emerging, with integral membrane proteins and fatty acid transporters standing out as pivotal elements operating at the dynamic plasma membrane–lipid droplet interface.

Integral membrane proteins and transporters: aquaporin-7. Aquaporins (AQP) are integral membrane proteins that function mainly as water channels. AQP7 belongs to the subfamily of aquaglyceroporins, which are permeable to both glycerol and water, being expressed in adipocytes[339–341]. Mouse and human AQP7 exhibit six prospective sites for PKA phosphorylation, suggesting a putative cAMP/PKA-dependent regulation. *Aqp7*-knockout mice show defective glycerol exit from fat cells, adipocyte hypertrophy due to TAG accumulation and moderate adult-onset obesity[342,343]. Short-term regulation and translocation of AQP7 to the plasma membrane is stimulated by catecholamines, while insulin exerts a long-term negative control. More recently, in addition to AQP7, the presence and functionality of other members of the aquaglyceroporin subfamily, AQP3 and AQP9, have been identified in adipose tissue and shown to be regulated by insulin and leptin via the PI3K/Akt/mTOR signalling cascade[344].

Integral membrane proteins and transporters: caveolin-1. Caveolae account for over 25 % of the adipocyte's membrane, being specialised plasma membrane microdomain invaginations involved in important cellular transport processes such as endo- and transcytosis as well as signal transduction[345]. Three classes of caveolae formed by caveolin-1, the scaffolding hairpin-like protein facing the cytosol, have been identified, with high-density caveolae taking up exogenous fatty acids and converting them to TAG. These TAG-metabolising caveolae serve as a platform for FABP4, fatty acid transport protein (FATP) 1 and 4 (FATP1 and FATP4), long-chain acyl-CoA synthetase 1 (ACSL1) and CD36 (also known as fatty acid translocase). Noteworthy, these caveolae contain FATP1 and FATP4 together with the enzymes needed for TAG synthesis[346–348]. Furthermore, HSL and perilipin have been shown to be associated to these caveolae[349], demostrating that TAG can be hydrolysed in them (Fig. 4). Caveolin-1 exerts an indirect structural role in caveolae formation, controlling surface availability or stability of CD36, a fatty acid transporter key to long-chain fatty acid uptake[350]. In response to NEFA, caveolin-1 reportedly translocates from the plasma membrane to lipid droplets. Caveolin-1 knockout mice lack caveolae in adipocyte plasma membranes, exhibiting increased circulating NEFA and TAG, reduced adipocyte lipid droplet size and resistance to diet-induced obesity[351]. Experiments with caveolin-1-null mouse embryonic fibroblasts indicate that caveolin-1 deficiency is followed by a total loss of caveolae, absence of CD36 plasma membrane expression and a reduction in fatty acid uptake, which is reverted by re-expression of caveolin-1[352]. Interestingly, caveolin-1 has been shown to exert inhibitory interactions with various proteins such as PKA, endothelial NOS and insulin receptors, with knockout mice exhibiting an attenuated lipolytic activity and decreased perilipin phosphorylation[349]. Caveolin-1 potently inhibits cAMP-dependent signalling *in vivo*, with a direct interaction between caveolin-1 and the catalytic subunit of PKA having been demonstrated both *in vitro* and *in vivo*.

Integral membrane proteins and transporters: fatty acid translocase (CD36). As mentioned above, CD36 localises to caveolae as well as to intracellular vesicles. CD36 is a glycoprotein belonging to the family of class B scavenger receptors predicted to have two transmembrane domains at the N- and C-terminal, a large extracellular domain loop and two short intracellular cytoplasmic tails[259]. CD36 is expressed in organs with high fatty acid metabolism rates, such as adipose tissue, operating as a NEFA scavenger. Insulin activation of the forkhead transcription factor and AMPK stimulation trigger CD36 translocation from intracellular stores to the plasma membrane, thereby enhancing NEFA uptake. CD36 deficiency is associated with increased basal lipolysis and responsiveness to the anti-lipolytic effect of insulin, with *Cd36*-null mice exhibiting an impaired fatty acid uptake in metabolic tissues (including adipocytes) and increased plasma NEFA and TAG concentrations[353,354]. Knockdown of CD36 by RNAi in 3T3-L1 adipocytes resulted in a profound reduction of both basal and insulin-stimulated NEFA uptake. Conversely, overexpression of CD36 led to mice with decreased adiposity and low circulating levels of NEFA, TAG and cholesterol, suggesting that a strict control of these molecules for an effective lipolysis is required.

Integral membrane proteins and transporters: adipose fatty acid binding protein. FABP4, also known as ALBP and aP2, is a cytosolic lipid-binding protein highly expressed in adipocytes involved in fatty acid and retinoic acid intracellular trafficking[259]. It acts as a molecular chaperone, facilitating NEFA uptake and lipolysis, interacting with HSL and shuttling fatty acids out of adipocytes (Fig. 4). Upon PKA activation the HSL–FABP4 complex translocates to lipid droplets. Consistently with this, in *Fabp4*-knockout mice basal and stimulated lipolysis are

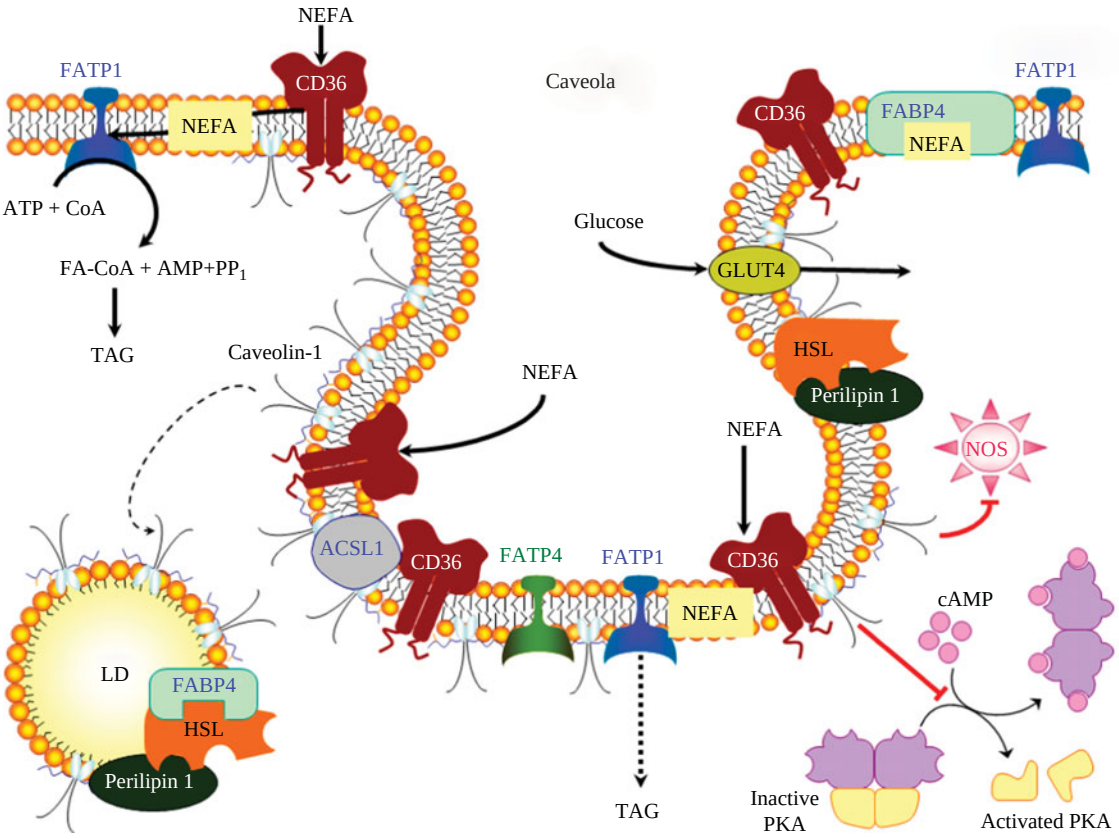

Fig. 4. Schematic diagram of a caveola present in the adipocyte's membrane and its participation in lipolysis. ACSL1, acyl coenzyme A synthetase 1; cAMP, cyclic AMP; CD36, fatty acid translocase; FA, fatty acid; FABP, fatty acid binding protein; FATP, fatty acid transport protein; HSL, hormone-sensitive lipase; LD, lipid droplet; NOS, NO synthase; PKA, protein kinase A; PP_1, pyrophosphate. (A colour version of this figure can be found online at http://www.journals.cambridge.org/nrr)

attenuated[291,355–357]. Interestingly, *Fabp4*-null mice have been shown to compensate FABP4 deletion by increasing the expression of other FABP, thereby highlighting that lipolysis seems to be linked to total FABP content rather than to a specific FABP type[4].

Integral membrane proteins and transporters: fatty acid transport protein 1. The underlying mechanism for fatty acid uptake by FATP1, an integral membrane protein of about 71 kDa with a hydrophobic domain at the N-terminal that may be membrane-anchored and other membrane-associated domains peripherally associated with the inner leaflet of the membrane, is still unknown. In response to insulin, FATP1 may translocate to structurally disordered non-lipid raft regions of the plasma membrane. Subsequently, FATP1 may extract fatty acid from the inner membrane leaflet and esterify it to CoA, thereby preventing its efflux and driving a NEFA concentration gradient across the membrane[358,359]. Most of the incoming fatty acids are converted into acyl-CoA and preferentially shunted into TAG synthesis (Fig. 4). Noteworthy, the conversion of incoming long-chain fatty acids to TAG takes place on or around the plasma membrane in rat adipocytes, plausibly linking in a mechanistic way fatty acid influx to TAG synthesis[259,360]. Knockdown and knockout experiments revealed an absolute requirement for FATP1 in insulin-stimulated fatty acid uptake, whereas FATP1 overexpression led to a fatty acid uptake increase.

Integral membrane proteins and transporters: fatty acid transport protein 4. FATP4 presents a 60 % identity to FATP1 and is expressed in adipose tissue, skin, heart, skeletal muscle, liver, as well as in the small intestine, where it was observed to work in intestinal lipid absorption[259,361]. FATP4 knockdown in 3T3-L1 adipocytes by RNAi did not affect basal and insulin-stimulated fatty acid uptake. FATP4 knockouts exhibit perinatal lethality due to restrictive dermopathy, suggesting a key role in the formation of the epidermal barrier rather than in fatty acid uptake and intestinal lipid absorption.

Integral membrane proteins and transporters: acyl-CoA synthetase long-chain 1. ACSL1, a 78-kDa membrane protein expressed in adipocytes and localised to various subcellular sites including the plasma membrane, lipid droplets, and GLUT4-containing vesicles, co-localises with FATP1[259]. ACSL1 was found to be involved in the reacylation of fatty acids released from the lipid droplets during basal and hormone-induced lipolysis[359]. Overexpression of ACSL1 in fibroblasts is followed by an increase in NEFA uptake, thereby supporting a co-operative role in fatty acid transport across the adipocyte plasma membrane[362]. However, knockdown of ACSL1 expression

by RNAi in 3T3-L1 adipocytes points to a role in fatty acid efflux but not influx.

Depot-specific differences

The main anatomical fat depots in humans include intra-abdominal (greater and lesser omental and mesenteric depots, also known as visceral fat), lower-body (gluteal, subcutaneous leg and intramuscular fat) and upper-body subcutaneous fat[363,364]. Subcutaneous adipose tissue constitutes the largest site for fat storage (about 80 % of total body fat), while under normal circumstances visceral adipose tissue accounts for a small fraction of body fat (about 20 % in men, and 5–8 % in women)[365]. Regional differences, including preadipocyte replication and differentiation, adipocyte size, blood supply, gene expression, basal metabolic activities and hormonal responsiveness, contribute to regional fat distribution[363–366]. Increased NEFA availability, resulting from increased effective adipose tissue lipolysis, plausibly undelies some of the visceral obesity-associated metabolic alterations[367,368]. Owing to its anatomical distribution, NEFA released from visceral fat are drained directly to the liver through the portal vein, whereas venous drainage of NEFA from subcutaneous adipose tissue is through systemic veins[369]. The venous drainage of fat via the portal system directly provides NEFA as substrates for hepatic lipoprotein metabolism or glucose production. Excess NEFA favours the onset of dyslipidaemia, hyperinsulinaemia and insulin resistance by reducing hepatic degradation of apoB and insulin as well as by increasing VLDL production[4].

Table 1 summarises regional variations in adipocyte lipolysis leading to increased NEFA release from visceral as compared with subcutaneous fat during hormone stimulation. Visceral adipocytes show the highest lipolytic responsiveness to catecholamines due to an increased function of the lipolytic β_1-, β_2- and β_3-adrenoceptors[370,371]. On the other hand, as mentioned above, several mechanisms have been linked to the weak lipolytic response to catecholamines in subcutaneous adipocytes, such as enhanced anti-lipolytic α_2-adrenoceptor activity, decreased lipolytic β_2-adrenoceptor responsiveness as well as reduced expression or function of HSL, FABP4 or perilipin[363,370].

The anti-lipolytic effect of insulin is more prominent in subcutaneous adipocytes compared with visceral fat cells[370,372]. Regional differences involve insulin receptor affinity, which is partly caused by variations in the insulin dissociation rate, but also by reduced insulin receptor phosphorylation and signal transduction via the IRS-1/PI3K pathway[370,372,373]. Testosterone has been reported to show both stimulatory[374] (i.e. up-regulation

Table 1. Depot-specific differences of diverse factors regulating adipocyte lipolysis

Regulatory factor	Activity	Main fat depot target	Reference
Catecholamines			
β_1-Adrenoreceptor	Lipolytic	Visceral adipose tissue	370
β_2-Adrenoreceptor	Lipolytic	Visceral adipose tissue	370
β_3-Adrenoreceptor	Lipolytic	Visceral adipose tissue	370
α_2-Adrenoreceptor	Anti-lipolytic	Subcutaneous fat	370
Insulin			
Insulin receptor	Anti-lipolytic	Subcutaneous fat	370
Growth hormone			
Growth hormone receptor	Lipolytic	Unknown	431
Ghrelin/obestatin			
Growth hormone secretagogue receptor	Anti-lipolytic	Visceral and subcutaneous fat	432, 433
Testosterone			
Androgen receptors	Anti-lipolytic	Subcutaneous fat	75
Oestrogens			
Oestrogen receptor-α	Anti-lipolytic	Subcutaneous fat	375
Endothelin			
Endothelin receptor A	Lipolytic	Unknown	217
Endothelin receptor B	Lipolytic	Visceral adipose tissue	216
TNF-α			
TNF receptor 1	Lipolytic	Unknown	434
IL-6			
IL-6 receptor and glycoprotein 130	Lipolytic	Visceral adipose tissue	131, 435
Lipopolysaccharide			
Toll-like receptor 4	Lipolytic	Unknown	436
Leptin			
Leptin receptor: OB-R	Lipolytic	Subcutaneous fat	376
Adiponectin			
Full-length adiponectin	Anti-lipolytic	Subcutaneous fat	164
Globular adiponectin	Anti-lipolytic	Visceral and subcutaneous fat	164
Trimeric adiponectin	Anti-lipolytic	Visceral and subcutaneous fat	164
Natriuretic peptides			
Atrial natriuretic peptide	Lipolytic	Visceral and subcutaneous fat	378
Brain natriuretic peptide	Lipolytic	Unknown	377
C-type natriuretic peptide	Lipolytic	Unknown	377

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of β_2-adrenoreceptors in visceral fat cells) and inhibitory[75] (i.e. down-regulation of β_2-adrenoceptors and HSL in subcutaneous adipocytes) effects on cathecolamine-induced lipolytic activity. Oestrogen attenuates the lipolytic response through up-regulation of a number of anti-lipolytic α_2-adrenergic receptors[375].

Leptin and adiponectin, the most abundant adipocyte-secreted factors, show opposite actions on lipolysis regulation[12]. Leptin produces a significantly greater stimulation of lipolysis in subcutaneous fat cells compared with omental adipocytes[376]. Adiponectin has recently emerged as an anti-lipolytic factor on binding adiponectin receptor type 1 and 2 (AdipoR1 and AdipoR2). Full-length adiponectin exerts an anti-lipolytic action in subcutaneous adipose tissue in non-obese subjects, while exhibiting no effect on visceral fat[163,164]. Atrial (ANP), brain (BNP) and C-type (CNP) natiruretic peptides also induce lipolysis in human abdominal adipocytes, with the potency order of the lipolytic effect being ANP > BNP > CNP[377]. ANP-induced lipolysis is not subjected to primary regional regulation in differentiated human subcutaneous and visceral fat cells[378]. Fat-depot differences in the lipolytic effect of BNP and CNP remain to be established.

In addition to the physiological depot-specific differences in the neuroendocrine control of adipose tissue, it is important to consider the role of body fat distribution in the development of cardiometabolic alterations[363–366]. Adipose tissue distribution varies with sex, age, genetic background, nervous and endocrine factors, nutritional and pharmacological influences as well as disease state, which impinge on preadipocyte replication and differentiation, developmental gene expression, vascularity, inflammation, adipokine secretion and apoptosis. The excess visceral fat observed in obesity is closely linked with metabolic and cardiovascular co-morbidities, whereas increased subcutaneous fat may even exert protective effects. However, how interdepot differences in the molecular, cellular, histological and pathophysiological properties translate into co-morbidity development needs to be fully unravelled[379–381].

Lipophagy: role of autophagy in lipid metabolism

Autophagy is a self-digestive process that entails the formation of double-membrane vesicles, termed autophagosomes, that sequester and target cytoplasmic cargo for lysosomal degradation[382–384]. In addition to quality control, autophagy also regulates lipid metabolism by degrading lipid droplets via lipophagy (Fig. 5). Small lipid droplets can be completely taken up by an autophagosome, or alternatively portions of large lipid droplets can be degraded[382]. Depletion of nutrients during starvation activates a second important cellular energy sensor, AMPK, that further activates unc51-like kinase 1 (ULK1) phosphorylation. Active ULK1 induces autophagy via the phosphorylation of beclin-1, a protein that recruits regulatory proteins to the VPS34 complex (class III PI3K), which is essential for the activity of the phagophore[385]. During the vesicle elongation process, ATG7 induces the conjugation of ATG12 to ATG5 as well as the conjugation of cytosolic light chain 3 (LC3)-I to phosphatidylethanolamine to generate LC3-II, one of the best-characterised components of autophagosomes. Once formed, autophagosomes engulf lipid droplets and eventually fuse with a hydrolase-containing lysosome, the lipases of which degrade lipids[382]. This process generates fatty acids that are released into the cytoplasm and can be oxidised in the mitochondria to generate ATP to maintain energy homeostasis. Under basal fed conditions, nutrients (particularly amino acids) or insulin and growth factors trigger the activity of class I PI3K that, in turn, activates mTOR, the best-characterised negative regulator of autophagy, and blocks autophagosome formation[386,387] (Fig. 5). As a result, lipid breakdown by autophagy is minimal in the fed state.

Autophagy also participates in adipocyte differentiation regulation[388]. Transgenic animals lacking the autophagy-related proteins ATG5 and ATG7 show a reduction in adipose mass, supporting that autophagy is essential for normal adipogenesis[389,390]. Analogously, *Atg5* and *Atg7* knockdown in 3T3-L1 adipocytes decrease intracellular lipid content and gene expression levels of the key adipogenic transcription factors, CCAAT/enhancer-binding protein α and β (C/EBPα and β) and PPAR-γ[389]. White adipocytes of *Atg7-d*eficient mice acquire some characteristics of brown adipocytes, such as higher mitochondrial content, multilocular lipid droplets and increased levels of the brown adipogenic factors PPAR-γ-coactivator 1α (PGC-1α) and uncoupling protein-1 (UCP-1), triggering adipose tissue fatty acid β-oxidation[390]. Interestingly, loss of *Atg7* disrupts brown fat differentiation and promotes the 'beige' (brown adipocyte-like) cell development in inguinal adipose tissue, thereby contributing to increased energy expenditure[391,392].

Human adipose tissue contains autophagosomes and obesity is associated with an altered expression of the autophagy-related molecules LC3-I, LC3-II, beclin-1, ATG5 and ATG7[200,393,394]. Markers of autophagy are correlated with whole-body adiposity, visceral fat distribution and adipocyte hypertrophy. However, the altered expression of autophagy in human obesity appears to be related to the degree of insulin resistance, rather than to excess adiposity[200]. In this sense, insulin constitutes a major inhibitor of autophagy, with insulin resistance being a potential activator of this process, since patients with type 2 diabetes show elevated formation of autophagosomes in subcutaneous adipose tissue[395]. Adipocyte autophagy is also regulated by TNF-α and ghrelin, showing opposite effects on the regulation of fat storage in human adipocytes[200]. TNF-α plays an important role in the pathophysiology of deranged lipid metabolism through both the suppression of LPL activity and enhancement of lipolysis in human fat cells[396]. In addition, TNF-α also triggers autophagy by increasing the transcript levels of *BECN1*

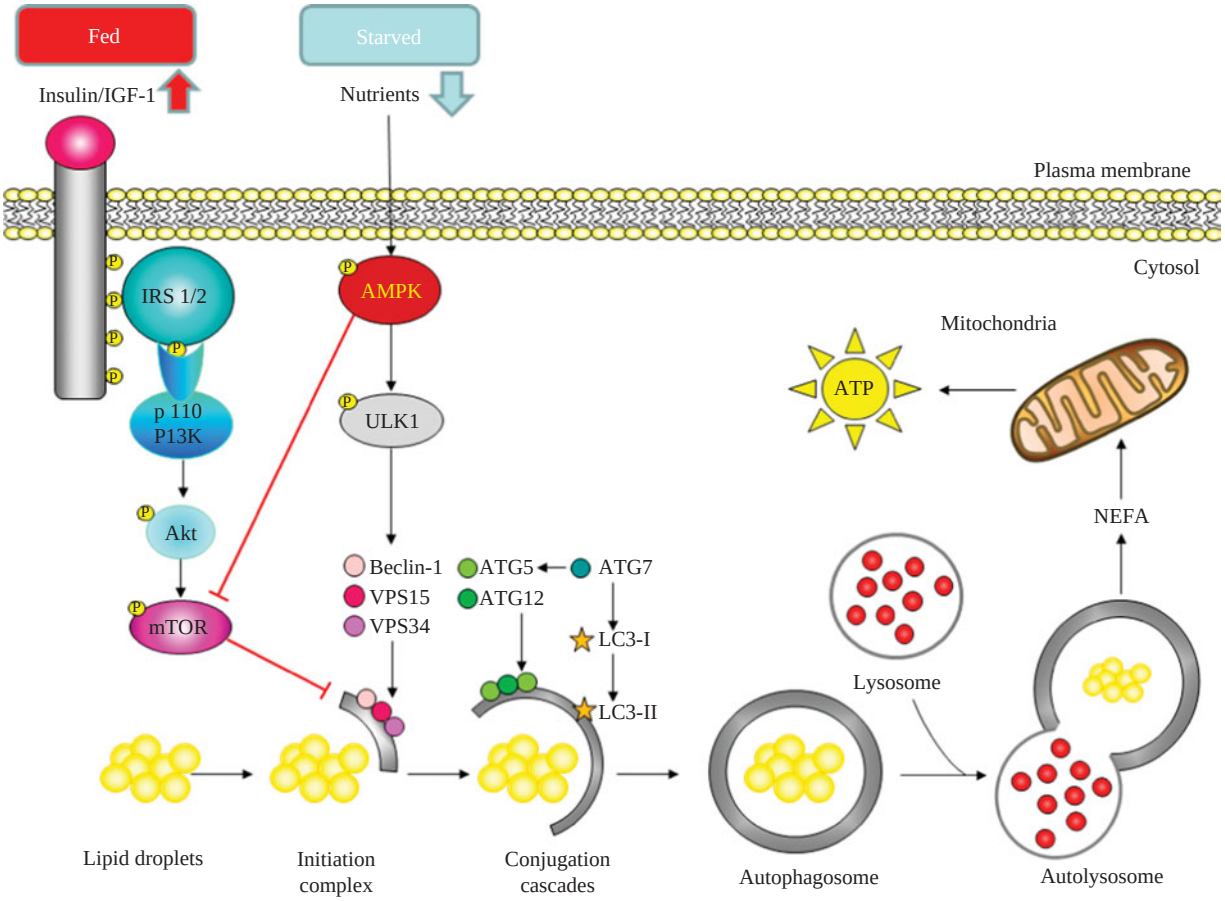

Fig. 5. Regulation of lipophagy. AMPK, AMP-activated protein kinase; Akt, protein kinase B; ATG, autophagy-related gene; IGF-1, insulin growth factor-1; IRS 1/2, insulin receptor substrate 1/2; LC3, light chain 3; mTOR, mammalian target of rapamycin; P, phosphate; PI3K, phospatidylinositol-3 kinase; ULK1, unc51-like kinase 1; VPS15, phosphoinositide-3-kinase, regulatory subunit 4; VPS34, class III phosphatidylinositol 3-kinase. (A colour version of this figure can be found online at http://www.journals.cambridge.org/nrr)

(beclin 1), required for the formation of the autophagosome initiation complex, as well as those of *ATG5*, and *ATG7*, the autophagy proteins involved in the conjugation cascades for autophagosome elongation in human adipocytes[200]. On the other hand, ghrelin is a gut-derived hormone that promotes adiposity through orexigenic and adipogenic actions[199,397]. Ghrelin isoforms (acylated and desacyl ghrelin) stimulate the expression of several fat storage-related proteins such as acetyl-CoA carboxylase, fatty acid synthase, LPL or perilipin through central mechanims[397] and directly acting on human adipocytes[199], thereby stimulating intracellular lipid accumulation. Besides its lipogenic action, acylated ghrelin reduces basal *ATG5* and *ATG7*, while desacyl ghrelin inhibits TNF-α-induced expression of *ATG5*, *ATG7* and *BECN1*. Taken together, ghrelin constitutes a negative regulator of basal and TNF-α-induced autophagy in human visceral adipocytes[200].

Novel fascinating findings in the field of adipocyte apoptosis have been recently reported[398,399]. White adipose tissue inflammation, a characteristic feature of obesity, results from the death of hypertrophic adipocytes that are subsequently cleared by macrophages, giving rise to crown-like structures (CLS). It has been recently shown that infiltrating macrophages actively take up remnant lipids of dead adipocytes[398]. Upon induction of adipocyte apoptosis, inflammatory cells infiltrate adipose tissue initially consisting of neutrophils followed by macrophages that are involved in CLS formation. Moreover, subcutaneous and visceral hypertrophic adipocytes obtained from obese mice exhibit ultrastructural abnormalities (cholesterol crystals and Ca accumulation), being more common in the hyperglycaemic *db/db v.* normoglycaemic *ob/ob* mice and in the visceral *v.* subcutaneous depots. Data indicate that white adipocyte overexpansion induces a stress state that ultimately leads to death with NOD-like receptor family, pyrin domain containing 3 (NLRP3)-dependent caspase-1 activation in hypertrophic adipocytes probably inducing obese adipocyte death by pyroptosis, a proinflammatory programmed cell death[399].

Lipolysis in human obesity

Obesity is characterised by a marked secretion of pro-inflammatory adipokines, including TNF-α, and a profound decrease in adiponectin synthesis[234]. The increased TNF-α production in adipose tissue triggers MAP kinase

activity in adipocytes, thus altering the action of perilipin and leading to an enhanced basal lipolytic rate[2,400]. Otherwise, adiponectin inhibits basal and cathecolamine-induced lipolysis in non-obese subjects, but this effect is lost in obesity[161]. The isoform-specific ability to prevent lipolysis is modified in obesity. While full-length adiponectin exerts an anti-lipolytic action in subcutaneous fat, without effect on visceral fat, in non-obese individuals, the lower adiponectin isoforms (globular and trimeric) become important actors in obesity, showing anti-lipolytic activity in obese subcutaneous and visceral adipose tissue, respectively[164].

Circulating NEFA and glycerol concentrations are elevated in obesity, suggesting an increase in overall lipolysis during fasting[344]. Several impairments in the control of lipolysis have been reported in obese individuals, including an altered responsiveness to catecholamines[2,4,53]. Obese subjects show a lower lipolytic effect of catecholamines in subcutaneous adipose tissue through decreased action of lipolytic β_2-adrenergic receptors and increased activity of the anti-lipolytic α_2-adrenergic adrenoceptors[370,401]. In this regard, a blunted lipolytic response has been shown in abdominal subcutaneous adipose tissue of obese individuals during intravenous infusion of the non-selective β-agonist isoprenaline[402]. On the other hand, catecholamine-induced lipolysis is markedly increased in visceral fat due to increased activity of β_3-adrenergic receptors and decreased activity of α_2-adrenoceptors[370,401]. In subjects with upper-body obesity these regional variations in the action of catecholamines on lipolysis are further enhanced[368,370]. These abnormalities in catecholamine function promote the release of NEFA from the visceral adipocytes through the portal system and might cause several of the metabolic complications of upper-body obesity. In addition, several polymorphisms in genes encoding β_1- (*ADRB1*), β_2- (*ADRB2*) and β_3- (*ADRB3*) adrenergic receptors have been associated with altered cathecolamine-induced adipocyte lipolysis and with obesity[403,404]. The polymorphisms in the *ADRB2* gene are highly frequent in obesity and associated with altered β_2-adrenergic function (Arg16Gly and Gln27Glu) and catecholamine-induced lipolysis in subcutaneous fat cells (Arg16Gly and Thr164Ile)[42,405,406]. However, the *ADRB1* (Ser49Gly and Arg389Gly)[404,407,408] and *ADRB3* (Trp64Arg)[409–411] polymorphisms do not appear to be major determinants of β_1- and β_3-adrenergic function for lipolysis or the pathophysiology of obesity.

It is not clear whether the anti-lipolytic effect of insulin is affected in obesity, since the altered catecholamine concentrations found in the obese state counteract the effect of insulin[2]. Consequently, normal, decreased and increased anti-lipolytic effects of insulin have been reported in obese patients[4]. Insulin sensitivity of adipose tissue lipolysis is normal or slightly impaired in the adipose tissue of obese individuals[4,412]. Modifications of other anti-lipolytic factors may also be altered in obesity.

The pathological enlargement of fat cells in obesity compromises angiogenesis and increases the formation of hypoxic areas that promote the apoptosis of adipocytes and induce the fibrotic and inflammatory programme[87]. Apoptotic adipocytes are surrounded by M1-stage macrophages that form CLS in the adipose tissue. This process is accompanied by a chronic inflammation due to the secretion by adipose tissue-embedded immune cells and the dysfunctional adipocytes of proinflammatory cytokines and acute-phase reactants, such as TNF-α, C-reactive protein, IL-6, IL-8, leptin, serum amyloid A (SAA) and monocyte chemotactic protein (MCP)-1[232,234]. As detailed in the Cytokines and other 'newcomers' section, the increase in proinflammatory adipokines, such as TNF-α or leptin, might be responsible for the high basal rate of lipolysis in obese patients.

Obesity is associated with a decreased expression and activity of HSL, but not ATGL, in visceral and subcutaneous adipocytes of obese individuals independently of age and sex, which may play an important role in the defective lipid mobilisation observed in obesity[413–415]. Furthermore, a decreased access of lipases to TAG due to alterations in lipid droplet-associated proteins cannot be ruled out[416–419]. In humans CGI-58 mutations have been identified in patients with Chanarin–Dorfman syndrome, a disorder characterised by the accumulation of abnormally large amounts of lipid droplets in several organs[420,421]. In these cases CGI-58 cannot be recruited to lipid droplets and fails to interact with perilipin, which may affect basal and PKA-stimulated lipolysis. Interestingly, CGI-58 gene silencing importantly reduces basal lipolysis by approximately 50 % but also completely abrogates PKA-stimulated lipolysis in a human white adipocyte model[255,422]. The exact and complex dynamics involving CGI-58, the diverse perilipins and ATGL in basal as well as PKA-stimulated lipolysis has yet to be completely unravelled.

Finally, changes in the molecules involved in lipolysis-derived metabolites, fatty acids and glycerol also contribute to lipolytic derangements in obesity. Several proteins like FABP, CD36 or FATP facilitate fatty acid transport across the membrane in adipocytes[423]. The transport of the other lipolysis-derived metabolite, glycerol, from adipocytes in response to the lipolytic stimuli is facilitated by AQP3 and AQP7 via their translocation from the cytosolic fraction (AQP3) or lipid droplets (AQP7) to the plasma membrane[341,344,424,425]. AQP7 expression is decreased in subcutaneous adipose tissue of obese subjects, resulting in an increase in intracellular glycerol accumulation, which is converted to glycerol-3-phosphate by the glycerol kinase enzyme and re-esterified into TAG, thereby promoting adipocyte hypertrophy[344,426]. On the other hand, the increased AQP3 and AQP7 expression in visceral fat in obese subjects suggests an overall increase in the lipolytic activity in this fat depot in obesity[344,426,427].

Concluding remarks and future perspectives

While adipose tissue elicited scarce interest for many decades[428], the identification in 1994 of leptin as an adipose-derived hormone[429] started a new era in adipobiology that recognises adipocytes as important dynamic endocrine cells. Essential lipolytic enzymes and a plethora of regulatory proteins and mechanisms have fundamentally changed our view of lipolysis and its impact, not only on adipose tissue but also more broadly on cellular metabolism[430]. Although the importance of lipolysis has been recognised for decades, many of the key proteins involved have been uncovered only recently. In this line, to further decipher the participation of lipolytic products and intermediates in many non-adipose tissues will be especially relevant to unravel previously underappreciated aspects of lipolysis and their relation to disease development. The regulation of lipolysis by numerous, and to some extent still incompletely identified, factors embodies the 'lipolysome', a complex metabolic network involved in ultimately controlling lipid mobilisation and fat storage. Information derived from the reactome linking the genome and metabolome via genome-sequence independent functional analysis of metabolic phenotypes and networks will be particularly fascinating. With the advent of systems biology a better integration of knowledge can be further expected to provide a more profound view of the true contribution of adipose tissue to health and disease.

Acknowledgements

The authors gratefully acknowledge the funding of the Spanish Instituto de Salud Carlos III, Fondo de Investigación Sanitaria – FEDER (project numbers CIBERobn CB06/03/1014, FIS PI10/01677 and PI12/00515) from the Ministerio de Economía y Competitividad, as well as the Plan de Investigación de la Universidad de Navarra (project PIUNA 2011-13). None of the funders had a role in the design, analysis or writing of this article.

All authors contributed fundamentally to the present paper. G. F. conducted the main review of the literature and drafted the manuscript. A. R. and L. M.-G. contributed significantly to the further drafting of the manuscript. All authors (G. F., L. M.-G., J. A. F.-F., S. F. and A. R.) made a critical review of the draft, provided input on data interpretation as well as commented on and approved the final manuscript.

The authors declare no conflicts of interest.

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2. Reduced hepatic aquaporin-9 and glycerol permeability are related to insulin resistance in non-alcoholic fatty liver disease

Article

Rodríguez A, Gena P, **Méndez-Giménez L**, Rosito A, Valentí V, Rotellar F, Sola I, Moncada R, Silva C, Svelto M, Salvador J, Calamita G, Frühbeck G.

Reduced hepatic aquaporin-9 and glycerol permeability are related to insulin resistance in non-alcoholic fatty liver disease.

Int J Obes 2014;38(9):1213-20.

Hypothesis

The hepatocyte basolateral membrane glycerol permeability as well as the expression of aquaglyceroporins in the liver of obese patients with varying degrees of insulin resistance might be related to the onset of NAFLD and NASH.

Objectives

- To analyze the prevalence of NAFLD and NASH in a cohort of morbid obese patients with normoglycemia, impaired glucose tolerance and T2D.
- To characterize the expression of aquaglyceroporins AQP3, AQP7, AQP9 and AQP10 in human liver.
- To study the impact of insulin-resistance on hepatocyte glycerol permeability and AQP9 expression in liver biopsies of morbid obese patients.
- To evaluate the impact of obesity-associated NAFLD and NASH on the expression of AQP9 in human liver according to the degree of steatosis and lobular inflammation.

Rodríguez A, Gena P, Méndez-Giménez L, Rosito A, Valentí V, Rotellar F, Sola I, Moncada R, Silva C, Svelto M, Salvador J, Calamita G, Frühbeck G. Reduced hepatic aquaporin-9 and glycerol permeability are related to insulin resistance in non-alcoholic fatty liver disease. International Journal of Obesity 2014;38(9):1213-20.

3. Leptin administration restores the altered adipose and hepatic expression of aquaglyceroporins improving the non-alcoholic fatty liver of *ob/ob* mice

Article

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Leptin administration restores the altered adipose and hepatic expression of aquaglyceroporins improving the non-alcoholic fatty liver of *ob/ob* mice.

Sci Rep 2015;5:12067.

Hypothesis

The beneficial effects of chronic leptin administration *in vivo* on hepatosteatosis are mediated via the coordinated regulation of aquaglyceroporins in adipose tissue and liver in wild type and leptin-deficient *ob/ob* mice.

Objectives

- To evaluate the effect of acute leptin treatment (4 h) *in vitro* on the expression and intracellular distribution of aquaglyceroporins in murine adipocytes.
- To study the impact of chronic *in vivo* leptin administration (1 month) on body weight, adiposity, hepatosteatosis and expression of aquaglyceroporins in subcutaneous WAT (AQP3 and AQP7) and liver (AQP9) of wild type and *ob/ob* mice.
- To evaluate the correlation of adipose as well as hepatic aquaglyceroporins with markers of adiposity, glucose and lipid metabolism and hepatic steatosis after leptin replacement.

SCIENTIFIC REPORTS

OPEN

Received: 27 January 2015
Accepted: 17 June 2015
Published: 10 July 2015

Leptin administration restores the altered adipose and hepatic expression of aquaglyceroporins improving the non-alcoholic fatty liver of *ob/ob* mice

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Glycerol is an important metabolite for the control of lipid accumulation in white adipose tissue (WAT) and liver. We aimed to investigate whether exogenous administration of leptin improves features of non-alcoholic fatty liver disease (NAFLD) in leptin-deficient *ob/ob* mice via the regulation of AQP3 and AQP7 (glycerol channels mediating glycerol efflux in adipocytes) and AQP9 (aquaglyceroporin facilitating glycerol influx in hepatocytes). Twelve-week-old male wild type and *ob/ob* mice were divided in three groups as follows: control, leptin-treated (1 mg/kg/d) and pair-fed. Leptin deficiency was associated with obesity and NAFLD exhibiting an AQP3 and AQP7 increase in WAT, without changes in hepatic AQP9. Adipose *Aqp3* and hepatic *Aqp9* transcripts positively correlated with markers of adiposity and hepatic steatosis. Chronic leptin administration (4-weeks) was associated with improved body weight, whole-body adiposity, and hepatosteatosis of *ob/ob* mice and to a down-regulation of AQP3, AQP7 in WAT and an up-regulation of hepatic AQP9. Acute leptin stimulation *in vitro* (4-h) induced the mobilization of aquaglyceroporins towards lipid droplets (AQP3) and the plasma membrane (AQP7) in murine adipocytes. Our results show that leptin restores the coordinated regulation of fat-specific AQP7 and liver-specific AQP9, a step which might prevent lipid overaccumulation in WAT and liver in obesity.

Non-alcoholic fatty liver disease (NAFLD) comprises a spectrum of liver disorders ranging from non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) to NASH-cirrhosis, and even hepatocellular carcinoma[1,2]. Obesity is a common well-documented risk factor for NAFLD and

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NASH[3–5]. The prevalence of NAFLD and NASH increases from around 20% and 3%, respectively, in the general population to 75% and 25–70%, respectively, in morbid obesity[3,6]. One of the main contributors leading to obesity-associated NAFLD is the increased adipose tissue lipolysis, the catabolic process leading to hydrolysis of triacylglycerols (TG) into free fatty acids (FFA), and glycerol-3-phosphate[7]. FFA released from visceral adipose tissue are collected into the portal vein and reach the liver at high concentrations, a step leading to excessive hepatic TG deposition and, ultimately, hepatocellular damage[8]. However, scarce is the attention on the relevance of hepatic import of glycerol, the other primary source (as glycerol-3-phosphate) of increased TG in hepatocytes[9].

Aquaglyceroporins (AQP3, 7, 9 and 10) are channel-forming integral membrane proteins that facilitate the movement of water and also small solutes, such as glycerol and urea, across cell membranes[9,10]. AQP7 is the main gateway facilitating glycerol release from adipocytes[10,11], although other glycerol channels such as AQP3, 9, 10 and the most recently described AQP11, also contribute to glycerol efflux from fat depots[12–14]. Circulating plasma glycerol is then introduced in hepatocytes by the liver-specific AQP9, where glycerol kinase (GK) catalyzes the initial step for its conversion into glucose (gluconeogenesis) and/or TG[15–17]. Thus, the coordinated regulation of aquaglyceroporins in adipocytes and hepatocytes plays a key role in maintaining the control of fat accumulation in adipose tissue and liver, as well as whole-body glucose homeostasis[9,18,19]. In this regard both obesity and NAFLD are associated with a dysregulation of aquaglyceroporins in adipose tissue and liver. Obese subjects exhibit high expression of AQP3 and AQP7 in visceral fat and low AQP7 levels in subcutaneous adipose tissue, a condition reflecting increased lipolysis and adipose tissue hypertrophy, respectively, in these fat depots[12,19–21]. On the other hand, NAFLD is associated with a down-regulation of AQP9 in experimental animals[22] and obese patients[23], suggesting a compensatory mechanism whereby liver prevents further TG accumulation and reduces hepatic gluconeogenesis.

Leptin is an adipocyte-derived hormone that exerts lipolytic effects by counteracting the adenosine deaminase-induced tonic inhibition[24]. Previous *in vitro* studies of our group have shown that leptin repressed AQP7 expression in differentiated human adipocytes via PI3K/Akt/mTOR signalling, suggesting a negative feedback regulation in lipolytic states to limit glycerol release from fat cells[12]. Notably, chronic leptin treatment reverts hepatic steatosis in patients with severe lipodystrophy by stimulating lipolysis in hepatocytes[25,26]. Thus, the aim of the present study was to analyze whether the beneficial effects of chronic leptin administration *in vivo* on hepatosteatosis are mediated via the coordinated regulation of aquaglyceroporins in adipose tissue and liver in wild type and leptin-deficient *ob/ob* mice.

Results

Acute leptin treatment *in vitro* regulates the expression and intracellular distribution of aquaglyceroporins in murine adipocytes. Acute leptin treatment increases lipolysis, leading to FFA and glycerol release from the adipose tissue[24,27]. We[12] and others[28–30] have reported that aquaglyceroporins AQP3 and AQP7 facilitate glycerol outflow from adipocytes in response to the lipolysis induced by the β-adrenergic agonist isoproterenol. Thus, in the present study, the direct effect of acute leptin treatment on aquaglyceroporin expression was analyzed by real-time PCR and Western blot in murine differentiated subcutaneous adipocytes. Upon 24-h leptin stimulation, *Aqp3* mRNA tended to decrease ($P = 0.072$) and *Aqp7* gene expression was down-regulated ($P < 0.05$) in murine subcutaneous adipocytes (Fig. 1A,B). Moreover, both AQP3 and AQP7 protein levels were reduced ($P < 0.05$) after leptin treatment (Fig. 1C,D). To gain more insight into the regulation of aquaglyceroporins by leptin, the subcellular localization of AQP was studied in differentiated 3T3-L1 adipocytes by confocal immunofluorescence microscopy (Fig. 1E). We previously described that after subcellular fractionation of quiescent 3T3-L1 adipocytes, AQP3 was located in the plasma membrane and cytosolic fraction, whereas AQP7 was expressed in the subfractions of lipid droplets and the rest of the cytoplasm[12]. In the present study, we confirmed that, under basal conditions, AQP3 was present mainly in the cell surface, although some punctuate labelling in the cytoplasm could also be observed, while AQP7 resided predominantly in the cytoplasm, surrounding lipid droplets of differentiated 3T3-L1 adipocytes. After 4-h leptin stimulation, AQP3 tended to surround lipid droplets more prominently, whereas AQP7 was translocated to the plasma membrane.

In order to test the functionality of aquaglyceroporins on the lipolytic effect triggered by leptin, murine subcutaneous adipocytes were exposed to leptin 10 nmol/L for 24 h in the presence of $HgCl_2$, a nonspecific AQP inhibitor[31], or to $CuSO_4$, a more selective AQP3 inhibitor[32], prior to determination of glycerol release to the culture media. The inhibition of AQP permeability with 0.3 mmol/L $HgCl_2$ alone induced a modest decrease in glycerol release in murine subcutaneous adipocytes (control 3.18 ± 0.19 *vs.* HgCl2 3.06 ± 0.30 mg/dL, $P = 0.729$). Nonetheless, mercury ions abolished around 50% of the leptin-induced glycerol release in murine subcutaneous adipocytes, while copper ions inhibited approximately 20% of the glycerol release caused by leptin (Fig. 1F). These data suggest that the major glycerol channel in murine adipocytes, AQP7 and, to a lesser extent, AQP3 mediate the glycerol efflux triggered by leptin in fat cells.

Chronic leptin administration *in vivo* reduces adiposity in parallel to a decrease in aquaglyceroporins AQP3 and AQP7 in adipose tissue. Leptin is an adipokine that reduces food intake and increases energy expenditure to maintain energy balance[33]. As expected, leptin-deficient *ob/ob* mice

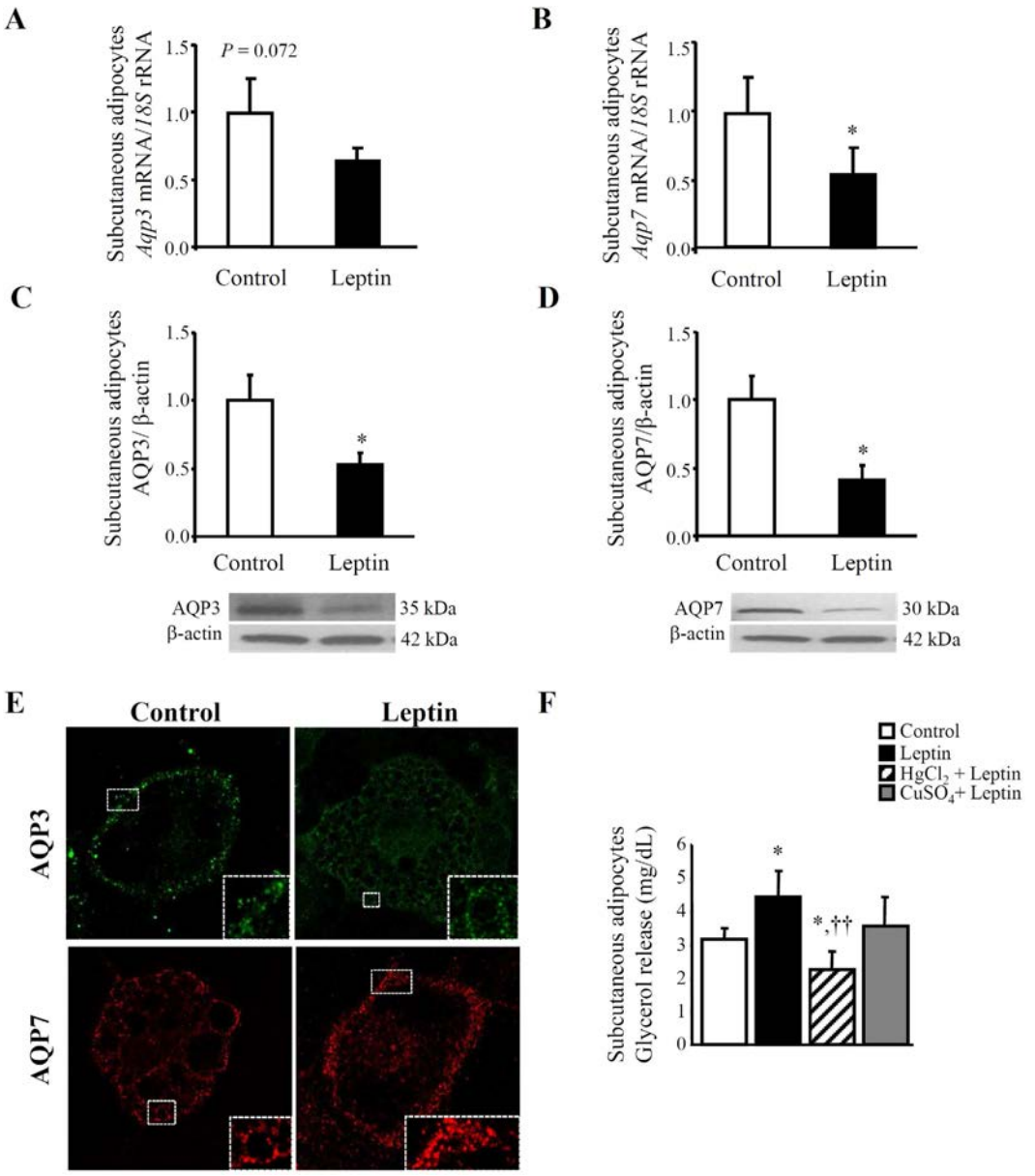

Figure 1. Effect of *in vitro* acute leptin treatment on aquaglyceroporins AQP3 and AQP7 expression and subcellular localization in murine adipocytes. Bar graphs show transcript and protein levels of AQP3 (**A**, **C**) and AQP7 (**B**, **D**) in differentiated murine adipocytes obtained from subcutaneous white adipose tissue (WAT) of wild type mice under basal conditions and after leptin (10 nmol/L) treatment for 24 h. The gene and protein expression in unstimulated cells was assumed to be 1. Representative blots are shown at the bottom of the figure. (**E**) Immunocytochemical detection of the AQP3 and AQP7 proteins in differentiated murine 3T3-L1 adipocytes (day 10) under basal conditions (*upper panels*) and after the stimulation for 4 h with leptin (10 nmol/L) (*lower panels*). Images were taken from the basal planes of the cells. Representative images of at least three separate experiments are shown. (**F**) Glycerol release from murine subcutaneous adipocytes under basal conditions (control) and after leptin (10 nmol/L)-induced stimulation without or with preincubation with $HgCl_2$ (0.3 mmol/L), a nonspecific AQP inhibitor, or with $CuSO_4$ (0.1 mmol/L), a selective AQP3 inhibitor. Differences between groups were analyzed by Student's *t* test or one-way ANOVA followed by Tukey's test. *$P < 0.05$ *vs.* control unstimulated cells; ††$P < 0.01$ *vs.* adipocytes stimulated with leptin.

exhibited severe obesity and hyperphagia (Table 1). Chronic leptin treatment corrected the obese phenotype of *ob/ob* mice, as evidenced by the lower body weight as well as epididymal, subcutaneous and perirenal fat mass via the reduction of food intake and the increase in rectal temperature. In the present study, chronic leptin administration was associated with a decrease in circulating FFA and glycerol, pointing to a lower lipolytic rate in leptin-treated animals.

	Wild type			*ob/ob*		
	Control	Pair-fed	Leptin	Control	Pair-fed	Leptin
n	9	10	9	10	10	9
Body weight (g)[a,b,c]	25.4 ± 0.2	22.6 ± 0.1[*]	22.6 ± 0.1[*]	49.2 ± 0.8[*]	33.6 ± 0.4[*,†]	24.9 ± 0.2[†,$]
Cumulative food intake (g)[a,b,c]	94 ± 2	88 ± 1	88 ± 1	191 ± 5[*]	66 ± 5[*,†]	66 ± 5[*,†]
Rectal temperature (°C)[c]	35.4 ± 0.2	36.0 ± 0.2	35.4 ± 0.1	35.7 ± 0.2	34.7 ± 0.3[†]	36.0 ± 0.2[$]
Epididymal WAT (g)[a,b,c]	0.19 ± 0.01	0.13 ± 0.02	0.04 ± 0.01	1.64 ± 0.10[*]	1.04 ± 0.05[*,†]	0.46 ± 0.06[*,†,$]
Subcutaneous WAT (g)[a,b,c]	0.17 ± 0.01	0.15 ± 0.02	0.09 ± 0.05	3.07 ± 0.32[*]	1.85 ± 0.11[*,†]	0.47 ± 0.06[*,†,$]
Perirrenal WAT (g)[a,b,c]	0.05 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.92 ± 0.05[*]	0.51 ± 0.04[*,†]	0.09 ± 0.01[*,†,$]
Total white adiposity (g)[a,b,c]	0.42 ± 0.04	0.31 ± 0.01	0.14 ± 0.05	5.62 ± 0.38[*]	3.40 ± 0.23[*,†]	1.03 ± 0.13[*,†,$]
FFA (mg/dL)[b]	44 ± 8	45 ± 5	32 ± 4	39 ± 5	55 ± 11	24 ± 2
Glycerol (mmol/mL)[a,b,c]	0.05 ± 0.01	0.03 ± 0.01	0.02 ± 0.01[*]	0.07 ± 0.01[*]	0.05 ± 0.01	0.01 ± 0.01[*,†,$]

Table 1. Markers of adiposity, body temperature and lipolysis of experimental groups. FFA, free fatty acids; WAT, white adipose tissue. Values presented as the mean ± SEM. Differences between groups were analyzed by two-way ANOVA or one-way ANOVA followed by Tukey's *post-hoc* test when an interaction between factors was detected. [a]$P < 0.05$, effect of genotype; [b]$P < 0.05$ effect of treatment; [c]$P < 0.05$ interaction between genotype and treatment. [*]$P < 0.05$ *vs.* vehicle-treated wild type mice; [†]$P < 0.05$ *vs.* vehicle-treated *ob/ob* mice; [#]$P < 0.05$ *vs.* pair-fed wild type mice; [$]$P < 0.05$ *vs.* pair-fed *ob/ob* mice.

To analyze the potential involvement of aquaglyceroporins in the changes observed on adiposity after chronic exogenous leptin administration (4 weeks), we first assessed the gene and protein expression of AQP3 and AQP7 in subcutaneous WAT of the experimental groups by real-time PCR, Western blot and immunohistochemistry (Fig. 2). As illustrated in Fig. 2A,B, the tissue distribution of AQP3 and AQP7 showed a predominant immunostaining in the stromovascular fraction and lower expression in mature adipocytes, as previously reported by our group and others[12,34]. In the multiple lineal regression analysis, AQP3 and AQP7 protein levels in subcutaneous WAT contributed independently to 51.0% ($P < 0.05$) and 51.2% ($P < 0.05$) to the circulating glycerol concentrations after controlling for body weight, suggesting an important role of these aquaglyceroporins in glycerol efflux from adipose tissue.

Leptin deficiency was associated with higher mRNA and protein levels of AQP3 and AQP7 in subcutaneous WAT (Fig. 2C–F). In line with these results, *Aqp3* and *Aqp7* mRNA levels were positively associated with markers of adiposity [body weight ($r = 0.33$, $P = 0.025$ and $r = 0.44$, $P = 0.001$) or subcutaneous WAT/body weight ($r = 0.33$, $P = 0.025$ and $r = 0.53$, $P = 0.001$)] and hepatosteatosis [liver/body weight ($r = 0.36$, $P = 0.013$ and $r = 0.35$, $P = 0.010$ and intrahepatic TG ($r = 0.40$, $P = 0.006$ and $r = 0.53$, $P < 0.001$)]. No differences in the transcript levels of *Aqp3* and *Aqp7* were detected after leptin administration, but a tendency towards a down-regulation of both glycerol channels was observed in leptin-treated *ob/ob* mice. Nonetheless, at the protein level, both leptin administration and caloric restriction reduced ($P < 0.05$) AQP3 and AQP7 in subcutaneous WAT of wild type and *ob/ob* mice.

Exogenous leptin replacement reduces the hepatic steatosis of *ob/ob* mice and upregulates AQP9 expression in the liver. Leptin-deficient *ob/ob* mice showed an increased ($P < 0.0001$) liver weight that was significantly reduced ($P < 0.0001$) by either caloric restriction or leptin replacement (Fig. 3A). Histological sections of leptin-deficient *ob/ob* mice were characterized by the presence of severe macrovesicular steatosis, but not advanced inflammation/fibrosis, that was completely reverted after leptin administration for 28 days (Fig. 3E). The analysis of intrahepatic triacylglycerol content revealed elevated TG levels ($P < 0.001$) in the liver of *ob/ob* mice that was prevented by leptin treatment ($P < 0.05$), but not by caloric restriction (Fig. 3B).

We next analyzed the expression of AQP9, the primary route for glycerol uptake in murine hepatocytes, by real-time PCR, Western blot and immunohistochemistry. As previously described by our group[22], two immunoreactive bands of 30–32 kDa, corresponding to the core and N-glycosylated forms of AQP9 protein, respectively, were observed in the immunoblots (Fig. 3D). Leptin deficiency was associated with similar expression of *AQP9* mRNA and whole (glycosylated and non-glycosylated) AQP9 protein signal than that observed in wild type mice, with leptin administration and caloric restriction increasing ($P < 0.05$) AQP9 gene and protein expression (Fig. 3C,D). *Aqp9* gene expression was positively associated with markers of adiposity [body weight ($r = 0.60$, $P < 0.001$) or subcutaneous WAT/body weight ($r = 0.44$, $P = 0.002$)] and hepatic steatosis [liver/body weight ($r = 0.69$, $P < 0.001$) and intrahepatic TG ($r = 0.28$, $P < 0.05$)]. Liver sections showed a strong immunoreactivity for AQP9 after leptin infusion, which was mainly localized in the plasma membrane of hepatocytes around the central veins (Fig. 3E).

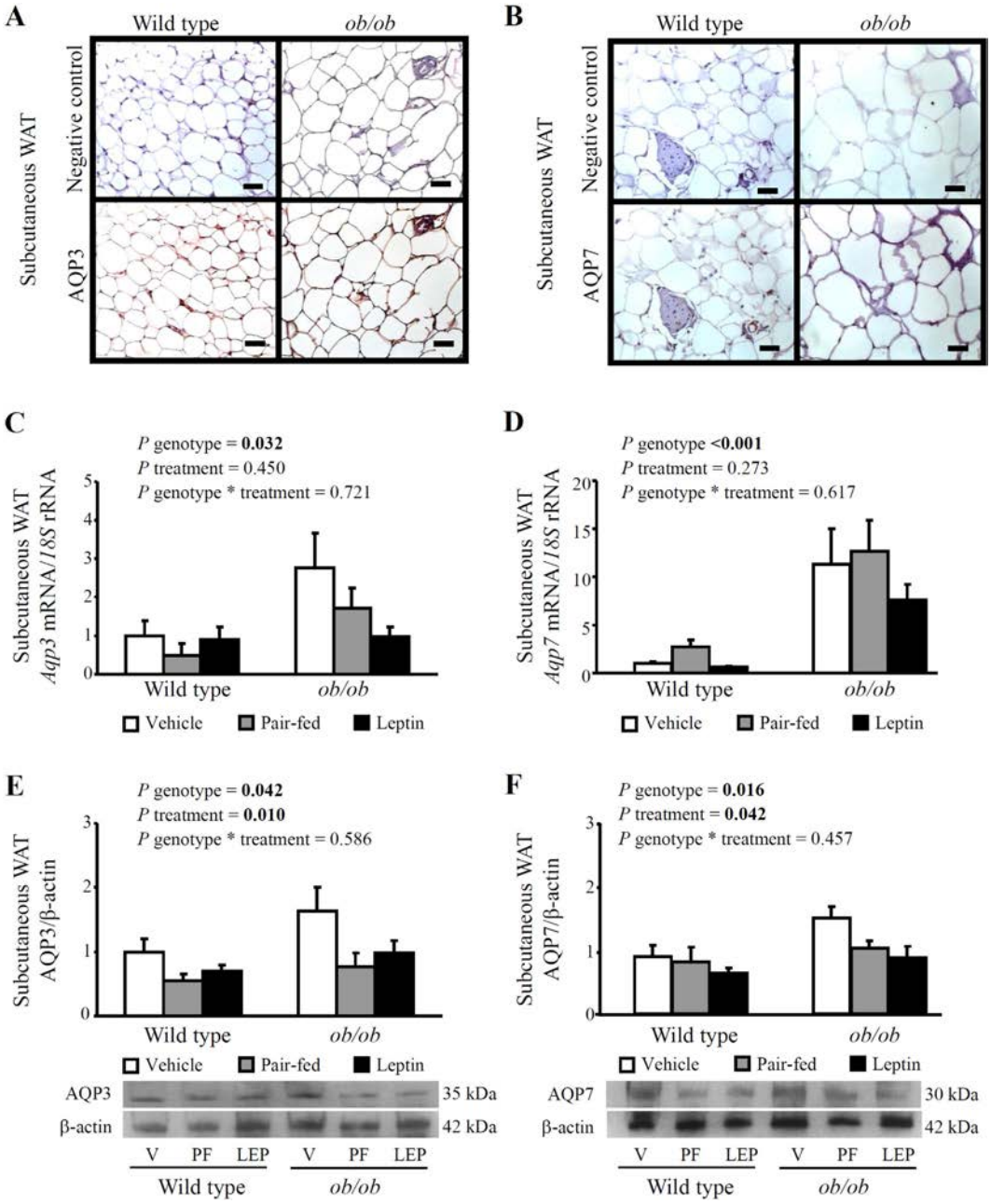

Figure 2. Effect of *in vivo* chronic leptin administration on aquaglyceroporins AQP3 and AQP7 expression and tissue distribution in adipose tissue of wild type and *ob/ob* mice. Immunohistochemical detection of AQP3 (**A**) and AQP7 (**B**) in subcutaneous white adipose tissue (WAT) of wild type (*left panels*) and *ob/ob* (*right panels*) mice (magnification 200X, scale bar = 50 μm). Bar graphs show transcript and protein levels of AQP3 (**C**, **E**) and AQP7 (**D**, **F**) in subcutaneous WAT obtained from vehicle-treated, pair-fed and leptin-treated wild type and *ob/ob* mice. The gene and protein expression in vehicle-treated wild type mice was assumed to be 1. Representative blots are shown at the bottom of the figure. Differences between groups were analyzed by two-way ANOVA.

Positive association of PPARγ with changes observed in the expression of aquaglyceroporins in adipose tissue and liver after leptin replacement. Peroxisome proliferator-activated receptor γ (PPARγ) represents a well-known lipogenic factor and, importantly, putative peroxisome proliferator response elements (PPRE) are present in the promoters of *Aqp3* and *Aqp7* genes[35,36]. In line with the observed excess adiposity and hepatic steatosis, leptin-deficient mice exhibited higher *Pparg* mRNA levels in the adipose tissue and liver that were reduced by leptin replacement and, to a lesser extent, by caloric restriction (Fig. 4A,B). As expected, gene expression levels of *Pparg* in subcutaneous WAT and liver were positively associated with markers of obesity [body weight ($r = 0.43$, $P < 0.001$ and

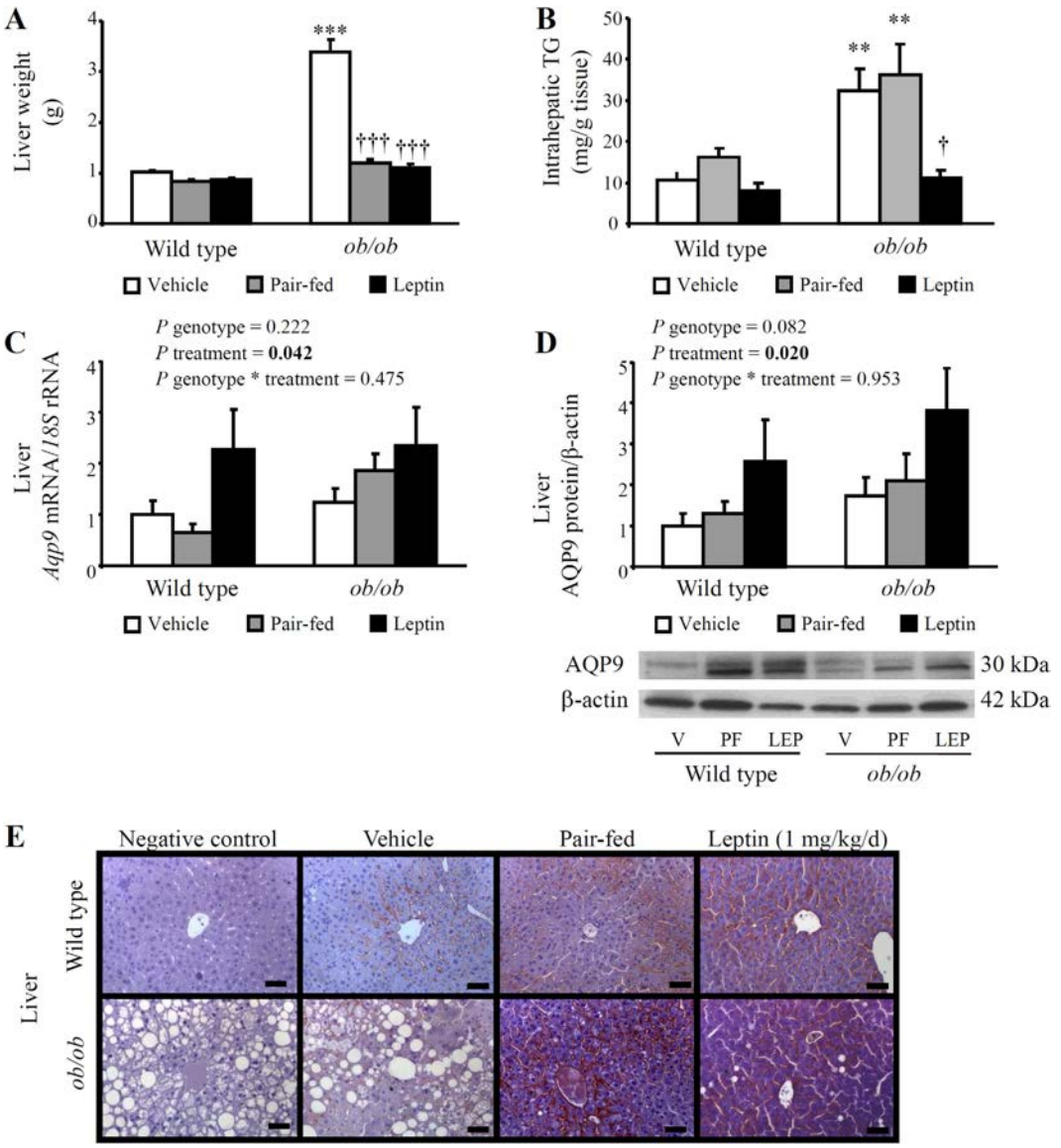

Figure 3. Effect of *in vivo* chronic leptin administration on fatty liver and hepatic aquaglyceroporin AQP9 expression in experimental animals. Bar graphs show the liver weight (**A**) intrahepatic concentrations of triacylglycerols (TG) (**B**) as well as gene (**C**) and protein (**D**) expression levels of AQP9 in the liver of vehicle-treated, pair-fed and leptin-treated wild type and *ob/ob* mice. The gene and protein expression in vehicle-treated wild type mice was assumed to be 1. Representative blots are shown at the bottom of the figure. (**E**) Inmunohistochemical detection of AQP9 protein in histological sections of liver of wild type (*upper panels*) and *ob/ob* mice (*lower panels*) (magnification, 200X; scale bar, 50 μm). Differences between groups were analyzed by two-way ANOVA or one-way ANOVA followed by Tukey's *post-hoc* test, if an interaction was detected. $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* vehicle-treated wild type mice; $\dagger P < 0.05$; $\dagger\dagger\dagger P < 0.001$ *vs.* vehicle-treated *ob/ob* mice.

r = 0.70, $P < 0.0001$) or subcutaneous WAT/body weight (r = 0.35, $P = 0.010$ and r = 0.70, $P < 0.0001$)], fatty liver [liver weight/body weight (r = 0.43, $P < 0.001$ and r = 0.65, $P < 0.0001$) and intrahepatic TG (r = 0.40, $P = 0.003$ and r = 0.45, $P = 0.001$)]. Moreover, a strong positive association was found between *Pparg* transcript levels and *Aqp7* mRNA in the adipose tissue as well as with *Aqp9* mRNA in the liver (Fig. 4C,D). *Pparg* mRNA was also correlated with *Aqp3* gene expression in subcutaneous WAT but to a lower extent (r = 0.43, $P < 0.001$).

To gain further insight into the plausible association of PPARγ with these glycerol channels after leptin treatment, we examined the effect of leptin stimulation on basal and PPARγ agonist rosiglitazone-induced expression of aquaglyceroporins in murine subcutaneous differentiated adipocytes and AML12 hepatocytes. As expected, rosiglitazone stimulation for 24 h upregulated 1.4- and 2.0-fold the transcription of *Pparg* gene in murine subcutaneous adipocytes and AML12 hepatocytes, respectively, although no

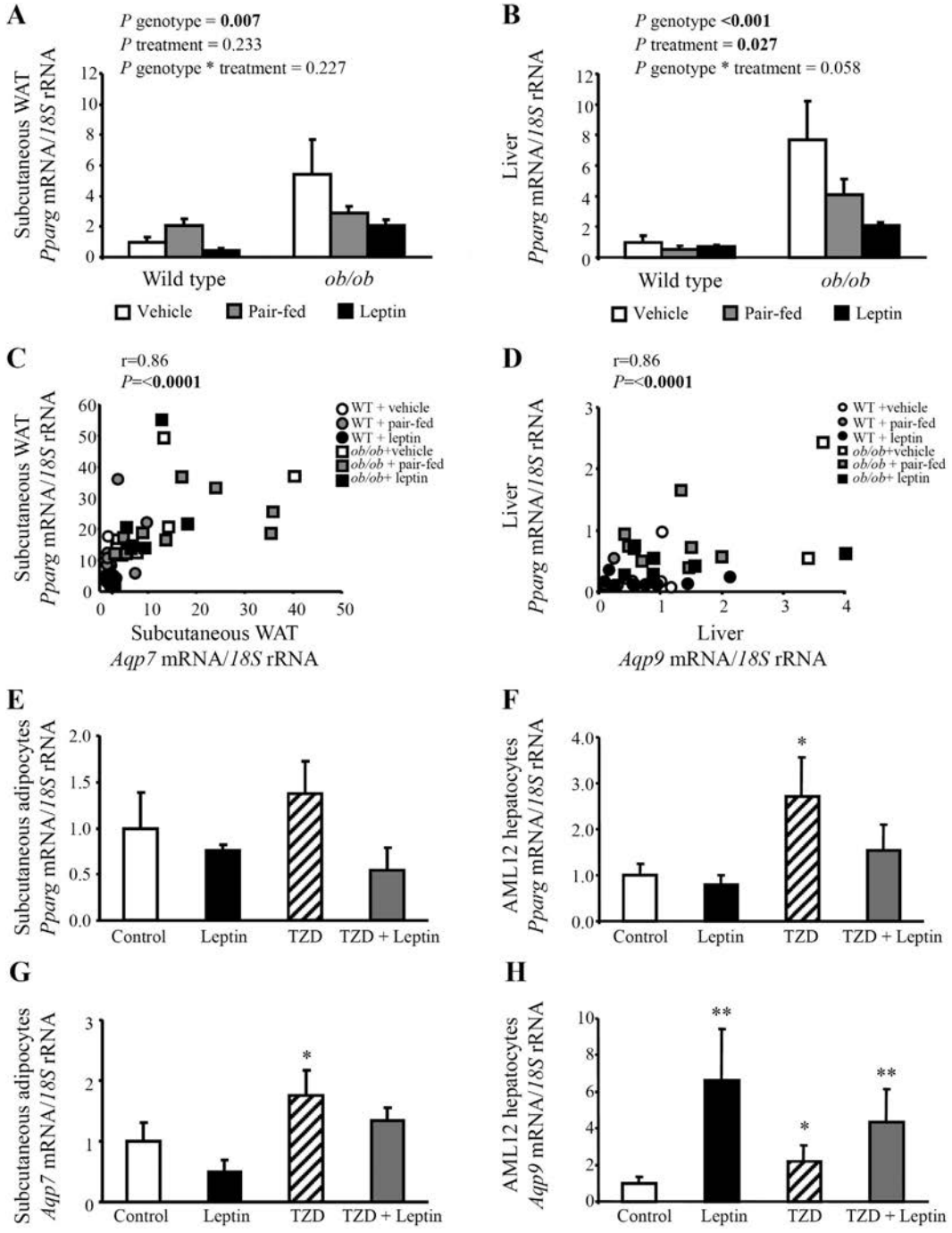

Figure 4. Positive association of *Pparg* transcript levels with aquaglyceroporins *Aqp7* and *Aqp9* in adipose tissue and liver of experimental animals. Bar graphs illustrate the changes in *Pparg* mRNA levels in subcutaneous white adipose tissue (WAT) (**A**) and liver (**B**) obtained from vehicle-treated, pair-fed and leptin-treated wild type and *ob/ob* mice. A positive correlation was found between *Pparg* and *Aqp7* mRNA in subcutaneous WAT (**C**) as well as between *Pparg* and *Aqp9* transcript levels in liver (**D**) of experimental groups. The Pearson's correlation coefficient (r) and *P* values are indicated. Effect of leptin stimulation for 24 h on basal and thiazolidinedione (TZD) rosiglitazone (10 μmol/L)-induced expression of *Pparg* (**E**, **F**) and aquaglyceroporins *Aqp7* and *Aqp9* (G, H) in murine differentiated subcutaneous adipocytes and AML12 hepatocytes. The gene expression in vehicle-treated wild type mice or unstimulated cells was assumed to be 1. Differences between groups were analyzed by two-way ANOVA or one-way ANOVA followed by Tukey's *post-hoc* test, where appropriate. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control unstimulated cells.

statistical differences between groups were found in fat cells ($P = 0.269$) (Fig. 4E,F). Moreover, the treatment with this TZD also increased the transcription of *Aqp7* in subcutaneous fat cells and of *Aqp9* in AML12 hepatocytes (Fig. 4G,H). The co-incubation with leptin tended to reduce both basal and TZD-induced mRNA expression of *Pparg* and *Aqp7* genes in subcutaneous adipocytes, although changes fell out of statistical significance ($P = 0.083$ and $P = 0.125$, respectively). A similar trend was observed for the effect of leptin on basal and rosiglitazone-induced expression of *Aqp3* gene in subcutaneous adipocytes (control 1.0 ± 0.4 A.U.; leptin 0.4 ± 0.1 A.U.; TZD 4.3 ± 1.6 A.U.; TZD + leptin 2.7 ± 0.8 A.U.; $P = 0.003$). However, leptin co-treatment induced a slight down-regulation of *Pparg* transcript levels ($P = 0.292$), while increasing ($P < 0.05$) the transcription of *Aqp9* in AML12 hepatic cells.

Discussion

Adipocyte lipolysis is the process that controls the breakdown of TG into glycerol and FFA, which are released into the circulation and used as energy substrates in metabolic organs[7,37]. AQP3 and AQP7 facilitate glycerol outflow from adipocytes in response to β-adrenergic receptor-stimulated lipolysis via its translocation from the cytosolic fraction (AQP3) or lipid droplets (AQP7) to the plasma membrane[12,28,29]. Basal lipolytic activity of adipocytes is conditioned not only by catecholamines, but also by other factors, such as atrial natriuretic peptides, insulin, leptin, adenosine, tumor necrosis factor α (TNF-α) or neuropeptide Y, among others[7]. The adipokine leptin exerts an autocrine/paracrine lipolytic effect on murine adipocytes[27]. In this sense, acute leptin treatment (1 h) reportedly increases basal lipolysis of wild type and *ob/ob* mice[27]. Here, we found that acute leptin treatment (4 h) stimulated AQP3 translocation from the plasma membrane to lipid droplets, a step that might reflect the glycerol efflux from lipid droplets after lipolytic response in differentiated subcutaneous murine adipocytes. Upon leptin stimulation, AQP7 was translocated from lipid droplets to the plasma membrane, and this finding suggests that this glycerol channel constitutes the main gateway for glycerol secretion to the bloodstream. Thus, we speculate that acute leptin treatment induces the translocation of AQP3 and AQP7 to lipid droplets and the plasma membrane, respectively, to facilitate glycerol mobilization after lipolysis. Nonetheless, the existence of further operative glycerol channels in subcutaneous adipocytes cannot be discarded.

Obesity is associated with increased lipolysis due to higher lipolytic activity of β_3-adrenergic receptors and reduced anti-lipolytic action of insulin, leading to elevated circulating concentrations of FFA and glycerol[38,39]. In the present study, we found that leptin-deficient obese *ob/ob* mice showed increased circulating glycerol together with higher subcutaneous fat expression of AQP3 and AQP7. Both chronic leptin treatment and caloric restriction significantly decreased circulating glycerol and AQP3 and AQP7 proteins in subcutaneous adipose tissue in *ob/ob* mice. The adipose tissue is composed not only by adipocytes, but also by SVFCs (i.e., macrophages, T lymphocytes, endothelial cells, fibroblasts, vascular smooth muscle cells or mesenchymal stem cells). Because SVFCs might contribute to the reduction of aquaglyceroporins in adipose tissue, we also studied the direct effect of leptin treatment on differentiated murine subcutaneous adipocytes. In line with the results obtained with the whole adipose tissue, 24-h leptin treatment decreased the gene and protein expression of AQP3 and AQP7 of differentiated murine subcutaneous adipocytes. In this regard, in a previous study, we found that *in vitro* 24-h leptin treatment downregulated AQP7 protein expression in differentiated human adipocytes via the PI3K/Akt/mTOR signalling pathway[12]. Taken together, both *in vivo* chronic leptin administration and caloric restriction limit glycerol release from adipocytes through the down-regulation of AQP3 and AQP7, suggesting a negative feedback regulation in lipolytic states to maintain intracellular glycerol and, therefore, to avoid the depletion of fat stores (Fig. 5).

Liver steatosis is a multi-factorial disease where abnormal TG accumulation in the hepatocytes can be triggered by metabolic, toxic, pharmacological or viral insults across a genetic predisposition[1,2]. Glycerol-3-phosphate constitutes a key metabolite for *de novo* synthesis of TG and derives from glycolysis, glyceroneogenesis as well as recycling of glycerol by GK[40,41]. AQP9 represents the main facilitative pathway for glycerol uptake as a substrate for gluconeogenesis and lipogenesis in hepatocytes[15–17]. Interestingly, a decrease in hepatic AQP9 and glycerol permeability has been observed in murine and human NAFLD, suggesting a defensive mechanism to prevent further development of hyperglycemia and hepatosteatosis[19,22,23]. Moreover, a dysregulation of AQP9 has been observed in several hepatic inflammatory derangements, such as extrahepatic cholestasis, alcoholic steatohepatitis and NASH[23,42–44]. However, little is known about the regulation of AQP9 in the context of NAFLD/NASH. In the present study, AQP9 was mainly localized in the sinusoidal domain of the plasma membrane of hepatocytes, which is in agreement with previous results[45,46] including ours[12,23]. Leptin-deficient mice, a murine model of NAFLD, displayed macrovesicular steatosis without changes in hepatic AQP9 mRNA and protein. In a previous study, a lower expression of AQP9 was found in liver samples of *ob/ob* mice[22]. In this regard, AQP9 expression in the liver is influenced by the degree of hepatic steatosis and inflammation[23] that might change the expression of this aquaglyceroporin during the ongoing NAFLD in adult *ob/ob* mice. Short-term leptin administration has been reported to exert profound effects on hepatic lipid metabolism of *ob/ob* mice by reducing *de novo* lipogenesis via repressing acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) or stearoyl-coenzyme A desaturase 1 (SCD1) expression, and through the activation of β-oxidation by increasing the transcript levels of acetyl-coenzyme A acetyl-transferase 1 (ACAT1) or carnitine palmitoyl transferase 1 (CPT1)[47]. We herein show that chronic leptin administration completely rescues the hepatosteatosis of *ob/ob* mice as evidenced by the normalization of intrahepatocellular

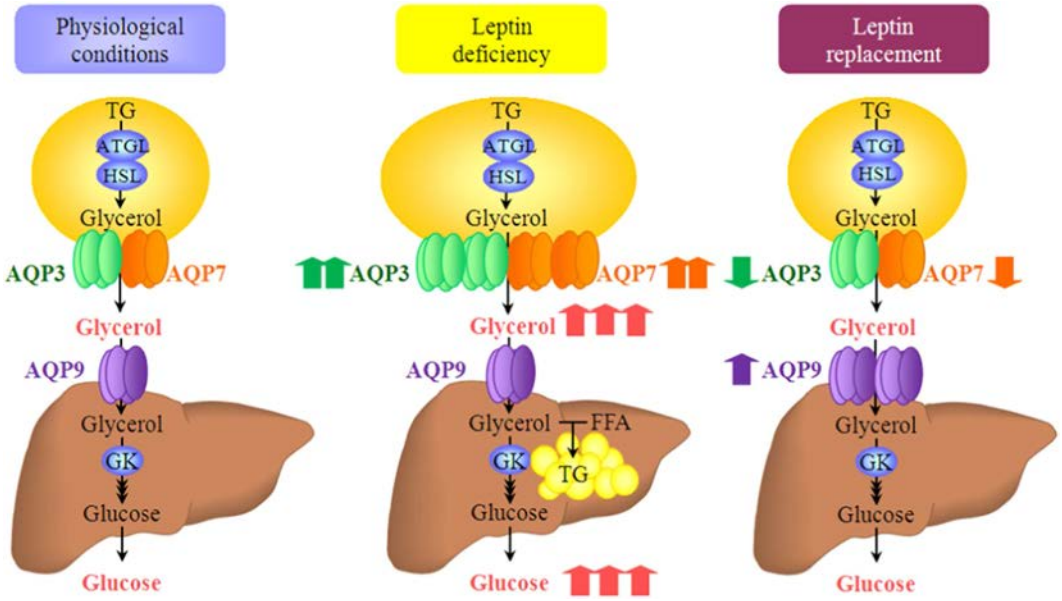

Figure 5. Proposed working model for the coordinated regulation of aquaglyceroporins in adipose tissue and liver by leptin.

hepatocytes and liver morphology. Moreover, a valuable result of this work regards the up-regulation of AQP9 after chronic leptin treatment in wild type and *ob/ob* mice. Taken together, similar or lower levels of AQP9 associated to leptin deficiency appear to reflect a defensive cell reaction of the steatotic hepatocyte. Interestingly, chronic leptin administration not only rescues the fatty liver, but also increases AQP9 in order to facilitate glycerol import into hepatocytes for maintaining the glycemia as well as an appropriate lipid metabolism.

The adipose tissue and liver from leptin-deficient *ob/ob* mice showed an induction of PPARγ, which is a critical transcription factor for the development of obesity and hepatic steatosis as previously reported by other authors[48–50]. In a previous study of our group[51], we found that the downregulation of PPARγ in adipose tissue and liver of diet-induced obese rats after bariatric surgery was strongly associated with a reduction in the transcription of aquaglyceroporins in these tissues. Nonetheless, the molecular mechanisms underlying this association were unclear. In the present study, we found that chronic leptin administration significantly decreased *Pparg* transcript levels in parallel with the improvement of adiposity and fatty liver. Interestingly, the promoters of *Aqp3* and *Aqp7* genes present putative PPRE with the expression of these aquaglyceroporins being up-regulated by PPARγ agonists[35,36]. In line with this observation, *Pparg* transcript levels were positively correlated with *Aqp3* and *Aqp7* in adipose tissue, but also with *Aqp9* in the liver. Moreover, leptin co-treatment tended to reduce the transcription of PPARγ and AQP7 induced by rosiglitazone stimulation, a well-known PPARγ-selective agonist, in murine subcutaneous adipocytes. Our results are in agreement with other reports showing that pioglitazone and rosiglitazone administration to rodents increase the expression of AQP7 in adipose tissue[35,52]. However, leptin increased both basal and rosiglitazone-induced transcription of *Aqp9* in AML12 hepatocytes, despite inducing a slight reduction *Pparg* mRNA levels in these hepatic cells. Thus, the mild action of leptin on rosiglitazone-induced up-regulation of aquaglyceroporins in adipocytes and hepatocytes suggests that other upstream molecules in addition to PPARγ might be involved in the regulatory effect of this adipokine.

The coordinated regulation of adipose and hepatic aquaglyceroporins is extremely relevant to maintain the control of fat accumulation and glycemia (Fig. 5)[12,18]. We herein report, for the first time, that chronic leptin administration regulates the altered expression of the adipose glycerol channels AQP3 and AQP7 and the liver-specific AQP9 in leptin-deficient obese *ob/ob* mice. Since glycerol is a key metabolite for lipid accumulation in fat depots and liver, the improvement of glycerol availability might be involved in the beneficial effects of leptin on obesity and NAFLD. Nonetheless, future *in vivo* studies are needed to fully demonstrate the requirement of AQP proteins for the improvement of these pathologies. Moreover, the time functional link between the regulation of AQP and leptin-dependent changes in lipid flux at the clinical level require the exact characterization of NAFLD and more advanced liver damage stages in patients with respect to weight changes and diet.

Methods

Animals. Ten-week-old male wild type (C57BL/6J) (n = 30) and genetically obese *ob/ob* mice (C57BL/6J) (n = 30) (Harlan Laboratories Inc., Barcelona, Spain) were housed in a room with controlled

temperature (22 ± 2 °C), and a 12:12 light-dark cycle (lights on at 08:00 am). Wild type and *ob*/*ob* mice were divided in control, leptin-treated (1 mg/kg/d) and pair-fed groups (n = 10 per group), as previously described[26]. The control and pair-fed groups received vehicle (PBS), while leptin-treated groups were intraperitoneally administered with leptin (Bachem, Bubendorf, Switzerland) twice a day at 08:00 and 20:00 for 28 days. Control and leptin-treated groups were provided with water and food *ad libitum* with a rodent maintenance diet (12.1 kJ: 4% fat, 82% carbohydrate and 14% protein, Diet 2014S, Teklad Global Diets, Harlan, Barcelona, Spain), while the daily food intake of the pair-fed groups was matched to the amount eaten by the leptin-treated groups the day before to discriminate the inhibitory effect of leptin on appetite. All experimental groups had an isoproteic intake consuming similar amounts of sodium and phytates[53]. Body weight and food intake were daily registered and rectal temperature was measured using a thermoprobe (YSI 4600 Series Precision Thermometers, YSI Temperature, Dayton, OH, USA) at the end of the experiment. Animals were sacrificed on the 28th day of treatment by CO_2 inhalation. Epididymal, subcutaneous and perirenal white adipose tissue (WAT) as well as the liver were rapidly dissected out, weighed, frozen in liquid nitrogen, and stored at −80 °C until mRNA and protein extraction. A piece of the tissues was fixed in 4% formaldehyde for immunohistochemical analyses. All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU) and were approved by the Ethical Committee for Animal Experimentation of the University of Navarra (041/08).

Blood and tissue assays. Blood assays were determined as previously described[26]. Intrahepatic TG content was measured by enzymatic methods, in accordance with previously published procedures[12]. Briefly, liver biopsies were homogenized and diluted in saline at a final concentration of 50 mg/mL. Homogenates were diluted (1:1) in 1% deoxycholate (Sigma, St. Louis, MO, USA) and incubated at 37 °C for 5 min. For TG measurements, samples were diluted 1:100 in the reagent (Infinity™ Triglycerides Liquid Stable Reagent, Thermo Electron Corporation, Melbourne, Australia) and incubated for 30 min at 37 °C. The resulting dye was measured based on its absorbance at 550 nm. Concentrations were determined compared with a standard TG curve (Infinity™ Triglycerides Standard, Thermo Electron Corporation). The protein content of the preparations was measured by the Bradford method, using bovine serum albumin (BSA) (Sigma) as standard. All assays were performed in duplicate.

RNA extraction and real-time PCR. RNA isolation and purification was performed as described earlier[19]. Transcript levels for *Aqp3* (NM_016689.2), *Aqp7* (NM_007473.4), *Aqp9* (NM_022026.2) and *Pparg* (NM_001127330.1) were quantified by real-time PCR (7300 Real Time PCR System, Applied Biosystems, Foster City, CA, USA). Primers and probes (Supplementary Table S1) were designed using the software Primer Express 2.0 (Applied Biosystems) and purchased from Genosys (Sigma). Primers or TaqMan® probes encompassing fragments of the areas from the extremes of two exons were designed to ensure the detection of the corresponding transcript avoiding genomic DNA amplification. The cDNA was amplified at the following conditions: 95 °C for 10 min, followed by 45 cycles of 15 s at 95 °C and 1 min at 59 °C, using the TaqMan® Universal PCR Master Mix (Applied Biosystems). The primer and probe concentrations were 300 and 200 nmol/L, respectively. All results were normalized for the expression of 18 S rRNA (Applied Biosystems), and relative quantification was calculated using the ΔΔCt formula[19]. Relative mRNA expression was expressed as fold expression over the calibrator sample. All samples were run in triplicate and the average values were calculated.

Western blot studies. Tissues and cells were harvested and homogenized in ice-cold lysis buffer (0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA·2 H_2O, 1 mol/L Tris-HCl, 150 mmol/L NaCl, 1% sodium deoxycholate, pH 7.40) supplemented with a protease inhibitor cocktail (Complete™ Mini-EDTA free, Roche, Mannheim, Germany). Lysates were centrifuged at 16,000 g at 4 °C for 15 min to remove nuclei and unbroken cells. Total protein concentrations were determined by the Bradford assay. Thirty micrograms of total protein were diluted in loading buffer 4X (20% β-mercaptoethanol, 40 mmol/L dithiothreitol, 8% SDS, 40% glycerol, 0.016% bromophenol blue, 200 mmol/L Tris-HCl, pH 6.80) and heated for 10 min at 100 °C. Samples were run out in 10% SDS-PAGE, subsequently transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked in Tris-buffered saline (TBS) (10 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.00) with 0,05% Tween 20 containing 5% non-fat dry milk for 1 h at room temperature (RT). Blots were then incubated overnight at 4 °C with goat polyclonal anti-AQP3, rabbit polyclonal anti-AQP7, goat polyclonal anti-AQP9 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or murine monoclonal anti-β-actin (Sigma) (diluted 1:5,000 in blocking solution). The antigen-antibody complexes were visualized using horseradish peroxidase (HRP)-conjugated anti-goat, anti-rabbit or anti-mouse IgG antibodies (diluted 1:5,000 in blocking solution) and the enhanced chemiluminescence ECL Plus detection system (Amersham Biosciences, Buckinghamshire, UK). The intensity of the bands was determined by densitometric analysis with the Gel Doc™ gel documentation system and Quantity One 4.5.0 software (Bio-Rad) and normalized with β-actin density values. All assays were performed in duplicate.

Immunohistochemistry. The immunodetection of AQP3, AQP7 and AQP9 in histological sections of subcutaneous adipose tissue and liver was performed by the indirect immunoperoxidase method[12].

Sections of formalin-fixed paraffin-embedded adipose tissue (6 μm) or liver (4 μm) were dewaxed in xylene, rehydrated in decreasing concentrations of ethanol and treated with 3% H_2O_2 (Sigma) in absolute methanol for 10 min at RT to quench endogenous peroxidase activity. Slides were blocked during 1 h with 1% goat serum (Sigma) diluted in TBS (50 mmol/L Tris, 0.5 mol/L NaCl; pH 7.36) to prevent nonspecific adsorption. Sections were incubated overnight at 4 °C with goat polyclonal anti-AQP3, rabbit anti-AQP7 (Santa Cruz Biotechnology) or rabbit anti-AQP9 (#AQP91-A, Alpha Diagnostic International, San Antonio, TX, USA) antibodies diluted 1:100 for AQP3 and AQP7 in subcutaneous WAT and 1:500 for AQP9 in liver in TBS. After washing three times (5 min each) with TBS, slides were incubated with HRP-conjugated anti-goat IgG diluted in TBS (1:500) or Dako Real™ EnVision™ HRP-conjugated anti-rabbit/mouse (Dako, Glostrup, Denmark) for 1 h at RT. The peroxidase reaction was visualized using a 0.5 mg/mL diaminobenzidine (DAB)/0.03% H_2O_2 solution diluted in 50 mmol/L Tris-HCl, pH 7.36, and Harris hematoxylin solution (Sigma) as counterstaining. Negative control slides without primary antibody were included to assess non-specific staining.

Cell cultures. Murine stromovascular fraction cells (SVFC) were isolated from subcutaneous adipose tissue from wild type mice as previously described[12]. SVFC were seeded at 2×10^5 cells/cm^2 and grown in adipocyte medium [DMEM/F-12 [1:1] (Invitrogen, Paisley, UK), 16 μmol/L biotin, 18 μmol/L panthotenate, 100 μmol/L ascorbate and antibiotic-antimycotic] supplemented with 10% newborn calf serum (NCS). After 4 days, the medium was changed to adipocyte medium supplemented with 3% NCS, 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 0.1 μmol/L dexamethasone, and 10 μg/mL insulin. After a 3-day induction period, cells were fed every 2 days with the same medium but without IBMX supplementation for the remaining 7 days of adipocyte differentiation.

The non-tumorigenic mouse hepatocyte cell line AML12 was purchased from ATCC (Manassas, VA). Murine AML12 hepatocytes were maintained in DMEM/F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium (Invitrogen), 40 ng/mL dexamethasone (Sigma), and antibiotic-antimycotic (Complete Growth Medium). AML12 cells were seeded at 2×10^5 cell/cm^2 and grown in Complete Growth Medium.

Differentiated subcutaneous adipocytes and AML12 hepatocytes were serum-starved for 24 h and quiescent cells were stimulated with recombinant murine leptin (10 nmol/L) (450-31, PeproTech EC, Inc., Rocky Hill, NJ, USA) or with TZD rosiglitazone (BRL49653, Cayman Chemical Ann Arbor, MI) 10 μmol/L for 24 h. One sample per experiment was used to obtain control responses in the presence of the solvent.

Measurement of glycerol release. Glycerol release to the culture media was evaluated according to previously described methods[12]. Briefly, differentiated murine subcutaneous adipocytes were stimulated with leptin 10 nmol/L for 24 h at 37 °C in the presence or absence of $HgCl_2$ (0.3 mmol/L) or $CuSO_4$ (0.1 mmol/L). The glycerol concentration in the culture media was measured by a quantitative enzymatic determination assay (Sigma). Intra- and inter-assay coefficients of variation were 1.5% and 4.2%, respectively.

Confocal immunofluorescence microscopy. 3T3-L1 cells were differentiated into adipocytes as previously described[12], grown on glass coverslips and stimulated with leptin (10 nmol/L) for 4 h. Cells were fixed in cold methanol (−20 °C), washed with PBS, permeabilized with blocking solution (PBS containing 0.1% Triton X-100 and 5 mmol/L glycine) for 1 h at RT and then incubated with goat polyclonal anti-AQP3 or rabbit polyclonal anti-AQP7 (Santa Cruz Biotechnology) antibodies diluted 1:100 overnight at 4 °C. After washing with PBS, cells were incubated with Alexa Fluor® 488-conjugated donkey anti-goat IgG or Alexa Fluor® 594 chicken anti-rabbit (Invitrogen) diluted 1:200 for 2 h at RT. After washing, coverslips were mounted on microscope slides and examined under a TCS-SP2-AOBS confocal laser scanning microscope (Leica Corp., Heidelberg, Germany).

Statistical analysis. Data are expressed as the mean ± SEM. Statistical differences between mean values were analyzed using two-way ANOVA (genotype x treatment) or one-way ANOVA followed by Tukey's post-hoc test, if an interaction was detected. Pearson correlation coefficients (r) and stepwise multiple linear regression analysis were used to analyze the association between variables. A P value < 0.05 was considered statistically significant. The statistical analyses were performed using the SPSS/Windows version 15.0 software (SPSS Inc,. Chicago, IL, USA).

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Acknowledgements

The authors gratefully acknowledge Patricia Autonell for her technical assistance. This work was supported by Fondo de Investigación Sanitaria-FEDER (FIS PI10/01677, PI12/00515 and PI13/01430) from the Spanish Instituto de Salud Carlos III, the Department of Health of the Gobierno de Navarra (61/2014) as well as by the Plan de Investigación de la Universidad de Navarra (PIUNA 2011-14). CIBER de Fisiopatología de la Obesidad y Nutrición (CIBERobn) is an initiative of the Instituto de Salud Carlos III, Spain.

Author Contributions

A.R. and G.F. designed the study. A.R., N.M., I.B., L.M.-G., S.B., V.C., J.G.A., M.M.M. and G.F. researched data. A.R. and G.F. wrote the manuscript. A.R., N.M., S.B., V.C., P.P., G.C., G.S., M.M.M. and G.F. contributed to the Discussion. A.R., N.M., I.B., L.M.-G., S.B., V.C., J.G.A., P.P., G.C., G.S., M.M.M. and G.F. reviewed/edited manuscript. A.R. and G.F. acquired funding for this study. G.F. is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Rodríguez, A. *et al.* Leptin administration restores the altered adipose and hepatic expression of aquaglyceroporins improving the non-alcoholic fatty liver of *ob/ob* mice. *Sci. Rep.* **5**, 12067; doi: 10.1038/srep12067 (2015).

4. Aquaporins in health and disease: new molecular targets for drug discovery

Article

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Aquaporins in health.

Aquaporins in health and disease: new molecular targets for drug discovery (ISBN: 9781498707831). Chapter 6. Ed. CRC Press Taylor & Francis Group, Publishing 2016.

Editor Graça Soveral, Søren Nielsen and Angela Casini. DOI: 10.1201/b19017-9.

Main objective

This book chapter focuses on the functional relevance of aquaporins in mammalian physiology and pathophysiology.

Specific objectives

- To describe the main expression sites and biological functions of aquaporins, aquaglyceroporins and superaquaporins.
- To review murine and human phenotypes of aquaporin deficiency.
- To outline the relevance of aquaporins in human metabolism and disease.

Rodríguez A, Méndez-Giménez L, Frühbeck G. Aquaporins in health. In: Soveral G, Nielsen S, Casini A, editors. Aquaporins in health and disease: new molecular targets for drug discovery. CRC Press, 2016, p. 103-124.