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THE ACTION OF METFORMIN IS MODULATED BY THE METABOLIC CONTEXT

FINAL BACHELOR RESEARCH PROJECT

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And lastly, I extend a special thanks to Marta Riera, for her endless support and dedicated involvement, without which this would have not been possible.

RESEARCH PLACEMENT

All research was undertaken at Unitat de Recerca Biomèdica (URB) as part of my internship. This research unit belongs to the Institut d'Investigació Sanitària Pere Virgili, and is located on the third floor of the old Hospital Universitari San Joan de Reus.

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The director of the research unit (URB) is Dr. Jorge Joven (jorge.joven@urv.cat), who together with Marta Riera (marta.riera.borrull@gmail.com), a predoctoral researcher at the unit, supervised this research project.

Due to the length of the study and the lack of experience with laboratory animals, some experimental procedures and analyses were already performed at the time of my arrival at the research unit. My contribution to this study began with the samples already collected at the time of the animal sacrifices. With permission, these results have been included in this project as background information to the study.

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ABSTRACT

Introduction: Obesity and its associated morbidities are a health concern no longer restricted to Western societies. Obesity occurs as a consequence of multiple factors and is closely associated with inflammation, insulin resistance and other features of the metabolic syndrome, which are a well-known risk factor for the development of chronic liver diseases, such as non-alcoholic fatty liver disease (NAFLD). Insulin sensitizers, and particularly, metformin, have been proposed as a supportive treatment in these patients.

Purpose: The aim of the present study was to determine the potential beneficial effects of this drug in low-density lipoprotein receptor (LDL) knockout mice (LDLr -/-) serving as a mouse model of NAFLD.

Methods: Male LDLr -/- mice were fed with either a low- or high-fat diet for 14 weeks and treated with either metformin (200 mg/kg) or placebo.

Results: Multiple and significant combinatorial effects between metformin and diet were found. Metformin therapy was associated with reduced body weight, anti-inflammatory processes and an amelioration of hepatic steatosis, white adipose tissue phenotype and a great number of metabolic parameters uniquely observed under a low-fat diet. In fact, the effects of metformin under a high-fat diet in this mouse model not only showed no improvement but also a worsening effect in many of the parameters evaluated. Furthermore, the beneficial effect of metformin may be at least partly dependent on AMPK activation and that the AMPK signalling pathway could be an important mediator of these effects.

Conclusion: Taken together, these results suggest that the action of the drug, specifically in this mouse model is modulated by the metabolic context in a low-caloric dependent-manner.

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) refers to an increasingly prevalent clinical problem among obese population and it has become the most common cause of chronic liver disease [1, 2]. NAFLD is characterized by a significant fat deposition in hepatocytes referred as hepatic steatosis (fatty liver disease) causing functional impairment and liver damage. When hepatic steatosis is further accompanied by inflammation, the condition is termed as steatohepatitis. Both pathological conditions are similar to those observed in alcohol-induced liver injury occurring in individuals who do not drink a significant consumption of alcohol [1-4].

The term NAFLD encompasses a wide spectrum of liver damage, ranging from a simple and macrovesicular steatosis, to its progressive stages through a non-alcoholic steatohepatitis (NASH). It is known that NASH can eventually progress into a cirrhosis state and related complications including hepatocarcinoma [3, 5].

The liver plays a central role in lipid metabolism; including *novo synthesis* and uptake of free fatty acids (FFA), which are then esterified and converted into triglycerides (TG) [6]. These non-esterified FFA are oxidized in the liver by β-oxidation, whereas TG are either stored as vacuoles in hepatocytes or assembled and released into the circulation as very low-density lipoproteins (VLDL). [6-8]

In NAFLD, there is an excess amount of FFAs delivered to the liver or that are being *de novo* synthesized on it. This situation leads to a saturation of the liver's oxidative capacity. As the triglyceride synthesis continues to increase, it also exceeds the amount that can be released as VLDL and therefore TG accumulate within the hepatocytes causing hepatic steatosis [6].

Initially, a "two-hit hypothesis" by Day *et al.* in [9] was proposed to explain the pathogenesis and progression of NAFLD. The 'first hit' claims that accumulation of excess TG in the liver (simple steatosis) is caused by insulin resistance and obesity which lead to a metabolic and molecular alteration that sensitizes the liver to the "second hit". This second hit is usually referred as the progression of steatosis into inflammation and fibrosis characterized by the release of pro-inflammatory cytokines and oxidative stress [1, 6, 10]. However, this two-hit model has been recently questioned as it has been suggested that accumulation of TG in the liver might be actually protective towards further hepatic damage and that FFAs are the toxic substances that lead to steatohepatitis and fibrosis [6, 11-13]

Although the exact pathogenesis of the disorder is not fully understood yet, development of obesity and obesity-associated insulin resistance are widely regarded as important risk factors that critically contribute in the development of NAFLD [4]. However, different studies have emphasized that insulin resistance is a characteristic feature of NAFLD in subjects that present metabolic obesity, defined by isolated increase in visceral fat in people who are not obese [6, 11, 14]. For this reason, NAFLD is now evolving as one of the principle hepatic manifestations of the metabolic syndrome, affecting up to almost a third of the general population. Nevertheless, the real prevalence is unknown since NAFLD is often undiagnosed [11].

There are currently no clear guidelines for the treatment of NAFLD [15]. The first-line strategies in NAFLD management are currently based on diet restrictions involving combined lifestyle modifications that have proven to improve liver functionality of these patients. However, despite extensive research, specific pharmacologic treatment for NAFLD is so far lacking [1, 16].

FACTORS CONTRIBUTING TO DEVELOPMENT AND PROGRESSION OF NAFLD

Obesity as a low-grade chronic inflammatory state

Obesity is now recognized as a major public health problem worldwide. Nowadays, the total burden of obesity and related conditions has been estimated to be of the order of approximately 1.5 billions of people obese or with overweight [2, 17]. Obesity is likely due to contributions from multiple factors including but not limited to genetic predisposition, sedentary lifestyle, impaired central appetite regulation and dietary factors [6, 18].

The incidence has alarmingly increased by the over-intake of calorie-rich diets in developed countries and its prevalence leading to decreased life expectancy is dramatically rising due to associated pathologies [19]. Obesity, particularly abdominal or central obesity, is therefore an important risk factor for insulin resistance that results in type 2 diabetes mellitus (T2DM) and other features of the metabolic syndrome such as dyslipidemia, high-cholesterol and hypertension [2, 19, 20]. As well, development of obesity or metabolic obesity is seen as the starting point of NAFLD [6].

Over recent years, more evidence has emerged that obesity is associated with inflammation that is casually involved in the development of insulin resistance. It has long been known that adipose tissue plays an endocrine function by producing large amounts of pro-inflammatory cytokines, called adipocytokines such as TNF-α, MCP-1 (also known as CCL2), IL-1β, IL-6, IL-8, among others [19]. It is noteworthy that not all white tissues might be the same; an increased volume of central white adipose tissue and its pro-inflammatory cytokine production seems to have a more important role in development of

insulin resistance when compared to subcutaneous adipose tissue. However, the primary cause of obesity-induced inflammation is not fully understood yet [6].

A potential explanation for its initiation is pointing towards endoplasmatic reticulum stress (ERS). The following sequence of events has been proposed: Overnutrition would cause ERS in liver and adipose tissue due to excess lipid accumulation and disturbed energy metabolism. This situation would then activate a stress response by signalling inflammatory reactions that in turn, would attract macrophages principally into adipose tissue. Once having infiltrated the tissue with macrophages, a ceaseless inflammatory cascade would be triggered by secretion of pro-inflammatory cytokines as the ones mentioned above. Besides, the recruitment of macrophages and the conversion of stellate cells into fibromyoblastic cell types are inevitably linked to the progression from steatosis to steatohepatitis [10, 19].

Obesity-induced insulin resistance

Obesity-associated insulin resistance is considered as a factor that critically contributes to the development of NAFLD. Insulin resistance is an impaired response to insulin of its main target organs (adipose tissue, liver and muscle) and it results in metabolic defects of both glucose and lipid metabolism.

To better understand the connection between insulin resistance and hepatic steatosis, the physiologic role of insulin will be concisely described. Insulin regulates uptake of glucose and circulating (FFA concentrations. In peripheral tissues, such adipose tissue and skeletal muscle, insulin mainly induces glucose uptake by stimulating the translocation of the glucose transporter (GLUT4) to the plasma membrane. In liver insulin suppress gluconeogenesis and stimulate glycogen synthesis by reducing or enhancing key enzyme activities [19]. Another important peripheral effect of insulin, principally in adipose tissue, is its lipogenic effect and suppression of lipolysis thereby, reducing FFA efflux from adipocytes. [10]

The effects of insulin take place when insulin binds its hepatic insulin membrane receptor. Very briefly, upon binding to the dimeric insulin receptor, autophosphorylation of the receptor takes place with subsequent tyrosine phosphorylation of the adaptor protein insulin receptor substrates (IRS-1 and IRS-2). Downstream of IRS proteins lead to activation of two major pathways: phosphatidylinositol-3 kinase (PI3K-AKT) and mitogen activated protein kinases (MAPK). These two pathways are largely responsible for insulin signalling [10].

Irregularities in insulin signalling pathways have been linked to atypical phosphorylation of IRS on critical serine sites, which prevents proper tyrosine phosphorylation and inactivates insulin signalling and consequently, downstream of the signalling pathways mentioned above [10]. Furthermore, there is evidence that serine IRS kinases are typical mediators of inflammatory signalling cascades, providing an inhibitory crosstalk between

inflammatory and insulin signalling on molecular level. Interestingly, high TNF-α levels can induce insulin resistance through activation of two main serine kinases: I-kappa-B-kinase-β (IKKβ) and Jun N-terminal kinase (JNK). JNK can directly cause insulin resistance by phosphorylating IRS-1 on a serine residue, whereas IKKB leads to activation of NF-κB via transcription that in turn, increases the expression of inflammatory mediators. Similarly, other pro-inflammatory cytokines like IL-6 and IL-1 lead to continuing cycles of JNK and NF-KB activation worsening insulin resistance [6, 19]

The consequence of an impaired tissue response to insulin is then a decreased glucose uptake by the peripheral tissues and a failure of insulin to stimulate glycogenesis and inhibit gluconeogenesis in the liver, causing hyperglycaemia [21].

Furthermore, insulin resistance causes an increase of the FFAs flux to the liver due to an increased lipolysis in peripheral adipose tissue. As previously described, high amounts of FFAs contribute to TG production in the liver, that in turn, saturate liver's oxidation capacity and its secretion as VLDL in the hepatocytes (*Figure 1*). It is noteworthy that, although this seems to be the primary cause of hepatic steatosis, disruption of other mechanisms like β-oxidation or VLDL assembly could also play a key role; and more importantly, that insulin resistance is implicated in most of these mechanisms [6].

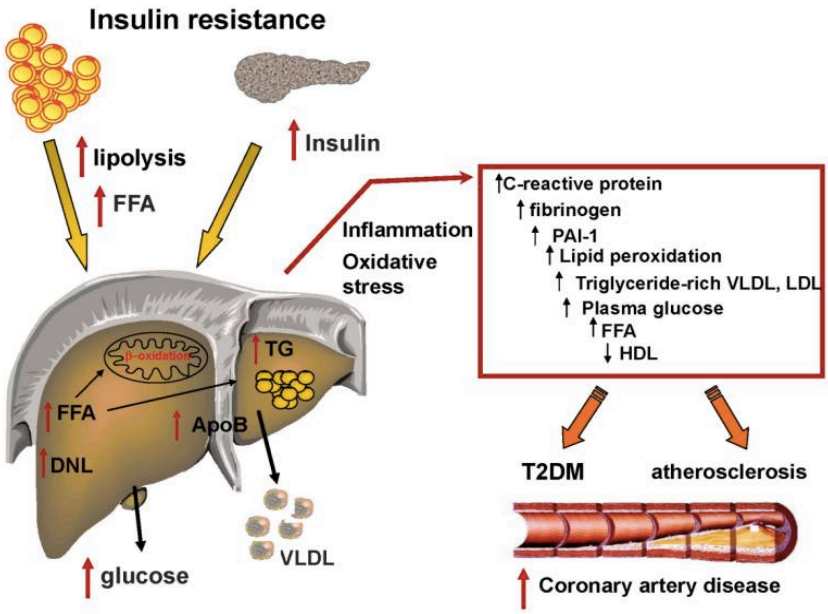

Figure 1. Link between insulin resistance and metabolic dyslipidemia. Source from Gaggini, M., et al. [11]

NAFLD subjects have increased secretion of inflammatory markers, plasma glucose and low-density lipoproteins (LDL) combined with decreased high-density lipoproteins (HDL), which represent a threat for the development of atherosclerosis and cardiovascular disease [11]. Finally, insulin resistance together with abnormalities in insulin secretion leads to T2DM [19].

To sum up, there are many factors that contribute to obesity development, which in turn, leads to insulin resistance via pro-inflammatory adipocytokines. This impaired insulin response increases lipolysis and an excessive delivery of FFAs to the liver that accumulate causing hepatic steatosis and lipotoxicity that eventually progresses to steatohepatitis and cirrhosis [6] (*Figure 2*).

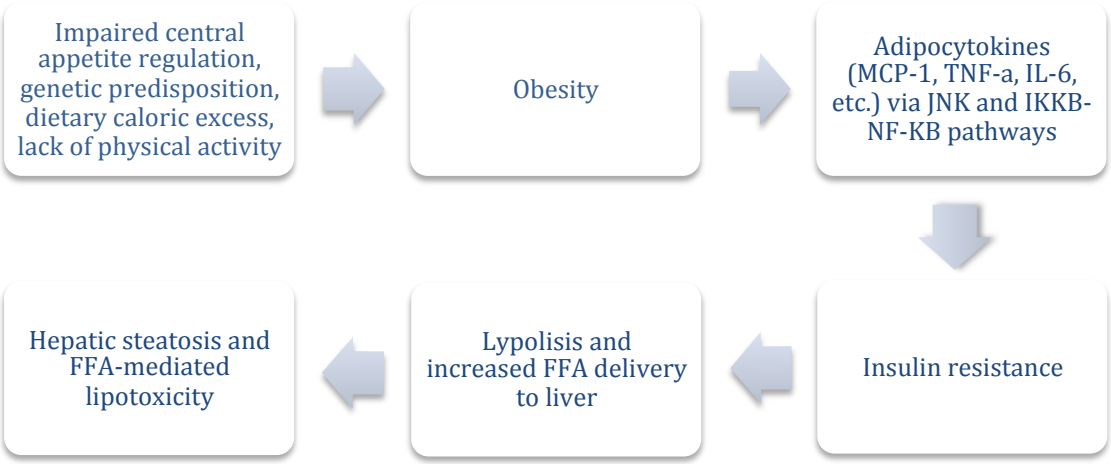

Figure 2. Development and progression of non-alcoholic fatty liver disease. Adapted source from Guturu, P. et al., [6]

In conclusion, NAFLD is increasing in prevalence and could become the most common cause of chronic liver disease in the near future in the Western World [6]. The relationship between obesity, inflammation and insulin resistance is of particular importance to finally understand the complex molecular mechanisms and mediators involved in order to develop new therapeutic targets for this disease.

To date, there is no specific treatment for the management of NAFLD, and no drug can substitute for lifestyle modifications. However, as already mentioned, NAFLD is strongly associated with insulin resistance and other components of the metabolic syndrome, and in view of this fact, it is not surprising that insulin-sensitizing agents have been proposed as a therapeutic strategy in NAFLD. Among insulin sensitizers, metformin has acquired a pivotal role in the treatment of T2DM and other disorders associated with insulin resistance, and moreover, many experimental and clinical studies have supported its use as a promising drug in the treatment of NAFLD [1, 5].

METFORMIN

Metformin is obtained from an herbaceous plant named *Galega officinalis* which is native to the Middle East but that has been naturalized in Southern Europe [22]. Metformin was introduced in clinical practice in the late 1950s as an oral hypoglycaemic drug in the biguanide class and since then, it has been the recommended as the first-line drug therapy of type 2 diabetes worldwide [1, 23-28]. This is down to several factors, including its efficacy, the safety profile of the drug and its low cost. In addition, a role in non-diabetic obese people has been also suggested on the basis of its effects on insulin resistance and weight loss [1, 5], and in the recent years, new indications for metformin use in clinical practice are emerging, including a reduction in cardiovascular disease and a possible reduction in cancer incidence. Although these results seem promising, more data are needed in order to assess drug efficacy and safety in such specific populations [23, 26, 29].

Metformin is not metabolised, and its excretion occurs entirely through the kidneys as an unaltered form. After oral administration, metformin is mostly absorbed in small intestine involving an active uptake process mediated by key organic cation transporters (OCT) primarily in the intestine, liver and kidneys [29]. Once absorbed, the drug does not bound to any plasma protein and its half-life in healthy subjects is around 5 hours [24]. These transporters determine the pharmacokinetics of metformin: the absorption across the gut epithelium (PMAT and OCT3), the transport into the hepatocytes (OCT1) and from the hepatocytes to the bile, and finally into the renal epithelial cells and tubules (OCT2) [23, 29]. Studies with OCT1 knockout mice revealed a reduced efficacy of metformin emphasising the critical role of the liver as the primary site of action of the drug [23, 29-31].

The effectiveness of metformin is explained by its anti-hyperglycaemic action, achieving a potent reduction of blood glucose levels by suppressing gluconeogenesis in the liver and stimulating peripheral glucose uptake. Metformin also increase fatty acid oxidation in adipose tissue [1, 16, 22, 24, 27, 32]. Although prescribed since over 50 years and despite extensive research the last years, metformin remains as an intriguing drug with multiple physiological and the mechanism(s) by which exert its therapeutic effects remain to be explained [28].

Mechanism of action and target of metformin

It has been suggested that metformin acts through inhibition of mitochondrial respiratory chain (complex I) and activation of AMP-activated protein kinase (AMPK) in hepatocytes, which acts as a cellular energy nutrient sensor, able to stimulate catabolic pathways [1, 27, 33, 34]. However, in contrast to these findings, it has also been suggested that metformin suppresses gluconeogenesis in a AMPK-independent manner and may be mediated by inhibition glucagon-induced elevation of cyclic adenosine monophosphate

(cAMP) and consequent activation of protein kinase A (PKA), inhibition of glycerolphosphate dehydrogenase, or it could even have an effect on gut microbiota [23, 35, 36].

The most widely accepted mechanism is that suppression of hepatic gluconeogenesis occurs principally as a consequence of mitochondrial inhibition [23, 37] (*Figure 3*). Metformin is transported into hepatocytes mainly via OCT1, resulting in an inhibition of the mitochondrial chain (complex I) through a currently unknown mechanism(s), which in turn, activates AMPK through decreases in hepatic energy charge (increasing [AMP]/[ADP] and/or [ADP]/[ATP] ratios) [24, 27]. Upon activation of AMPK, the resulting energy stress is adjusted by reducing the energy consumption in the cell, mainly suppressing gluconeogenesis directly in the liver and increasing glucose utilization in peripheral tissues [24, 37]. Besides, it inhibits glycogen synthase, thereby inducing a reduction in glycogen synthesis. The activation of AMPK by metformin exerts beneficial effects also in lipid metabolism; AMPK inactivates acetyl-CoA carboxylase (ACC) and HMG-CoA reductase, decreases fatty acid synthase (FAS) expression, and activates manolyl-CoA carboxylase; resulting in a reduction in fatty acid and cholesterol synthesis [1, 23].

In the other hand, ADP-/AMP- independent effects may also contribute to the inhibition of gluconeogenesis and to the therapeutic effects of the drug [23]. For example, it has been proposed that increased AMP levels can allosterically inhibit fructose 1,6-biphosphatase (FBPase), a key glucogenic enzyme, or suppress adenylate cyclase that results in cAMP-PKA signalling inhibition [1, 23, 38].

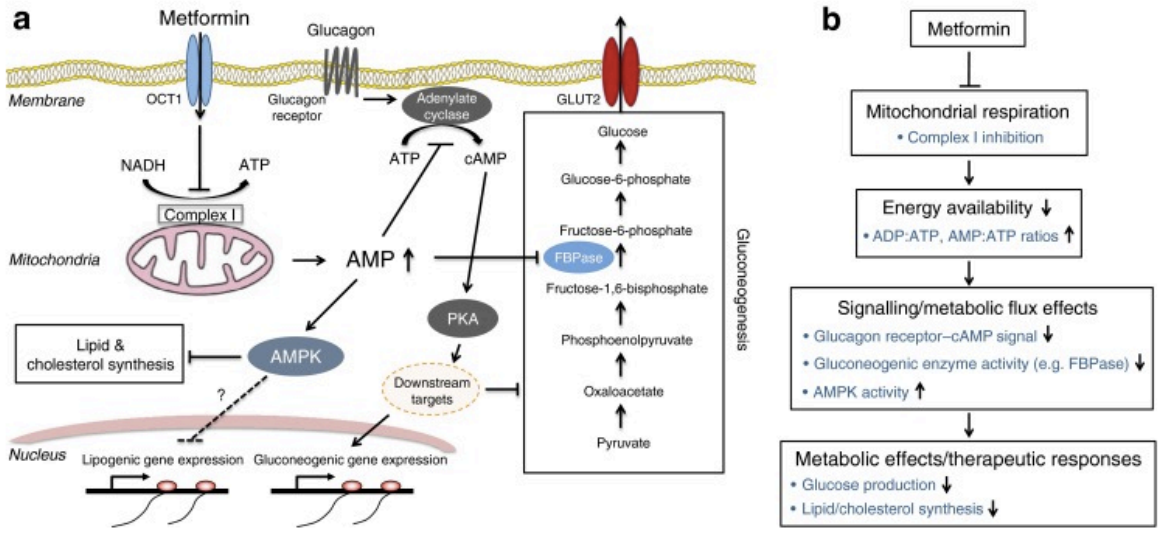

Figure 3. (**a**, **b**) Schematic diagram of a possible anti-hyperglycaemic mechanism of action of metformin on liver cells. Part (**b**) shows a simplified version of (**a**). Source from Rena, G. et al., [23]

ANIMAL MODEL

Over the past century, researchers have used an array of innovative technologies to produce genetically modified mice (GM mice). The ability to genetically customise a particular aspect of these mice makes it possible to examine the function of targeted genes [39]. Furthermore, exploring the molecular basis of the hepatic alterations associated with the metabolic syndrome is highly dependent on the availability of GM mice capable to mimic human conditions and develop diseases comparable to those found in humans [40, 41]. The research of GM mice has, therefore, proved highly useful for the development of preventive and ameliorative treatments for humans.

In the current study, the mice were deficient for the receptor of low-density lipoprotein ($LDLr^{-/-}$). Specifically, the strain of mice used was homozygous for the *LdLr*^{tm1Her} mutation with the same genetic background (C57BL/6J). They were obtained by inbreeding of mice purchased from the Jackson Laboratory (Bar Harbor, ME.) [42, 43] (*Figure 4*).

Figure 4. Knock-out $LDLr^{-/-}$ mice model.

These particular mice display a modestly elevated serum cholesterol level of 200-400 mg/dl compared to wild-type normal levels (80-100mg/dl). On high fat diet feeding, they not only show very high serum cholesterol levels (>2,000 mg/dl) but also develop hypertriglyceridemia. [43] Thus, this strain presented an altered lipid metabolism called hyperlipidemia which, has been a well-established model to study hepatopathologies characterized by fat accumulation in the liver, as in NAFLD. Given this, LDLr -/- mice, serving as a mouse model of NAFLD, were used to examine the therapeutic effects of metformin, as well as the underlying mechanisms.

OBJECTIVES AND HYPOTHESIS

Metformin has been proposed as a drug that improves steatosis and liver damage in animal models and in humans, suggesting a potential therapeutic agent for managing NAFLD. In addition to its widely used anti-diabetic effects, recent evidence has shown that metformin exerts various potentially beneficial effects such as weight loss, liver anti-inflammatory processes and changes in adipose tissue phenotype among others [3, 44-46].

Accordingly, the hypothesis postulated in this study is that the administration of metformin in non-diabetic and hyperlipidemic mice together with a low- or high-fat diet (serving as a mouse model of NAFLD), prevents and ameliorates pathologies associated with lipid metabolism. And therefore, the aim of the present study is to determine in LDLr knockout mice (LDLr -/-) the effects of this drug on different aspects including: mice body weight with respect to their dietary intake, insulin sensitivity and serum concentrations of different metabolic parameters, and also hepatic steatosis and adipose tissue phenotype. Furthermore we examined if there were any anti-inflammatory effects of metformin and at the molecular level, some of its possible mechanisms of action in hepatic tissue. Finally, the metformin impact on the overall expression level of cytokines was also analysed in liver homogenates of mice.

MATERIALS AND METHODS

ETHICAL STATEMENT

The animal study was carried out in accordance with the guidelines on the Ethical Committee for Animal Experimentation (AEC) issued by the Institut d'Investigació Sanitària Pere Virgili. [47] All interventions and sacrifices were performed under intraperitoneal anesthesia, and all efforts were made to minimize suffering.

EXPERIMENTAL DESIGN

The strain of mice used in the current study was deficient for the receptor of low-density lipoprotein ($LDLr^{-/-}$), a well-established model to study hepatopathologies characterized by fat accumulation in the liver, as in NAFLD. A comparative study was then designed according to previous data.

Male mice were housed under standardised conditions (constant room temperature 22ºC, 55% air humidity and light/dark cycles of 12 hours). Feeding was based on a *chow diet, CD* (14% protein rodent commercial diet, Harlan, Barcelona, Spain) for the 10 first weeks of age. Water and food was made given available to the mice *ad libitum*.

At 10 weeks of age, the 32 animals under study were randomly distributed into four different groups (n=8). Two of the groups were maintained under the same feeding regime, fed with a low-fat or chow diet (CD), whereas the other two received a *high-fat diet,* (HFD) (21% carbohydrates, 19% protein and 60% lipids, ssnif Spezialdiäten GmbH, Germany). In addition, mice were supplied with either normal drinking tap water or a solution of metformin (Dianben, 850 mg, Merck S.L.) that was prepared by dissolving the solid compound in tap water so that it could be administered orally to the mice. The metformin dose given was 166 mg/kg/day (0,2% w/w). The study lasted for 24 weeks and during that time, food intake and body weight were monitored weekly. *Figure 5* illustrates the scheme of the study.

Figure 5. Schematic distribution of the different animal groups. (n=8) Mice were fed with either chow diet (CD) or high-fat diet (HFD) for 14 weeks and treated with metformin or placebo.

EVALUATION OF INSULIN SENSITIVITY

Approximately a week before the animal sacrifice at 24 weeks of age, mice in all groups underwent a glucose tolerance test. This was conducted in order to evaluate insulin resistance and was administered after the mice were given a four hours fast. The glucose tolerance test was based on the intraperitoneal administration of 2 grams of glucose per kilogram body weight.

Blood samples were drawn from the tail vein at 0 (Before the administration), 15, 30, 60 and 120 minutes for measurement of glucose concentrations in plasma. A small drop of blood was placed on a test strip and inserted in an Accu-Chek Comfort glucose meter (Roche Diagnostic, Barcelona, Spain). The meter quickly calculates and displays the blood sugar level of the sample in mg/dl.

SAMPLING AND CONSERVATION

All four groups were sacrificed at 24 weeks and some tissue and blood samples were obtained. After four hours fasting, the mice were euthanized with an overdose of xylazine/ketamine (Xilagesic 2% Laboratorios Calier Sa, Barcelona; Ketolar 5 mg/mL, Pfizer, Madrid). Before proceeding with the obtainment of the samples, a foot reflex test was used to confirm that the anaesthesia overdose was lethal.

The blood was then extracted by cardiac puncture and collected in different tubes; some of them containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant and some of them without in order to obtain plasma and serum samples respectively. The blood in the circulatory system of the mouse was then replaced with saline solution (0,1 %NaCl) directly perfusing fixative throughout the natural vascular network. The goal of fixation is to rapidly and uniformly preserve tissue in a like-like state.

Next, tissue samples were removed, weighted and stored according to the different analysis procedures to be done later on. Not all tissues were meant to be used in the current study, however, they were still extracted for possible analysis in future. Some of them and some halves were snap frozen in liquid nitrogen for later homogenisation and the rest were formalin-fixed to avoid tissue degradation and paraffin-embedded for immunohistochemical analysis.

BIOCHEMICAL ANALYSIS OF SERUM

Various serum biochemistry parameters (glucose, cholesterol, triglycerides and bilirubin) were performed by an auto-analyser (Cobas Mira Plus, *Roche Diagnostics, Basel Switzerland*) on blood samples obtained in the mouse sacrifices.

Furthermore, in order to confirm and expand the data obtained in the study of the lipid content, the distribution of lipids over serum lipoproteins fractions (VLDL, LDL and HDL) was determined using fast protein liquid chromatography (FPLC). Serum was pooled per group, and 200 μl of each pool were injected onto a Superose 6 10/300 GL column (BioLogic DuoFlow, Bio-Rad, Madrid, Spain) and eluted at a constant flow rate of 0.3-0.4 ml/min.

In order to create a lipid profile that allowed us to discriminate different lipoproteins, the fractions collected were assayed for total cholesterol (TC) and triglyceride levels (TG) using the commercially available enzymatic kits and protocol: CHOL 300 and TG 300 (Synchron Systems, Beckman Coulter Inc.). The results were then read in the Synergy HT Microplate reader (Bio Tek) at 520 nm. Changes in absorbance were used to calculate and express cholesterol and triglyceride concentrations [48].

HISTOLOGICAL EVALUATION AND IMMUNOHISTOCHEMISTRY

The hepatic tissue and white adipose tissue (WAT) samples removed at the time of the sacrifice were then dehydrated and embedded in wax paraffin for tissue processing.

Tissue sections of 2μm thick were obtained by cutting with a microtome and mounted on a glass microscope slide. Haematoxylin and eosin staining was performed using standard protocols in order to assess the measurement of adipocytes size, and a semiquantitative estimation of the steatosis degree in liver sections. It also served immunohistochemical analysis on hepatic tissue. Sections were assessed and scored blindly.

▪ SEMIQUANTITATIVE ESTIMATION OF THE STEATOSIS DEGREE

Liver histological examinations were performed with an optical microscope (Eclipse E600, Nikon) to evaluate the presence of lipid droplet using the AnaliSYS programme (Soft Imaging System, Münster, Germany).

Steatosis was semi-quantitatively evaluated according to the NAFLD Activity Score (NAS) shown in *Table 1*. This standard criteria proposed by Kleiner *et al.* is a modified histological *scoring system* based on the non-alcoholic steatohepatitis (NASH) grading on Brunt 1999 and Neuschwander-Tetri and Caldwell [49]. It is expressed in values from 0 to

3 (none, mild, moderate and severe, respectively) depending on the percentage of hepatocytes affected [4, 49].

Table 1. Histological scoring system for NAFLD
(*from Kleiner et al. 2005)*

Grade	% of hepatocytes affected
0	< 5 %
1	5% to 33 %
2	33 % to 66 %
3	> 66 %

▪ IMMUNOHISTOCHEMICAL ANALYSIS

The hepatic proportion of macrophages was determined by immunohistochemistry. The protein of interest was the F4/80, an antigenic determinant highly expressed on the surface of various macrophages, (Kupffer cells among them). The antibodies utilized are shown in *Table 2*:

Table 2. Antibodies used in the immunohistochemical analysis.

Primary antibody	Secundary antibody
Rat α-mouse F4/80 (Serotec, Oxford, UK, MCA497R)	Goat α-rat IgG (Vector, Burlingame, CA, USA, BA-9400)

The analysis was performed on 2-μm-thick formalin-fixed paraffin-embedded hepatic sections as described earlier. Tissues were incubated with the primary antibody (1/500 dilution) at 4ºC overnight, followed by incubation with the anti-rat biotinylated secondary antibody (90 min. 1/200 dilution) and the avidin-biotin complex (Vector PK-4000, Vectastain ABC Complex Kit, Burlingame, California) (45 min. 1/200 dilution) protecting from light. Detection was accomplished with diaminobenzidine chromogenic substrate (DAB, Dako, Glostrup, Denmark). Tissue sections were then washed, dehydrated, stained with Mayer's haematoxylin and mounted.

▪ ASSESSMENT OF ADIPOCYTE SIZE

To measure and quantify the area of the stained adipocytes on WAT, the optical microscope (Eclipse E600) and AnaliSYS programme mentioned before were used. Three consecutive scans of each tissue sample were taken and the area of 20 adipocytes

was measured in each of them. The absolute pixel area of each section was calculated and converted to μm^2.

WESTERN BLOTTING

Fifty milligrams of snap-frozen liver of each mouse were lysed with 250 μl of lysis buffer containing: 20 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1% CHAPS. Moreover, in order to protect our proteins of interest from proteolysis, 10 μl of phosphatase inhibitor cocktail together with 1,25 μl of irreversible serine protease inhibitor were added. (1mM Pefabloc, 1% Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich, Steinheim, Germany). Tissues were homogenized by using the Precellys 24 (Bertin Technologies, France) and the protein content was determined using the 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA).

Electrophoresis was carried out in different gels (NuPAGE Novex Bis-Tris and Tris-Gly gels, Invitrogen, Barcelona, Spain) depending on the molecular weight of the proteins of interest. Electroblotting was then used to transfer proteins from the gel into a nitrocellulose membrane by the iBlot Transfer system (Invitrogen, Barcelona, Spain). Membranes were blocked for 1 h at room temperature in (TRIS-buffered saline Tween 20 buffer with 5% non-fat dry milk) followed by overnight incubation with the primary antibody shown in *Table 3*. Blots were then exposed for 1 hour with the secondary antibody also shown in *Table 3*. Same membranes were used several times to detect different proteins by adding a strong antibody removal between detections. Bands were visualised by enhanced chemiluminescence (ECL reagent, Thermo Scientific Pierce ECL, GE Healthcare, Barcelona) and quantified using the Image Lab software by ChemiDoc (Bio-Rad, Madrid, Spain).

Table 3. Proteins detected and antibodies used in the Western Blotting

Protein	Primary antibody	Secondary antibody
pAMPK 60 kDa	Rabbit α-pAMPK (Thr172, Cell Signaling Tech.)	α-rabbit-HRP (Dako, Glostrup, Denmark)
AMPK 60 kDa	Rabbit α-AMPK (2532, Cell Signaling Tech.)	α-rabbit-HRP (Dako, Glostrup, Denmark)
Arginase 30 kDa	Rabbit α-Arginase (ab91279, Abcam)	α-rabbit-HRP (Dako, Glostrup, Denmark)
FASN 200 kDa	Rabbit α-FASN (C20G5,Cell Signaling Tech.)	α-rabbit-HRP (Dako, Glostrup, Denmark)
Actin 49 kDa	Rabbit α-actin (H-300, Santa Cruz Biotech.)	α-rabbit-HRP (Dako, Glostrup, Denmark)

MAGNETIC BEAD-BASED CYTOKINE ASSAY

In order to study the anti-inflammatory effect of metformin in the liver, a multiplex biometric immunoassay was performed for cytokine measurement in liver homogenates (Bio-Plex Pro mouse cytokine 8-plex, Bio-Rad Laboratories Inc., Madrid, Spain). The principle behind is similar to the sandwich ELISA immunoassay. It is built in a 96-well plate that incorporates magnetic polymer beads conjugated with monoclonal antibodies specific for the quantification of eight cytokines offered as a premix panel. In this case, it determined the following cytokines: IL-1β, IL-2, IL-6, IL-10, IL-12, MCP-1, IFN-γ and TNF-α. The four groups of mice were analysed according to the manufacture's instructions.

Each magnetic bead is colour-dyed that permits the simultaneous detection of up to 100 cytokines in a single well at the same time. The microplate was then read with the Luminex's laser based fluorescent analytical test instrumentation (Bio-Rad Laboratories Inc., Madrid, Spain). The analyte concentration was calculated analysed usingthe Bioplex Manager Software provided by the manufacter [50-52].

STATISTICAL ANALYSIS

The analysis, the organization and the presentation of the data obtained through the experiments was presented with the statistical software *Graph Prism* (4.0 version, GraphPad Software Incorporation, California, USA). As the number and the nature of parameters are flexible and not fixed in advance, the statistical analysis was performed following the Mann-Whitney U test, a non-parametric test. All data are expressed as mean ± SEM and differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

METFORMIN ADMINISTRATION ACCOMPAINED WITH CALORIC RESTRICTION MODIFIES BODY WEIGHT DEVELOPMENT

Male LDLr -/- mice were fed with either a low- or high-fat diet for 14 weeks and treated with either metformin (200 mg/kg) or placebo. The body weight of each mouse was measured weekly for 14 weeks. In addition, weekly measurements of food intake were taken by subtracting the weight of the remaining diet from the initially supplied diet.

Mean body weight increased steadily as a function of age in all groups (*Figures 6. A* and *B*). Considering the type of diet, the body weight of the mice fed with HFD was constantly higher than the CD group. Within the groups fed with a chow diet, body weight significantly reduced in a treatment-dependent manner. Differences were already noticeable from 14 weeks of age between these two groups (*Figure 6.A*). Contrary, body weight of mice under a HFD remained practically the same (*Figure 6.B*).

Weight loss in the chow diet group could have been due to a minor daily intake, but as it can be observed in *Figure 6.C* daily food consumption within diets was identical for the first week and remained similar throughout the study. As we expected, food intake is lower in a high-fat diet attributable to a more filling diet.

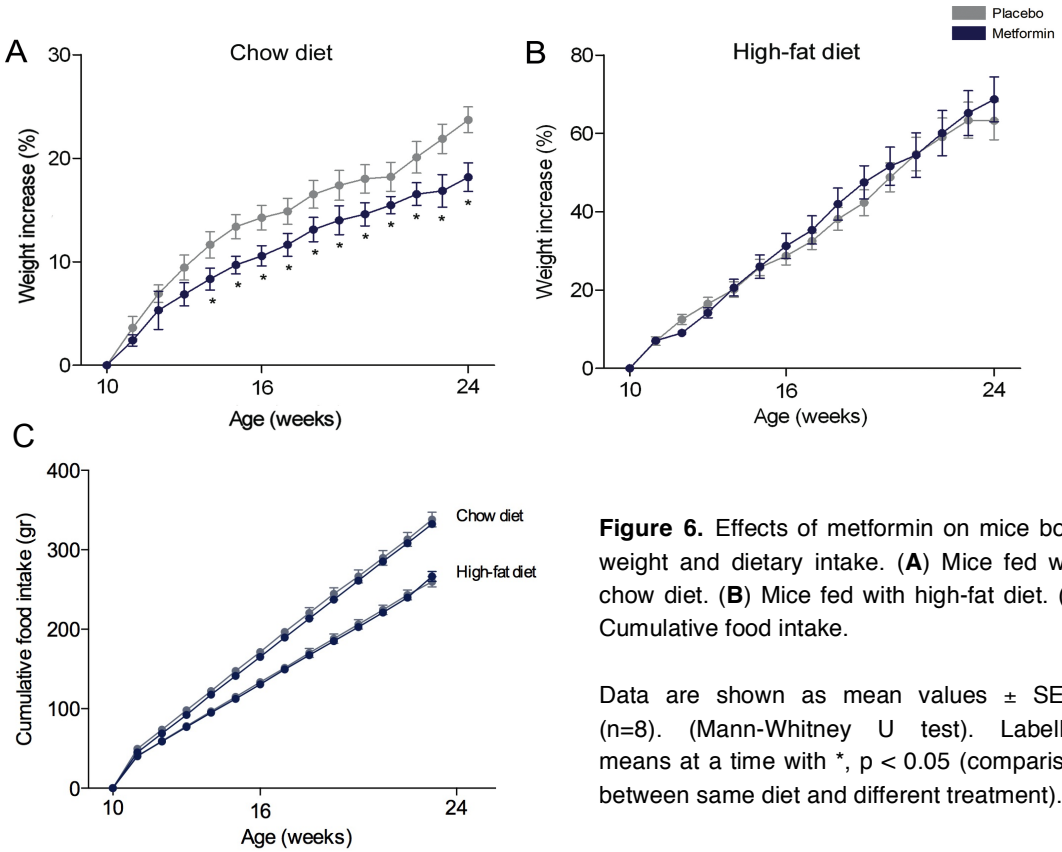

Figure 6. Effects of metformin on mice body weight and dietary intake. (**A**) Mice fed with chow diet. (**B**) Mice fed with high-fat diet. (**C**) Cumulative food intake.

Data are shown as mean values ± SEM. (n=8). (Mann-Whitney U test). Labelled means at a time with *, $p < 0.05$ (comparison between same diet and different treatment).

POOR GLYCAEMIC RESPONSE TO METFORMIN

In order to evaluate the effect of long-term treatment with metformin on insulin resistance, the mice in all groups underwent a glucose test which results are shown in *Figure 7.B*. As expected, marked increases in plasma glucose levels were observed in the HFD-fed mice compared with in CD-fed animals at all points of measurement (before 0 min) and after glucose loading, reaching a peak at 30 and 15 minutes respectively. Thus, marked glucose intolerance was shown in the HDF-fed groups.

Similarly, at this stage, plasma glucose levels in both groups treated with metformin presented slightly increased levels to their respective placebo counterparts. However, CD-fed and metformin-treated mice displayed lower glucose levels to the respective placebo group from around the 60 minutes mark. On the other hand, fasting serum glucose levels at the time of the sacrifice were also measured for each mouse (*Figure 7.A*). Once again, a high-fat diet markedly increased glucose levels respect to the low-fat diet groups. Although metformin lowered glucose levels in a low-fat diet, treatment with metformin failed to decrease glucose levels in obese mice.

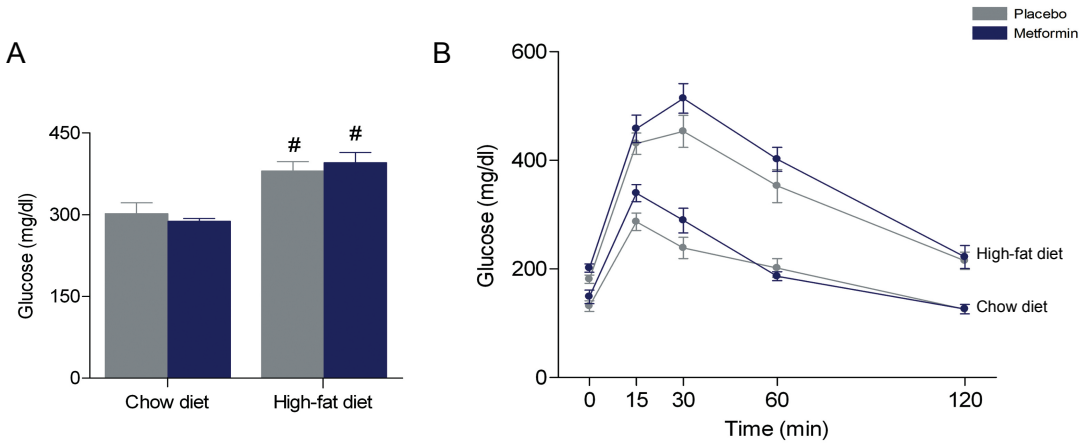

Figure 7. Effect of metformin on glucose plasma levels of LDL -/- mice fed with either high-fat diet or normal chow diet. **(A)** 4 h-fasted serum glucose levels at the time of sacrifice. **(B)** Glucose tolerance test.

Data are shown as mean values ± SEM. (n=8). (Mann-Whitney U test). Labelled means at a time with #, $p < 0.05$ (comparison between same treatment and different treatment).

THE ADMINISTRATION OF A LOW-FAT DIET WITH METFORMIN IMPROVES LIPOPROTEIN PROFILE AND LIPID SERUM SURROGATES

Lipid metabolism was visibly affected by treatment with metformin and diet intervention. Total cholesterol and triglycerides levels were measured in serum samples from each mouse (*Figure 8. E-F*).

As we could expect, lipid metabolic responses increased in a HFD compared to the CD-fed groups. Overall, these responses decreased in a treated-dependent manner with caloric restriction, reaching statistical significance at both: total cholesterol (TC) and triglycerides (TG) levels. Metformin did not greatly affect any of the parameters measured under a HFD.

Furthermore, the distribution of lipids over serum lipoprotein fractions was determined using Fast-Protein Liquid Chromatography (FPLC) from pooled serum samples of each group. The three main lipoproteins were separated: very-low density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). The individual fractions were then analyzed for cholesterol and triglycerides levels in order to create a lipid and protein profile content. (*Figure 8. A-D*)

Within the CD-fed animals (*Figure 8.A and 8.C*), metformin markedly lowered LDL-TC and LDL-TG levels. However HDL-TC and HDL-TG tended to be slightly decreased suggesting a worsening effect of metformin. Contrary, under a HFD metformin increased TC and TG levels on both LDL and HDL fractions *(Figure 8.B and 8.D*). In the other hand, VLDL-TG levels slightly decreased when treated with metformin under a CD; contrary, an increase of VLDL-TG can eventually be suggested in the HF-fed and metformin treated group. VLDL-TC remained rather constant in both diets and treatments.

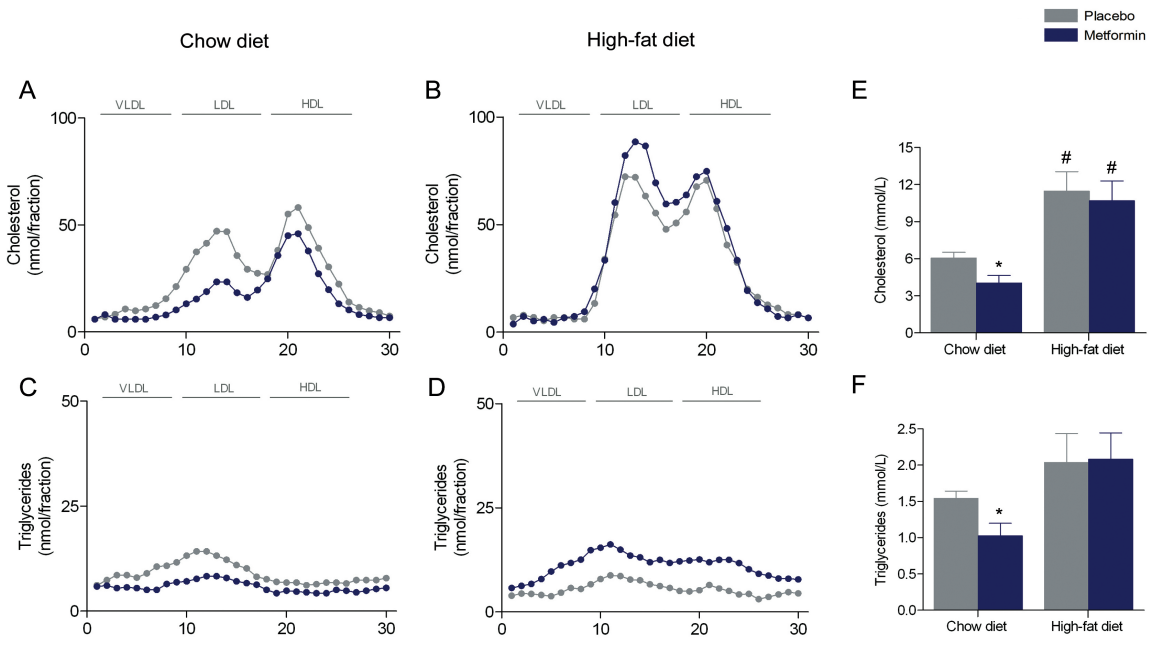

Figure 8. Effect of metformin on serum cholesterol, triglyceride levels and lipoprotein distribution. Blood samples from mice were collected at sacrifice. Serum TC (**E**) and TG (**F**) were determined from each mouse. Serum samples were pooled group wise (n=8) and size fractionated by FPLC. The individual fractions were analysed for cholesterol (**A-B**) and TG (**C-D**).

Data are shown as mean values ± SEM. (n=8). (Mann-Whitney U test). Labelled means at a time with *, $p < 0.05$ (comparison between same diet and different treatment) or #, $p < 0.05$ (comparison between same treatment and different treatment).

FAT DEPOSITION IN HEPATOCYTES AND LIVER DAMAGE: METFORMIN TREATMENT IS LINKED WITH DIET

In the liver, a high intake of cholesterol results in steatosis. Representative histological findings at 24-week-old mice livers are shown in *Figure 9.A.* These show the effect of metformin on normal chow diet and high fat diet on hyperlipemic LDLr -/- mice.

As expected, HF-fed mice displayed severe hepatic steatosis indicated by a huge amount of lipid droplets. This was significantly increased when compared to mice fed with a low-fat diet. The steaosis score is summarized in *Figure 9.B.* Upon treatment with metformin, fat deposition was uniquely reduced under caloric restriction, although it failed to reach statistical significance. Contrary, metformin did not ameliorate the degree of steatosis under a HF diet.

Furthermore, in order to check liver function in mice and match for signs of liver disease, serum bilirubin levels were measured. Treatment with metformin markedly reduced them, reaching statistical significance in the low-fat-fed groups.

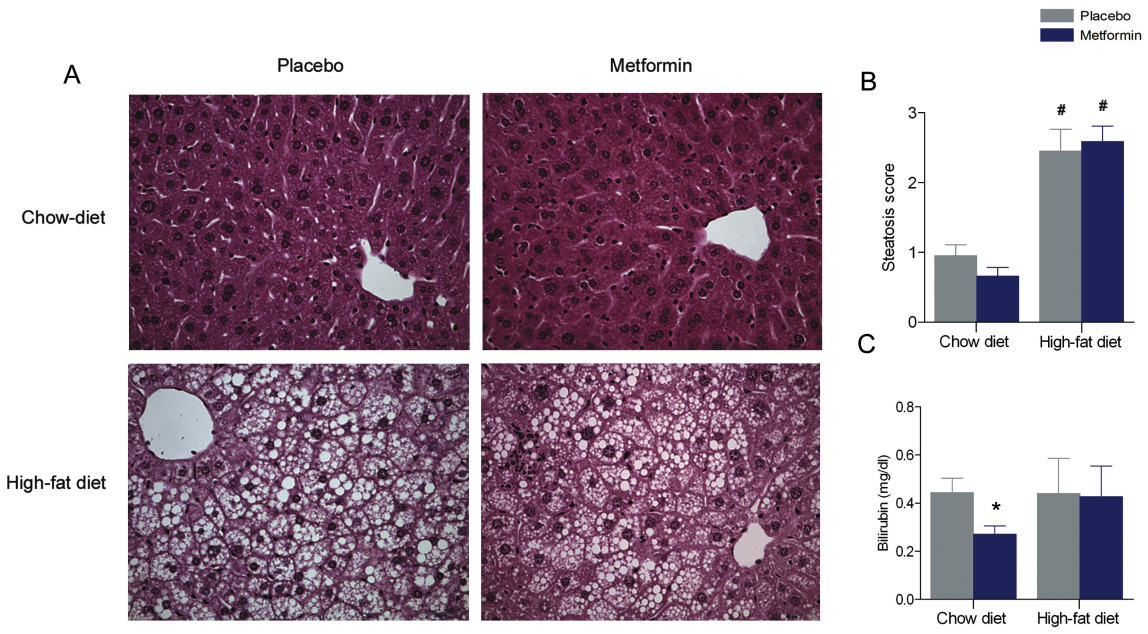

Figure 9. Effect of metformin on the content of lipid droplets in liver tissue and liver damage. (**A**) Representative photomicrographs (20x) of liver sections of LDLr -/- mice stained with hematoxilin/eosin. (**B**) Evaluation of the steatosis degree. (**C**) Serum bilirubin levels of mice at the time of sacrifice.

Data are shown as mean values ± SEM. (n=8). (Mann-Whitney U test). Labelled means at a time with *, $p < 0.05$ (comparison between same diet and different treatment) or #, $p < 0.05$ (comparison between same treatment and different treatment).

METFORMIN HAS A PROMINENT EFFECT ON EPIDIDYMAL WHITE ADIPOSE TISSUE

During obesity, non-functional adipose tissue critically contributes to the development of NAFLD [53]. Thus, changes in epididymal white adipose tissue (eWAT) phenotype were observed (*Figure 10.A*). Furthermore, at the time of sacrifice of mice, the weight of the eWAT was also registered. In this way, as plotted in *Figure 10.B,* some differences between groups could be observed.

Quantitative measurements of the area of the adipocytes on eWAT were performed on each mouse to compare the adipocyte size between groups. The adipocyte areas are summarized in *Figure 10.C.* Groups fed with a HFD showed a significantly superior adipocyte size to the CD groups. When comparing treatments within CD diet groups, treatment with metformin displayed a significant reduction in adipocyte size to its placebo counterpart. Additionally, the treatment with metformin did not ameliorate HFD-induced adiposity. The size of the adipocytes was significantly increased in HFD fed animals treated with metformin when compared to the group treated with placebo.

Furthermore, mice with a HFD showed an expected increase of the eWAT weight at sacrifice when compared to the CD groups. Remarkably, metformin showed opposite effects within diets. The weight of eWAT on CD groups was markedly lower in those treated with metformin compared to non-treated mice. Contrary, metformin showed significantly worsening effects in HFD fed mice.

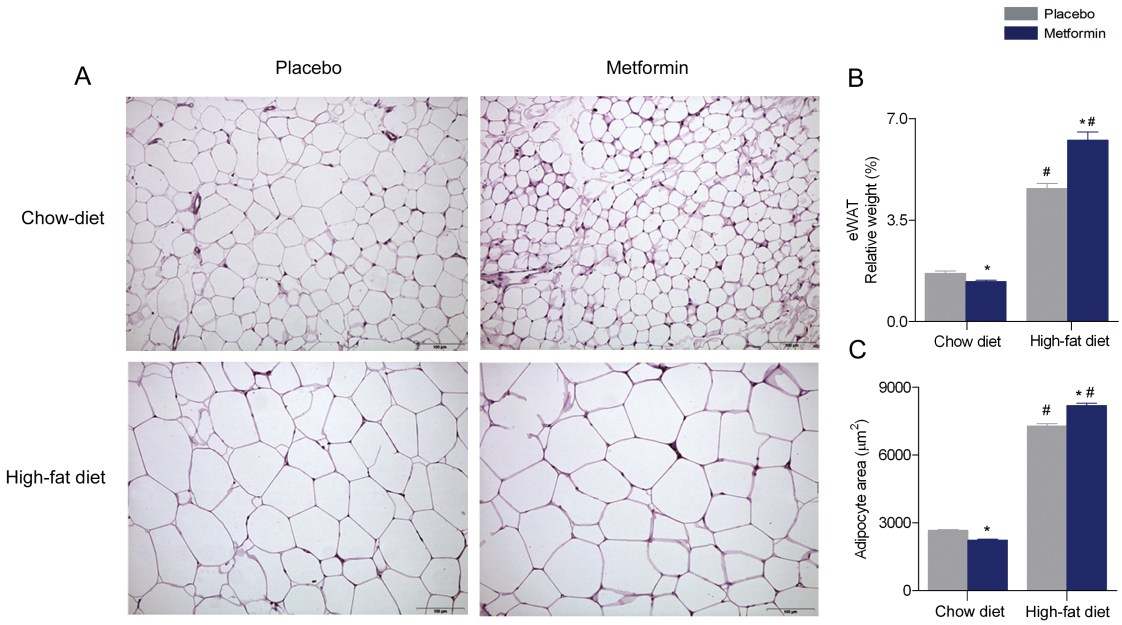

Figure 10. Effects of metformin on the weight and the adipocyte size of eWAT at the time of sacrifice.
(**A**) Representative photomicrographs (10x) of eWAT sections stained of LDLr -/- mice with hematoxilin/eosin. (**B**) eWAT relative weights. (**C**) Representation of the adipocytes area

Data are shown as mean values ± SEM. (n=8). (Mann-Whitney U test). Labelled means at a time with *, $p < 0.05$ (comparison between same diet and different treatment) or #, $p < 0.05$ (comparison between same treatment and different treatment).

METFORMIN FAILS TO REDUCE INFLAMMATORY RESPONSE IN THE LIVER

Inflammation is the key factor that drives the progression of simple steatosis to NASH. In order to study the direct effect of metformin on liver inflammatory response of mice, immunohistochemical analyses were assessed to determine the hepatic proportion of macrophages. Macrophages play a central role in the development of inflammation and they highly express an epitope of our protein of interest on their surface, F4/80.

An elevated presence of macrophages can be observed at both groups nourished with a HF diet in hepatic sections stained with hematoxilin/eosin (*Figure 11.A*). Surprisingly, metformin-treated groups also showed significantly increased levels compared to their corresponding control groups in both diets. The mean area of the macrophages of different groups under study is shown in *Figure 11.B*.

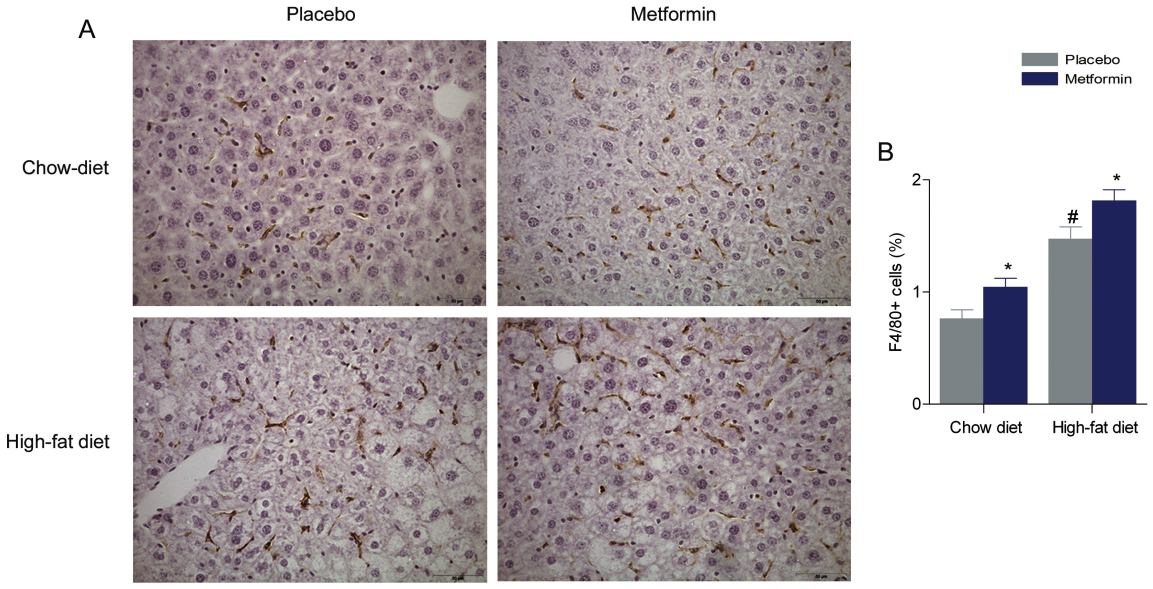

Figure 11. Effect of metformin on the inflammatory response of liver tissue in LDLr -/- mice. (**A**) Immunohistochemistry of macrophages. Representative photomicrographs (20x) of liver tissue sections stained with hematoxilin/eosin for F4/80+ cells. (**B**) Percentage of the content of F4/80+ cells (macrophages) in liver of mice.

Data are shown as mean values ± SEM. (n=8). (Mann-Whitney U test). Labelled means at a time with *, $p < 0.05$ (comparison between same diet and different treatment) or #, $p < 0.05$ (comparison between same treatment and different treatment).

METFORMIN EXERTS ITS BENEFICIAL EFFECTS VIA AMPK ACTIVATION

To further investigate the molecular mechanism by which metformin acts, key protein levels were determined in order to evaluate the activation of enzymes and signal transduction proteins that play a role in cellular energy homeostasis and lipid metabolism, such AMP-activated protein kinase (AMPK) and *Fatty Acid Synthase* (FAS) respectively. Likewise, the arginase enzyme was also determined, which behaves promoting anti-inflammatory responses in macrophages [54].

As shown in *Figure 12.A*, changes in liver AMPK phosphorylation, which might underlie the beneficial effects of metformin [3], were examined. Metformin increased AMPK activation (pAMPK levels) in mice fed with CD. The ratio between pAMPK and total AMPK reached a statistical difference when compared to its placebo counterpart (*Figure 12.B*). One limitation of this study is that due to the amount of fat accumulated in the liver extracts of mice under a HFD, placebo-treated samples were delipidated before the gel electrophoresis and no comparison to the metformin group could be made.

Arginase levels were significantly incremented by metformin when comparing between HF-fed mice groups, whereas no major differences were observed under a chow diet. In the other hand, metformin showed a considerable trend to decrease FAS enzyme expression under a CD whereas the other two groups remained almost at the levels of control.

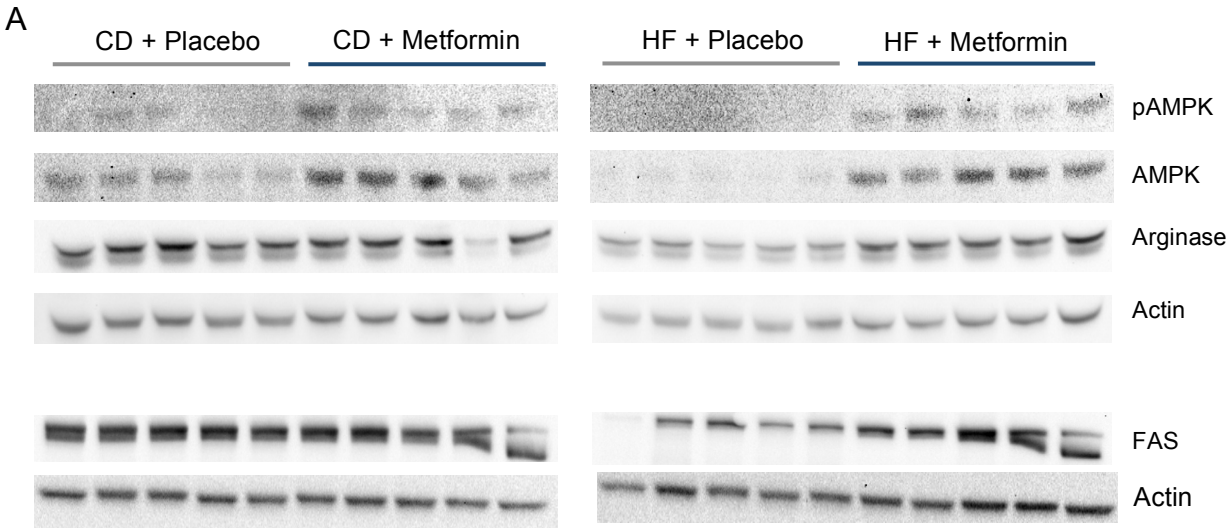

Figure 12.A. Effects of metformin on activation and expression of key proteins involved in cellular energy homeostasis and lipid and inflammatory signalling. Liver extracts of LDLr -/- mice were subjected to Western Blot analyses followed by densitometric quantification. Actin expression was used as internal housekeeping protein.

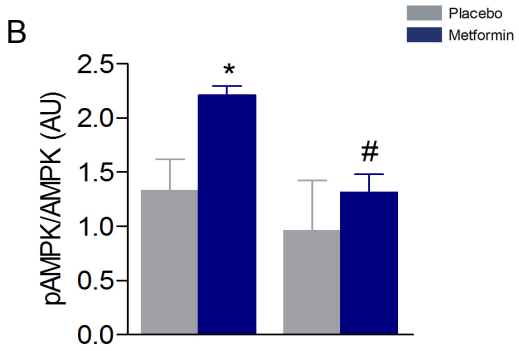

Figure 12.B. pAMPK/AMPK ratio. The phospho-to-total ratio was calculated after densitometric quantification. (AU, Arbitrary units)

Data are shown as mean values ± SEM. (n=8). (Mann-Whitney U test). Labelled means at a time with *, $p < 0.05$ (comparison between same diet and different treatment) or #, $p < 0.05$ (comparison between same treatment and different treatment).

POST TREATMENT WITH METFORMIN DECREASES PRO-INFLAMMATORY RESPONSE

To study the anti-inflammatory effect of metformin in the liver, the expression level of eight different cytokines was measured in liver homogenates of each mouse. In this case, the following cytokines were determined: IL-1β, IL-2, IL-6, IL-10, IL-12, CCL2 (also known as MCP-1), IFN-γ and TNF-α.

The results shown in *Figure 13* revealed that the overall expression level of each cytokine tested among the four groups was diminished in those treated with metformin with the exception of IL-12 and CCL2 which slightly increased in a HF diet. Under a CD, metformin significantly lowered IL-1β, IL-2, IL-6, IL-10 and TNF-α levels.

In addition, among the HFD group, IL-6 and TNF-α were also significantly minor when treated with metformin. Contrary, CCL2 expression reached a significant rise whereas this cytokine remained the same in mice fed with CD.

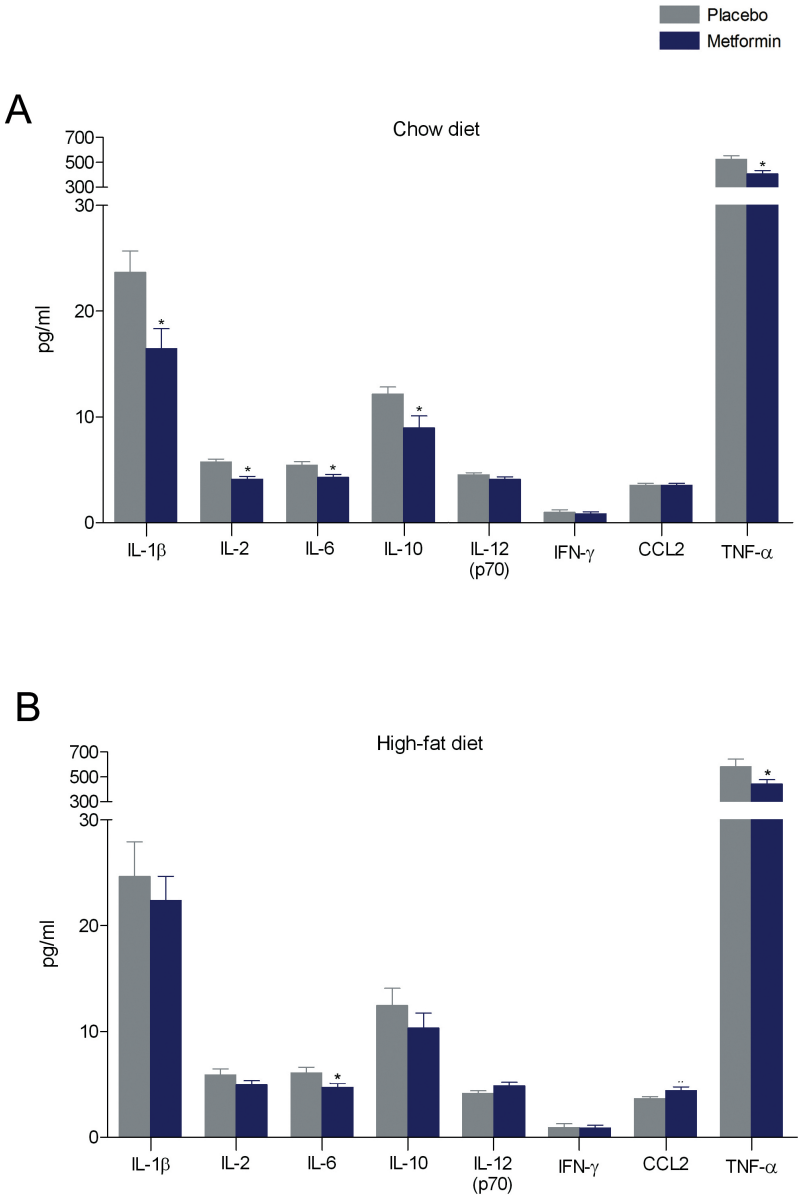

Figure 13. Effect of metformin on inflammatory cytokines levels of liver homogenates of LDLr -/- mice. (**A**) Cytokine concentrations present in mice fed with (**A**) chow diet (**B**) and high-fat diet.

Data are shown as mean values ± SEM. (n=8). (Mann-Whitney U test). Labelled means at a time with *, $p < 0.05$ (comparison between same diet and different treatment)

DISCUSSION

In recent years, obesity has become an epidemic problem that is no longer restricted to Western societies. Obesity and its associated morbidities have sharply increased due to the over-intake of high-fat diets and lack of physical activity [17, 18]. Obesity occurs as a consequence of multiple factors but is commonly associated with a chronic low-grade inflammation, insulin resistance and other features of the metabolic syndrome, which are a well-known risk factor for the development of chronic liver diseases, such as non-alcoholic fatty liver disease [17, 55].

Lifestyle changes involving diet restrictions are essential in the management of these patients, while specific pharmacologic treatment for NAFLD is so far lacking [56]. Significant weight loss is very difficult for most people to achieve in their day-to-day lives, especially amongst those who exhibit obesity-associated insulin resistance. Since this is considered a major factor that critically contributes to the development of NAFLD [19], insulin sensitizers have been proposed as a supportive treatment in these patients [56]. Several studies support a potential role of metformin in NAFLD treatment [5].

In the present study, the efficacy of metformin was evaluated (200 mg/kg) as a pharmacological treatment in combination with either a low- or high-fat diet in LDLr knockout (LDLr -/-) mice. These particular mice developed an altered lipid metabolism resulting in hyperlipidemia, which has been a well-established model to study NAFLD. Given this, LDLr -/- mice were used to examine the therapeutic effects of metformin, as well as the possible underlying mechanisms. To date, there seem to be no other studies evaluating the effects of metformin in this specific mouse model serving as a model of NAFLD.

Upon treatment with metformin, body weight reduction was only observed on mice fed with chow diet when compared to non-treated mice. In general, weight loss during metformin treatment has been attributed to decreased net caloric intake probably through gastrointestinal effects of metformin and its effect on appetite reduction [1, 18, 57]. However, contrary to some studies [5, 18, 57], daily food intake was unchanged by treatment with metformin, suggesting that metformin exerted its beneficial effects in weight reduction independently of food consumption, and thus no anorectic effect was observed. As expected, food intake was lower in a high-fat diet attributable to a more filling diet.

Contrary to many results obtained from both human subjects and rodent models [3, 5, 44, 58] in the present study, metformin treatment did not improve insulin sensitivity nor glucose tolerance in any of the mice groups. Although the clinical significance of these glycaemic profiles is undetermined, these results suggest a failure of metformin effects on improving insulin sensitivity in this particular mouse model. However, metformin-treated mice under normal diet exerted a minimal reducing effect on fasting serum glucose levels. Furthermore, differences on metformin effects data might occur due to the variability on

the dose of drug administered in vivo, which ranges from 50 to 500 mg/kg, as well as observations of chronic versus acute responses to metformin [36].

Several authors have shown that metformin improves lipid and lipoprotein profiles. [5, 44, 59] In this study, the results also clearly showed that a metformin dose of 200 mg/kg, together with a chow diet is sufficient to induce significant favourable effects on metabolic parameters. Compared with placebo, metformin treatment significantly lowered serum total cholesterol (TC) and triglycerides levels (TG) in low-fat-fed mice, potentially via decreased cholesterol synthesis by AMPK activation. Metformin also tended to reduce TC levels on high-fat diet whereas TG levels slightly increased in the liver.

Additionally, the distribution of cholesterol and triglycerides over serum lipoprotein fractions was very similar in both diets. However, metformin displayed completely different effects within diets. Unlike the lipoprotein profile in wild-type mice, LDLr -/- mice are mildly hypercholesterolemic due to the absence of LDL receptors and therefore cholesterol is mainly confined in the LDL fraction [41]. On one hand, metformin successfully reduced LDL-cholesterol and mildly raised HDL-cholesterol compared to control group in low-fat fed mice. Furthermore, VLDL fractions, primarily loaded with TG, were also reduced. In this study, experiments were performed in fasted mice, thereby excluding any significant contribution of intestine-derived chylomicrons to the change observed in circulating concentrations [59]. Such effects of the drug might be due to subtle changes of key enzymes of cholesterol synthesis and VLDL-TG production in the liver [60].

On the other hand, however, the effects of metformin on lipoprotein distribution under high-fat diet were very different from the observed on normal fed mice. Metformin rather increased LDL-TC, HDL-TC and VLDL-TG levels compared to control. In comparison, in the placebo groups VLDL-TG distribution under this HF diet displayed lower levels than its respective counterpart under a chow diet. This could be due to an impaired functionality of hepatic VLDL-TG production, and therefore we interpreted that metformin might have ameliorated the VLDL-TG synthesis in the liver and key enzymes such as LPL, resulting in elevated triglycerides and increased proportion of cholesterol respectively, that in turn, leads to an increased LDL-TC. However, recently in [59], Geerling, J.J. *et al.,* demonstrated that metformin does not affect hepatic VLDL-TG production but instead selectively promotes VLDL-clearance by brown adipose tissue (BAT), an effect associated with very high rates of VLDL-TG uptake by a mechanism that still remains to be fully characterized. Further direct studies should assess whether metformin alters lipid export from the liver.

In the present study, it was found that metformin therapy was associated with disappearance of hepatic steatosis when animals were fed with a low-fat diet, although the drug was not significantly more effective than its placebo counterpart. Experimental evidence in several studies indicate that reduction of intrahepatic lipid contents under metformin treatment could be explained by the activation of AMPK, which leads to reduced activity of acetyl-CoA carboxylase (ACC), HMG-CoA reductase (HMGCR) and the lipogenic transcription factor SREBP-1; resulting in a reduction of fatty acid and

cholesterol synthesis [1,23]. As expected, HF-fed mice displayed severe hepatic steatosis and treatment with metformin failed to reduce the amount of lipid droplets in the liver. Previous unpublished results from our research group with the same mouse model have shown that a caloric restriction altogether with metformin treatment can significantly reverse HF diet-induced steatosis. Accordingly with the present results, we believe that metformin effects are strikingly linked to diet in this mouse model, and it can only exert its beneficial effects under caloric restriction. The reason(s) and mechanism(s) of why metformin cause such worsening effect is yet to be elucidated.

Inflammation is the key factor that drives simple steatosis into steatohepatitis, and macrophages play a central role in controlling this progression [3, 61]. Liver inflammatory response was examined as indicated by the percentage of mature macrophages (F4/80+ cells) in livers of mice. Liver macrophage infiltration in HFD-fed groups was markedly increased compared with that in CD-fed mice. Upon treatment with metformin, liver macrophage infiltration was significantly increased in both diets. Shih-Lung Woo *et al.* reported similar results, in cases where non-treated groups contained fewer numbers of F4/80+ cells than treated ones. These results suggest that liver content of F4/80+ is not an ideal indicator of liver inflammatory responses [3]. However, several studies support a potential link between AMPK and reduced liver inflammation in obesity-associated NAFLD, where metformin has a direct effect on reducing hepatocyte fat content as discussed above [3].

Changes in adipose tissue phenotype were next examined. Dysfunctional adipose tissue causes an increase of the FFAs flux to the liver that critically contributes to development of hepatic steatosis. Because of this, it was also postulated that anti-steatosis effect of metformin could ameliorate adipose tissue phenotype [3]. In the current study, metformin treatment significantly reduced mice adipocyte size exclusively under a chow diet. Additionally, these results correlated with the weight-loss effect of metformin described above, which in turn, is closely tied to a significant reduction of epididymal white adipose tissue weight. These results may be attributable to the effect that metformin treatment altered adipose tissue AMPK phosphorylation. In the other hand, HFD-treated mice displayed increased adipocyte size, suggesting once again that metformin effects are strikingly linked to a low-fat diet and that metformin effects cannot reverse the HFD-induced damage in this particular mouse model.

At the molecular level, an important aspect of the present study was to assess the effect of metformin on liver AMPK, as several authors claim that the effects of the drug are due to the activation of this enzyme [1, 3, 44]. In our case, a greater AMPK phosphorylation, indicative of AMPK activity, was observed after treatment with metformin in both low and high fat diet, an effect that is expected to promote the acceleration of fatty acid oxidation in the liver and several anti-lipogenic effects.

Consistently, changes in liver AMPK phosphorylation were positively correlated with a decreased expression of the Fatty acid synthase (FAS) enzyme. This enzyme catalyses the last step in the fatty acid biosynthetic pathway and is believed to be a determining factor of the hepatic capacity to generate FA by *de novo* lipogenesis [44, 62]. In the

current study, treatment with metformin decreased the expression of FAS enzyme under a chow diet, whereas it remained unchanged under a high-fat diet. These results are consistent with the ones obtained by Dorn *et al.* that revealed a significant correlation between FAS expression and the degree of steatosis in *in vivo* and *in vitro* experiments with NAFLD [62]. In our case, the low-fat diet and metformin-induced reduction of hepatic FAS may have also contributed to the hepatic steatosis in our model, as mentioned above. Moreover, Dorn *et al.* also demonstrated that the expression levels of SREBP1, the main transcriptional factor of FAS, paralleled the FAS expression levels in the liver, suggesting that this enzyme expression is regulated at the transcriptional level [62].

We also examined the potential anti-inflammatory effects of metformin by studying the arginase expression in the liver, which has been demonstrated to promote anti-inflammatory responses in macrophages. In this study, treatment with metformin in high-fat fed mice significantly increased arginase levels. However, under low-fat diet no major differences were observed on metformin treated-mice. This last result could be explained by the fact that in the absence of immune stimulation, myeloid cells do not express this enzyme [63], suggesting a potential link between diet-induced liver inflammation and the beneficial anti-inflammatory effect of metformin in high-fat diet groups.

Besides, compelling evidence suggests that in NAFLD cytokines play a key role in inflammatory processes and mediate a range of their pro-inflammatory effects by activating NF-κB. Taking that into account, the concentration of several pro-inflammatory and anti-inflammatory molecules were analysed in the liver of mice. Upon treatment with metformin in both diets, the results obtained displayed an significant overall decrease of the following cytokines: IL-1β, IL-2, IL-6, IL-10 and TNF-α. These results are coherent with several studies describing the beneficial effects of metformin via AMPK activation and subsequent inhibition of cytokine-generating pathways such as NF-κB, JNK or PI3K, which play an important role in insulin resistance and eventually NAFLD development [6, 19, 64-66]. As an exception, IL-12 and CCL2 that slightly decreased in low-fat treated-mice were slightly increased when fed with a HF diet. IFN-γ was unaffected by metformin in both diets.

To conclude, the results presented in this study are consistent with a model in which the beneficial metabolic effects are mediated by increased AMPK activation in the liver. However, as a suggested direction for future research, further mRNA expression analyses in liver homogenates should be undertaken to assess whether AMPK phosphorylation results in suppression of hepatic lipogenic genes such as SREBP-1, Fas, HMGR and ACC, among others. This last enzyme catalyzes the biosynthesis of malonyl-CoA from acetyl-CoA, an initial substrate for *de novo* fatty acid biosynthesis [27]. Recently metformin has been proposed to activate AMPK through decreases on cellular ATP levels in hepatocytes owed to its inhibition of the mitochondrial respiratory chain (complex I) [23,27,28], as such, increases in cellular AMP:ATP and AMP:ADP ratios and the expression of key mitochondrial proteins involved could also be measured in future analysis. Furthermore, considering that metformin also exerted beneficial effects in adipose tissue phenotype in our results, it could also be interesting to study the activation of AMPK and related mitochondrial proteins in brown and white adipose tissue, as well as

metformin anti-inflammatory effect on them. Finally, most recent studies proposed that AMPK-independent pathways may also contribute to the therapeutic effects of the drug and therefore, on going work in this area could be useful to provide more insight into the molecular mechanism of metformin and a greater potential for targeted therapy [23, 38].

CONCLUSIONS

In the current study, multiple and significant combinatorial effects between metformin and diet were found. Metformin therapy was associated with reduced body weight, anti-inflammatory processes and an amelioration of hepatic steatosis, white adipose tissue phenotype and a great number of metabolic parameters uniquely observed under a low-fat diet. In fact, the effects of metformin at dose of 200 mg/kg under a high-fat diet in this mouse model not only showed no improvement but also a worsening effect in many of the parameters evaluated. Furthermore, our findings suggest that the beneficial effect of metformin may be at least partly dependent on AMPK activation and that the AMPK signalling pathway could be an important regulator in this pathological condition.

Therefore, from the current point of view, we can conclude that this study provides evidence to support that the action of the drug specifically in this mouse model, is modulated by the metabolic context in a low-caloric dependent-manner. As such, metformin treatment in combination with a caloric restriction could be an effective approach for treatment and prevention of NAFLD and lipid metabolism associated pathologies.

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