INFLAMMATION AND ENERGY METABOLISM IN OBESITY: THE SEARCH FOR BIOMARKERS AND NOVEL THERAPEUTIC STRATEGIES.

Esther Rodríguez Gallego

Dipòsit Legal: T 1337-2015

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PhD Thesis Dissertation

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I STATE that the present study, entitled **"Inflammation and energy metabolism in obesity: the search for biomarkers and novel therapeutic strategies"**, presented by **Esther Rodríguez Gallego** for the award of the degree of Doctor, has been carried out under my supervision at the **Department of Medicine and Surgery** of this university.

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“The important thing is not to stop questioning”

Albert Einstein

Abbreviations

AGB: Adjustable gastric band

ALT: Alanine transaminase

AMPK: AMP- activated protein kinase

ARG: Arginase

AST: Aspartate transaminase

ATG: Autophagy-related genes

BAT: Brown adipose tissue

BMI: Body mass index

BPD-DS: Biliopancreatic diversion with duodenal switch

CAC: Citric acid cycle

CCL2: C-C chemokine ligand 2

CCR2: C-C chemokine receptor type 2

ChREBP: Carbohydrate-responsive element-binding protein

CK18: Cytokeratin-18

CMA: Chaperon-mediated autophagy

CRP: C-reactive protein

CT: Computed tomography

DARC: Duffy antigen receptor for chemokines

EGLN3: Egl nine homolog 3

FFA: Free fatty acids

GAS-6: Growth arrest-specific 6

GLUT4: Glucose transporter type 4

GM-CSF: granulocyte-macrophage colony-stimulating factor

Hsc70: Heat shock protein 70 kDa

IGF1: Insulin growth factor-1

IKKβ: Inhibitor kappa kinase beta

IL: Interleukin

INFγ: Interferon gamma

INHBA: Inhibin beta A

iNOS: Inducible nitric oxide synthase

IP_3: Inositol triphosphate

IRS-1: Insulin receptor substrate-1 protein

JNK1: c-Jun NH2 terminal kinase 1

LAMP-2A: Lysosomal-associated membrane protein 2

LC/GC-QTOF-MS: liquid/gas chromatography-quadruple time-of-flight-mass spectrometry

LC3: Mictrotubule-assosciated protein light chain 3

LPS: Lipopolysaccharide

MCP-1: Monocyte chemoattractant protein-1

MCPIP: MCP-1 inducted protein

M-CSF: macrophage colony-stimulating factor

MRS: Magnetic resonance spectroscopy

MTOR: Mechanistic target of rapamycin

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NF-κβ: Nuclear factor kappa beta

PDGF: Platelet-derived growth factor

PE: Phophatidylethanolamine

PGC1α: Peroxisome proliferator-activated receptor gamma co-activator 1-alpha

PKC: Protein Kinase C

PLC: Phospholipase C

PPAR: Peroxisome proliferator-activated receptor

ROS: Reactive oxygen species

RYGB: Roux-en-Y Gatric Bypass

SG: Sleeve Grastrectomy

SREBP1: Sterol regulatory element-binding protein 1

T2DM: Type-2 diabetes mellitus

TCA: Tricarboxilic acid cycle

TDZs: Thiazolidinediones

TE: Transient elastrography

TNFα: Tumor necrosis factor alpha

VLDL: Very low density lipoproteins

WAT: White adipose tissue

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ABSTRACT

Abstract

Obesity is an epidemic and still growing health problem around the world and represents the major challenge to chronic disease prevention. Demographic, economic and epidemiological changes have driven to unacceptable rates of obesity among adults and children. The accompanying non-alcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease and it is associated with high morbidity and mortality.

NAFLD encompasses a wide spectrum of histological findings ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and advanced fibrosis, which could progress to cirrhosis, hepatocellular carcinoma and liver failure. Given the extremely high prevalence of NAFLD, the significant number of individuals which are asymptomatic until their liver begins to fail and the association between NAFLD and an increase of all-cause mortality, there is a great need for the development of non-invasive diagnostic methods which could reduce liver biopsy and overcome its drawbacks.

Current laboratory tests are insufficient and unreliable for the determination of NAFLD presence. Moreover, non-invasive imaging techniques to diagnose NAFLD are expensive and not suitable for morbidly obese patients. For this reason we aimed to discover a rapid and non-invasive method that could improve clinical care. Accordingly, **Study 1**, published in *International Journal of Obesity*, describes a non-targeted metabolomics approach on the plasma from morbidly obese patients undergoing bariatric surgery to gain a comprehensive measure of metabolite levels. Furthermore, on the basis of these findings, we developed a method for the accurate quantification of plasma α-ketoglutarate to explore its potential as a novel biomarker for the detection of NAFLD. This study elucidated that plasma α-ketoglutarate is superior to common liver function tests in obese patients as a surrogate biomarker of fatty liver disease. Thereby, the measurement of this biomarker may potentiate the search for therapeutic approaches, may decrease the need for liver biopsy and may be useful in the assessment of disease progression.

Excessive energy intake is a part of the current human lifestyle. This energy surplus alters metabolic homeostasis and leads to a state of low-grade chronic inflammation

which has an important role in the pathogenesis of obesity and NAFLD. Accordingly, the immune system and metabolism are closely interconnected and an energy excess management could compromise this relationship. For this reason, we reasoned that searching for an adequate animal model might allow us to better understand disease pathogenesis.

Chemokines are promising candidates for the design of such a model. Chemokines, especially Chemokine (C-C Motif) Ligand 2 (CCL2), not only governed the migration of immune cells during inflammation but also have a variety of additional functions that are involved in the correct functioning of metabolism. Moreover, they are overexpressed in non-communicable disease such as obesity and NAFLD so they could become a biomarker. Therefore, we hypothesised that challenging an animal model that systematically overexpresses CCL2 combined with diets rich in fat and cholesterol could help to assess the role of chronic inflammation in response to excessive energy. In **Study 2**, published in *Mediators of Inflammation,* we present the results obtained from the generated targeted CCL2 transgenic mice which overexpress CCL2 in all tissues. These data corroborated that CCL2 modifies lipid and glucose metabolism, contributes to hepatic steatosis and promotes changes in macrophage function and plasticity, mitochondrial biogenesis and autophagy. Thus, this study contributed to the knowledge about the relationship between inflammation and metabolism and suggested a number of mechanistic questions for further studies.

On the bases of these findings, and in order to determine if the deleterious metabolic effects caused by a continuous and ubiquitous expression of CCL2 combined with energy surplus could be counteract by the absence of its receptor (CCR2), we created a novel animal model, a double genetically modified mouse, CCL2 overexpressor and CCR2 knockout mice. Accordingly, in **Study 3** we present the preliminary results obtained from these mice which suggest that all metabolic disturbances observed in transgenic mice which overexpress CCL2 could be reverted by the inhibition of CCL2/CCR2 axis biological function. All this information could be really important to establish CCR2 modulators as a new class of therapeutic agents to the management of metabolic diseases.

INTRODUCTION

1. Obesity: the epidemic of the twenty-first century

Obesity is one of the most common diseases worldwide and has become the greatest public health challenge. The World Health Organization (WHO) has considered obesity as the "epidemic of the twenty-first century".

It is typically defined as the state of having excess body weight. However, this simple definition misrepresents an etiologically complex phenotype mainly associated with excess adiposity which can manifest metabolically and not just in terms of body size [1, 2].

Globally, the worldwide prevalence of obesity has considerably increased since the early 1980s. According to the WHO, in 2014 more than 1.9 billion of adult population was overweight and, of these, over 600 million had obesity (39% and 13% of total population, respectively) [3]. In European Union member states approximately 35% of all adults are overweight and approximately 17% are classified as obese. In addition, most available trend data suggest that, unfortunately, obesity rates are continuing to rise [4].

There is also an alarming increase in globally prevalence rates of overweight and obesity among children and adolescent population. Severe obesity in childhood is unfortunately not uncommon. In 2013, 42 million children under the age of 5 were overweight or obese [3].

In Europe, around 1 in 3 children aged 6-9 years old were overweight or obese in 2010. This is a worrying increase in regard to the experts estimates on 2008, when they were talking about 1 in 4 (estimates based on data from the WHO's Childhood Obesity Surveillance Initiative, COSI) [5-7].

Although overweight and obesity are considered a high-income country problem, their prevalence is increasing in lower and middle-income countries, particularly in urban surroundings. In these developing countries with emerging economies, the rate of this health problem is more than 30% higher than in developed countries [3].

1.1 Classification

The most commonly used method in major guidelines for classifying an individual as overweight or obese is based on body mass index (BMI), defined as the body weight in kilograms divided by height in meters squared.

In 1997, the WHO standardized the definition of normal weight, overweight and obesity and most studies have adopted these definitions [8]. Moreover, the WHO distinguishes several BMI categories based on increasing health risks (**Table 1**) [9]. Given this definition, in adults, overweight is defined by a BMI ≥25.0 kg/m^2, and a BMI ≥30.0 kg/m^2 is categorized as obesity [10-12].

Table 1. Classification of obesity based on increasing health risk. Adapted from WHO guides [9].

Weight category	BMI (kg/m^2)	Associated health risks
Underweight	<18.5	Low (but risk of other clinical problems)
Normal weight	18.5-24.9	Average
Overweight	≥25.0	Increased
Obese	≥30.0	
Obese class I	30.0-34.9	Moderately increased
Obese class II	35.0-39.9	Severely increased
Obese class III	≥40.0	Very severely increased

1.2 Obesity-inducing factors

Obesity arises as the result of an imbalance in energy homeostasis, when caloric intake exceeds energy expenditure during an extended period of time and leading to excess body weight.

The increasing prevalence of obesity is influenced by a complex interaction between genetic, metabolic, behavioral and environmental factors and also seems to be related to a countless social and economic changes inherent to modern society [13-16]. All of these factors create an obesogenic environment. This term has been coined to express the sum of influences, opportunities, or conditions of life that produce and support overweight and obesity through several intersecting mechanisms [17, 18].

These obesogenic shifts linked with the globalization and modernization include growing availability of abundant, cheap, energy-dense, highly palatable foods and

sugar-sweetened beverages. Gathering all of these factors together with improved food distribution and highly pervasive and persuasive marketing create a "push effect" that drives over-consumption of calories. Moreover, in the past decades, environmental changes have reduced physical activity via our service-based economy and labor-saving devices which have had a cumulative impact in decreasing daily energy expenditure. At the same time, energy expended in leisure-time physical activities has decreased because people spend more time doing sedentary activities rather than participating in activities that require greater amounts of energy expenditure. In addition, the frequent disruption of sleep and circadian rhythms and a variety of other cultural and economic factors also predispose to weight gain. Finally, hereditary factors such as genetics, family history and racial/ethnic differences also lead to the development of obesity [19-23].

The relative contribution of each of these factors has been studied extensively. The majority of studies conclude that behavioral and environmental factors, such as sedentary lifestyles combined with excess energy intake, are primarily responsible for the dramatic increase in obesity since population-wide genetic alterations do not occur in this relative short period of time in which obesity reached epidemic proportions [1, 11]. A summary of major risk factors and determinants of obesity is shown in **Figure 1.**

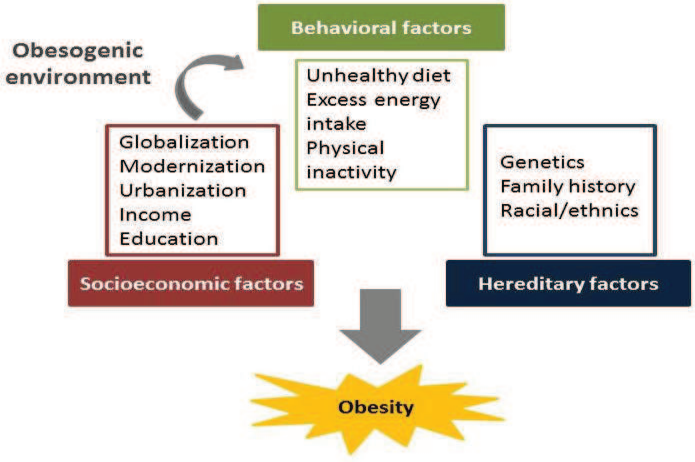


Figure 1. Major risk factors of obesity. Apart from hereditary factors, both socioeconomic and behavioral factors are modifiable. In addition, behavioral factors are the key in the prevention of obesity.

To sum up, body weight regulation is and should be viewed as a complex interaction between environmental, socioeconomic and genetic factors. However personal behaviors in response to these conditions continue to play a dominant role in preventing obesity. Importantly, apart from genetics, every risk factor discussed below could be modified.

1.3 Costs of obesity

Comorbidities

Obesity is considered one of the key risk factors for other chronic diseases together with smoking, high blood pressure and high blood cholesterol [24].

The increasing prevalence of obesity and overweight is associated with the incidence of several comorbidities including type-2 diabetes mellitus (T2DM), dyslipidemia, hypertension, metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease, nowadays the leading cause of death [24, 25]. Furthermore, many studies have examined the link between obesity and cancer and have reported that obesity is associated with multiple types of cancer including colorectal, liver, esophageal, pancreatic, breast, endometrial, cervical, ovarian, brain, renal, kidney, prostate, lymphoma and multiple myeloma [26, 27]. Finally, there is also a relationship between obesity and asthma, sleep apnea syndrome, infertility related to hormonal disturbances and many of psychiatric disorders, obese individuals suffer from social stigmatization, discrimination, and low self-steem [16].

Although obesity is associated with several metabolic disturbances, all obese humans are not equal and approximately 20% of patients with severe obesity have normal metabolic profile. The authors called these obese individuals “metabolically healthy” obese. However, most obese patients are “metabolically unhealthy”. The reasons of these two phenotypes are unknown. Differences in glucose tolerance, inflammatory response, adipose tissue distribution, adipokine secretion pattern and age may be an explanation to this phenomenon. Thus, obesity could be considered as a heterogeneous disorder with variable risk profile [28, 29].

Mortality

It is well known that obesity is associated with an increased risk of death. Recent estimates show that around 2.8 million deaths per year in the European Union result from overweight and obesity-related causes [30]. However, the relationship between body mass index and longevity remains an area of interest and controversy.

Numerous studies have demonstrated a 'U-shaped' association between BMI and risk of death (**Figure 2**), in other words, lower and upper BMI categories (underweight or BMI ≤18 kg/m^2 and morbidly obesity or BMI ≥40 kg/m^2) are associated with higher mortality than in middle categories. The main point of controversy concerns the increased risk of mortality at the low end of the BMI continuum. It is inconsistent with the generally relation between BMI and indicators of morbidity. Moreover, it stands in contrast to the fairly consistent observation that weight loss reduces risks factors for a variety of illnesses. In response to this counterintuitive finding, several authors suggested that the elevated risk of mortality at lowest BMI categories is an artifact. The two confounding variables most frequently cited are smoking and pre-existing "occult" disease which might contribute to weight loss and thus increased mortality in underweight people [31-34].

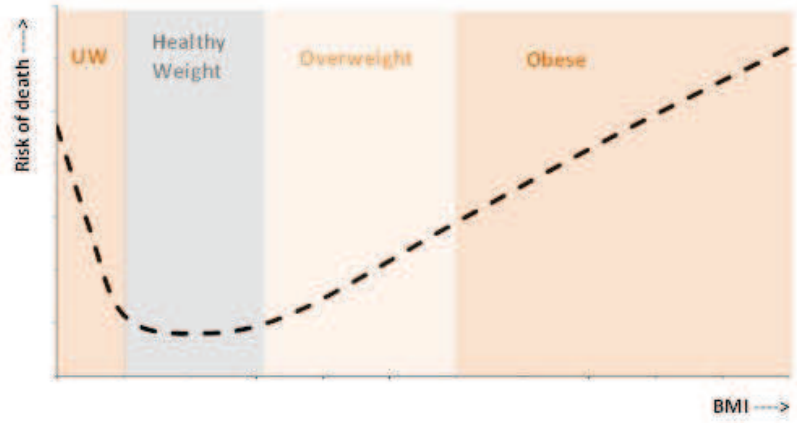


Figure 2. Schematic illustration of the association between mortality and BMI. UW, underweight; BMI, Body Mass Index.

The association between overweight or grade I obesity and obesity-induced comorbidities is not clear. Numerous studies have shown an unexpected and paradoxical inverse relationship with better prognosis in this patient group than in normal-weight group, the so-called "obesity paradox" [35-39]. They revealed that all-cause mortality among overweight or grade I obesity subjects is significantly lower or not higher, compared with normal-weight subjects in general population. For this reason, the debate on the "obesity paradox" in different comorbidities such as coronary heart disease, heart failure, hypertension, peripheral artery disease, stroke, thromboembolism, kidney and pulmonary disease among others is still open [40-56]. Conversely, there are numerous meta-analyses that have shown the opposite, an increased mortality in obese adults compared with normal weight range [57], so this scientific debate is not yet closed.

Nevertheless, the discussion over the existence of the obesity paradox cannot lead to an underestimation of obesity as a crucial risk factor for the development of cardiovascular and metabolic diseases that requires comprehensive prevention and management strategies. Thereby, it should be emphasized that the obesity paradox cannot be the argument against professional treatment of overweight or grade I obesity in subjects diagnosed with cardiovascular diseases or other associated comorbidities. However, in each case, the decision should be made individually with the assessment of potential benefits, including the effect of weight loss on related comorbidities and on quality of life [58].

1.4 Obesity management

The global increasing prevalence of obesity and its metabolic complications and consequently its health costs emphasize the global need for improved strategies in obesity prevention and treatment.

There is a growing demand that governments and international bodies such as United Nations and the WHO take action to reduce the burden of obesity and consequently all of its comorbidities.

As many other chronic diseases, optimal management for obesity consist of different intervention steps, starting with the least invasive and progressing to more invasive approaches as show in **Figure 3** [59].

Figure 3.Three-stepped intensifications of care approaches to weight management.

Lifestyle interventions

As obesity is the major contributor to many metabolic disorders, there is good reason to consider weight loss as a primary therapy. Accordingly, obesity preventive and therapeutic interventions are focused on the modification of risk factors promoting healthy eating, reducing caloric density, glycemic index and overall calorie intake, increasing aerobic and resistance exercise, promoting stress-reduction techniques and increasing the quality of nightly sleep. These approaches are lifestyle interventions, which are the first step and should be the basis of all obesity preventive and therapeutic strategies. Moreover, they are the most accessible and economical option because of their non-invasive nature and their weight-independent benefits [60-63]. Lifestyle interventions can be divided in three broad categories: behavioral, community and environmental interventions [64].

- **Behavioral interventions** have formed the cornerstone of obesity prevention and especially of its treatment. They are focused on current behavior-related aspects with a particular focus on increasing energy expenditure and reducing energy intake to achieve weight loss. These interventions could be self-directed but when they prove inadequate, professional guidance can be particularly helpful providing ongoing assessment and feedback about progress and identifying the most prominent stressors that are likely to contribute to obesity and helping to mitigate them.

- **Community interventions** are implemented in neighborhoods, schools, communal sites, social care facilities and cultural centers. They combine behavioral measures and local environmental changes to address the supply and demand for food and/or physical activity. Some examples of this type of interventions are: comprehensive community-based life-style programs (health education, seminars, community events, worksite programs), the creation of a local environment that emphasized and supported a physical active lifestyle, school based interventions, changes in travel behavior (to and from school/worksite) and after-school physical activity sessions among others.

- **Environmental interventions** modify a target population's environment and are often outside the healthcare sector. They have the potential to reach large number of individuals simultaneously and may have a more lasting effect on the behavior changes as they become incorporated into structures, systems, policies and sociocultural norms: taxes on unhealthy food and beverages, front-of-pack traffic light nutrition labeling, regulation of advertising of junk food and beverages to children and the implementation of educational mass media campaigns to increase health information and knowledge.

The response to this type of interventions is highly variable, some patients exhibit a substantial and durable weight loss, but many others are unable to achieve long-term weight maintenance. Accordingly, adjunctive, alternative and more intensive approaches are required.

Pharmacotherapy is the second-line approach recommended when lifestyle interventions are ineffective in yielding significant weight loss. This type of treatment is approved in patients with a BMI ≥30 kg/m^2 or BMI ≥27 kg/m^2 with obesity related comorbidities [59].

Pharmacotherapy

There are two groups of approved drugs that can be used: 1) medications approved from obesity management *per se* (appetite suppressants or satiety enhancers and gastrointestinal blockers and 2) medications that affect body weight for obese patients who have metabolic complications and are receiving them for chronic disease management (i.e. T2DM) [59, 65, 66]. Medications of these two groups approved by U.S Food and Drug Administration (FDA) are listed in **Table 2** and in **Table 3**, respectively.

History of drug treatment of obesity has experienced the rise and fall of several therapeutic agents which despite showing promising efficacy in body weight reduction and touted as "magic pills" for addressing the obesity epidemic, had to be withdrawn from the market due to serious adverse effects [67]. In 1930s, amphetamines were first introduced as anorectics. However, amphetamine was addictive and had euphoric side effects. By modifying the structure of amphetamines, the anorectic effect of the parental compound was maintained while the stimulatory properties and potential for addition were reduced. With this strategy, in 1960 four noradrenergic agents (phentermine, benzphetamine, diethylpropion, phendimetrazine) were approved as adjuncts in the management of obesity. Phentermine remains the most often prescribed drug for short-term use (up to 12 weeks) for weight loss in the United States [59, 68].

Among the currently approved anti-obesity drugs, orlistat was accepted by FDA in 1999 as the first lipase inhibitor for obesity management. In the past, it was the unique drug available for long-term treatment of obesity, and nowadays, it remains as the only approved obesity therapy in Europe [67-69]. Subsequently, after a long gap of more than ten years, two new therapies, lorcaserin and phentermine/topiramate, were approved in 2012. In 2014, FDA finally approved the combination of bupropion/naltrexone. All of these new pharmacological treatments provide additional options for the management of obesity [59, 67, 69, 70].

Table 2. Weight loss medications approved by U.S. FDA for treatment of obesity.

Family and generic name	Action
Short-term use	
Noradrenergic agents	
▪ Phentermine ▪ Benzphetamine ▪ Diethylpropion ▪ Phendimetrazine	Enhance satiety by inhibiting the reuptake of noradrenalin in the synapse and consequently, an increase of hypothalamic noraderenaline levels.
Long-term use	
Gastrointestinal lipase inhibitor	
▪ Orlistat	Reduces body weight by binding and inhibiting lipases produced by pancreas and stomach reducing the absorption of ingested dietary fat (approximately 30%).
Serotonin-2C receptor activation	
▪ Lorcaserin	Stimulates proopiomelanocortin (POMC) producing neurons in the hypothalamus resulting in generation of α-melanocortin stimulating hormone, which acts on melanocortin receptors to decrease food intake and enhances satiety.
Combined therapy	
▪ Phentermine-Topiramate extended release (ER)	Phentermine reduces appetite trough increasing noradrenaline in the hypothalamus. However, topiramate effects on reducing the appetite are not thoroughly understood. It is thought that it has some effects on γ-aminobutyric acid (GABA) receptors.
▪ Bupropion/Naltrexone	Bupropion reduces food intake by acting on adrenergic and dopaminergic receptors in the hypothalamus. Naltrexone is an opioid receptor antagonist which inhibits food intake.

Moreover, given the relative dearth of effective anti-obesity agents and the lack of prospects for new drug development, obesity researchers and clinicians are increasingly turning to a drug repositioning strategy in order to expand therapeutic options. For this reason, in obesity management nowadays there is an increasing utilization of medicaments traditionally used for the treatment of obesity-related comorbidities if they also produce weight loss (**Table 3**) [66, 71]. For example, metformin is an antihyperglycaemic agent approved for the treatment of type 2 diabetes in adults and children aged ≥10 years. There are a lot of clinical trials which demonstrated that metformin therapy reduced both energy intake and body weight [72-76].

Table 3. Other medications studied off-label for obesity prevention or treatment.

Family and generic name	Indication	Action
Biguanide ▪ Metformin	Treatment of type 2 diabetes	Enhances insulin sensitivity. Produces small sustained weight loss of about 2%.
Glucagon-like peptide-1 ▪ Exenatide ▪ Liraglutide		Reduce fasting and post-pandrial glucose levels, slow gastric emptying and decrease food intake by 19%.
Antidepressant drug ▪ Bupropion	Treatment of neurobehavioral disorders	Reduces food intake by acting on adrenergic and dopaminergic receptors in the hypothalamus.
Anticonvulsant drug ▪ Topiramate		Induces appetite suppression and satiety via GABA receptors mediated inhibitory activity.

To sum up, the ideal anti-obesity agent would selectively reduce body fat stores by ameliorating the regulatory or metabolic disturbances involved in the pathogenesis of obesity. Furthermore it should exhibit only minor, if any, side effects, be preferentially administered orally for long-term use and be widely accessible [66]. Care, consideration and close monitoring are essential when prescribing these medications but it is obvious that the progression of pharmacotherapy for obesity treatment gives us a chance to manage weight problems more effectively.

Bariatric surgery

Bariatric surgery is the third-line approach recommended when the multiple attempts at weight loss through lifestyle interventions and/or pharmacotherapy are not successful. Currently, bariatric surgery is reserved for patients with severe or complex obesity (BMI ≥40 kg/m^2 or BMI ≥35 kg/m^2 in the presence of at least one obesity-related comorbidity). This treatment approach seems to be the most effective treatment in this population; in terms of amount of weight loss achieved and weight maintenance, as well as ameliorating obesity-related comorbidities [77-80]. Although the short-term results of surgery are easily apparent in copious amounts of literature, long-term data about the benefits of bariatric surgery are also emerging. The Swedish Obese Subjects study has shown that, compared with conventional treatment in contemporaneously matched obese controls, bariatric surgery is also associated with a long-term reduction in all-cause mortality [78, 81].

There are four types of bariatric surgery which are usually performed laparoscopically: adjustable gastric band (AGB), sleeve gastrectomy (SG), Roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion with duodenal switch (BPD-DS) (**Figure 4**). Classically, bariatric surgery has been described as 1) restrictive, which aimed to reducing food intake by limiting gastric volume, or 2) restrictive with some malabsorption, which reduces stomach size and creates a physiological condition of malabsortption. The first group includes AGB and SG and the second includes RYGB and BPD [82-84].

All surgeries have advantages and disadvantages, but all procedures are safe and effective. Choice of procedure depends on many factors including local expertise and experience in the different bariatric surgery procedures and their aftercare, and the complexity and reversibility of the procedure. The following describes each of these surgical procedures:

- **Adjustable gastric band (AGB):** an adjustable band is placed around the fundus of the stomach. The restriction of the band is adjusted with fluid via a port, connected to the tubing and placed subcutaneously on the anterior abdominal wall. This causes early satiety once the pouch is full, and the amount of food is restricted until ingested food-stuff has passed through the band (**Figure 4A**).

- **Sleeve gastrectomy (SG):** the stomach is reduced to about 15% of its original size due to the stomach is removing leaving a thin tube of lesser curve (banana shape) (**Figure 4B**). This procedure is not reversible.

- **Roux-en-Y gastric baypass (RYGB):** with this technique stomach is divided to create a small pouch. The smaller stomach is joined directly to a loop of jejunum around one meter distal to the duodenal-jejunal flexure, bypassing the rest of the stomach and the upper portion of the small intestine (duodenum). The redundant stomach and jejunum are then re-anastomosed to the jejunum at a variable distance downstream where digestive juices join food (**Figure 4C**). In normal digestion, food passes through the stomach and enters the small intestine where most of the nutrients and calories are absorbed. Thus, with this surgery, food is not absorbed and the amount of food is restricted by limited size of gastric pouch.

- **Biliopancreatic diversion with duodenal switch (BPD-DS):** it involves a gastric restriction by a sleeve gastrectomy and malabsorption results from the small intestinal bypass. The duodenum is transected and anastomosed to an alimentary limb of ileum. The biliopancreatic limb, which consists of the distal duodenum, jejunum, and proximal ileum, contains the biliopancreatic secretions and is attached to the alimentary limb (**Figure 4D**). The BPD-DS has a malabsorptive component more important than RYGB surgery.

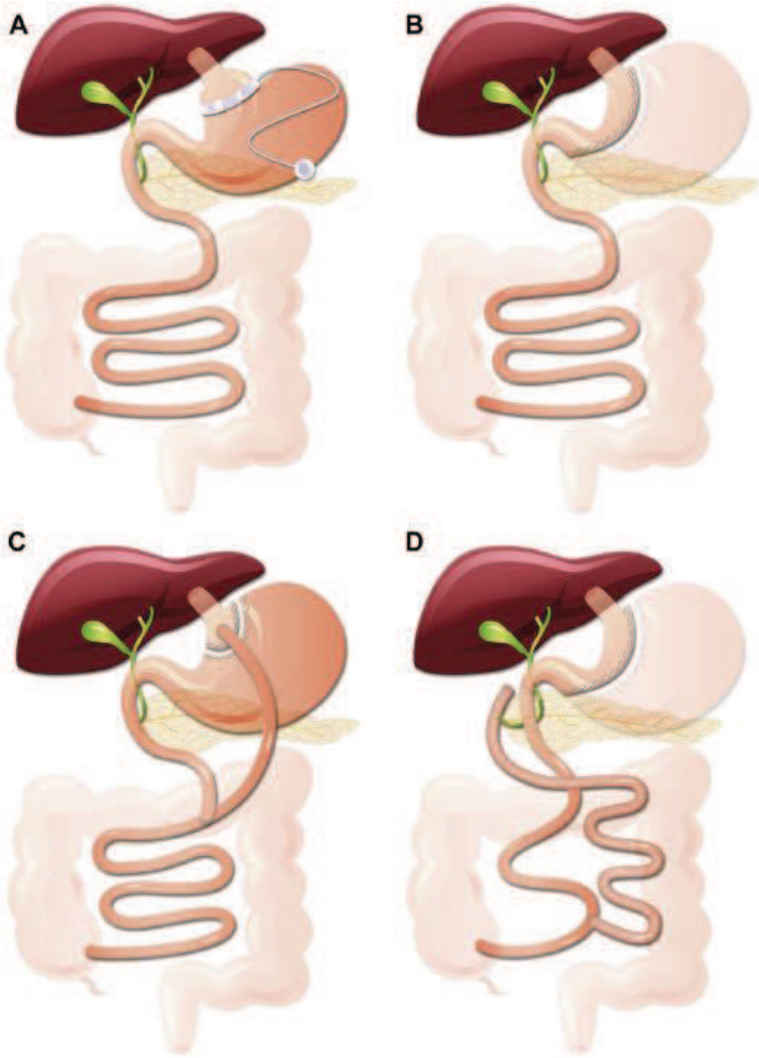


Figure 4. Graphical representation of the most common types of bariatric surgery. A Adjustable gastric band. **B** Sleeve gastrectomy. **C** Roux-en-Y gastric bypass and **D** Biliopancreatic diversion with duodenal switch (BPD-DS). Adapted from Piche et al. [82].

To conclude, bariatric surgery has increased in popularity because of its higher ability to induce long-term weight loss than medical and pharmacological strategies in magnitude and durability. Moreover, bariatric surgery is safe and beneficial in severely obese patients as it induces long-term metabolic benefits. Because of the low risk of surgery and the unequivocal sustained benefits of surgical-induced weight loss, it is likely that bariatric surgery will continue to evolve and to have an expanding role in the prevention of obesity and its related comorbidities [85].

2. NAFLD: the hepatic manifestation of metabolic syndrome

The rapidly increasing prevalence of obesity in both children and adults has also lead to the rise in NAFLD, recognized worldwide as the most common cause of chronic liver disease [86, 87]. Furthermore, it is thought that NAFLD is set to replace viral hepatitis as the primary cause of end-stage liver disease and liver transplantation over the next decade [88].

NAFLD is defined by the presence of a significant amount of lipid accumulation in the liver parenchyma (at least in 5% of hepatocytes), also known as hepatic steatosis, in the absence of excess alcohol consumption. These hepatic fatty deposits result in a wide spectrum of liver damage ranging from simple steatosis with no symptoms to non-alcoholic steatohepatitis or NASH (the presence of fat in liver parenchyma with inflammation, hepatocyte ballooning and lobular inflammation) through to fibrosis and cirrhosis which can result in hepatocellular carcinoma and liver failure (**Figure 5**) [89, 90].

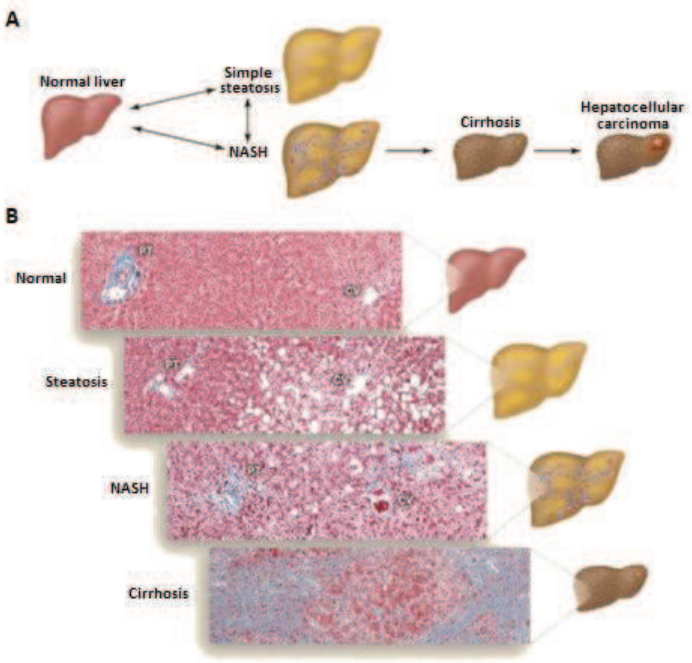


Figure 5.Disease spectrum of NAFLD. **A** Schematic representation of NAFLD progression **B** Histological sections of normal liver, steatosis, NASH, and cirrhosis. Adapted from Cohen et al. [90].

Original histopathologic descriptions of NAFLD date back from 1958 when the disease was characterized by Westwater and Fainer in a group of obese patients [91]. In 1980, Ludwig et al. described 20 patients who lacked a history of significant alcohol consumption but in whom liver histology mimics alcoholic liver disease [92]. They first coin the term of "non-alcoholic steatohepatitis" for this condition. After much debate, the entity of NASH became accepted [93].

2.1 Epidemiology

The worldwide incidence rate of NAFLD is unknown. This disease is usually clinically silent and there is a wide variation in the criteria and diagnostic methods used, consequently its impact has most likely been underestimated [87, 94, 95]. The prevalence of NAFLD in normal weight individuals without any metabolic risk factors is around 16%, rising to 43-60% in patients with diabetes, to 91% in obese patients undergoing bariatric surgery and up to 90% in patients with hyperlipidemia [89, 96].

NAFLD is associated with insulin resistance and other metabolic risk factors such as diabetes mellitus, central abdominal obesity, dyslipidemia and cardiovascular disease. The prevalence of NAFLD also increases with the age and is influenced by genetics. In addition, gender, ethnicity, race and chronic infections seem to be other risk factors of this liver disease [86, 87, 97]. Moreover, NAFLD is associated with the increase of all-cause mortality, contributed by liver related deaths as well as non-liver related causes [89, 98].

2.2 Pathogenesis and natural history

The liver is a metabolic organ that performs important biochemical functions necessary for metabolic homeostasis and it is one of the principal regulators of glucose and lipid metabolism. Insult to any of these processes can lead to liver disease. In the case of NAFLD, numerous disorders can be found in liver's capacity to process lipids and it has been linked to multifactorial alterations in genetics, diet, adipose tissue, hormone regulation, the immune system and gut microbiota [96, 99].

The amount of lipids present in hepatocytes represents a complex interaction between: 1) hepatic fatty acid uptake of plasma free fatty acids (FFA) released from lipolysis in adipose tissue and from the hydrolysis of circulating triglycerides, 2) *de novo* lipogenesis, 3) fatty acid oxidation and 4) fatty acid exportation within very low density lipoproteins (VLDL) [96, 99]. **Figure 6** shows a schematic representation of the sources and fates of liver fat. Accordingly, in NAFLD the accumulation of fat is the result of increased uptake of fatty acids and of *de novo* lipogenesis and impaired fatty acid elimination through oxidation or secretion of triglycerides into VLDL [100, 101].

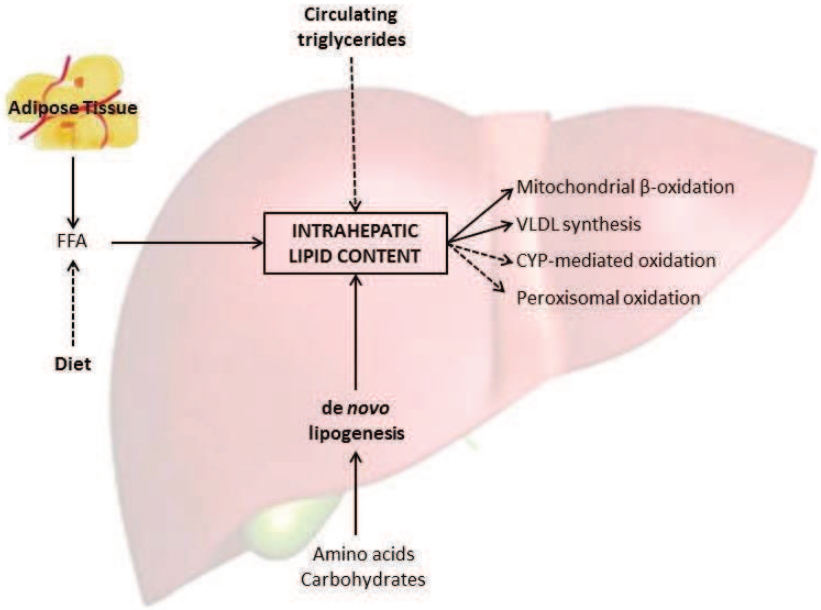


Figure 6. **Sources and fates of intrahepatic lipid content.** FFA: free fatty acids. VLDL: very low density lipoproteins. Adapted from Tiniakos et al. [102].

In obesity, caloric balance is positive. Initially, some of the excess energy is stored in matured adipocytes which increase in size (hypertrophy). Subsequently, adipogenesis, the differentiation of preadipocytes into new adipocytes, is triggered. However, the capacity to store energy in adipose tissue is limited. For this reason, if chronic positive caloric balance persists, adipogenesis may be overwhelmed [103, 104]. Thus, reduced capacity for adipogenesis, coupled with increased energy storage demand in obesity may account for the switch from hyperplastic (the

increase of adipocyte number) to hypertrophic adipocytes which have negative implications [105-108].

Adipocyte hypertrophy entails deregulated adipokine secretion (the balance of pro- and anti-inflammatory adipokines is shifted to a pro-inflammatory state), free fatty acid secretion, hypoxia, cell death and macrophage infiltration [105, 109-111]. Adipose tissue macrophages can be classified in two groups based on their surface marker expression and/or their chemokine secretion profile. On the one hand, there are "classically activated" macrophages or M1, which promote inflammation and, on the other hand, "alternatively activated" macrophages or M2 with anti-inflammatory functions. Accordingly, as obesity progresses, the number of macrophages in adipose tissue increase and it is accompanied by macrophage polarization from the M2 to the M1 phenotype. Consequently, there is an increased expression of pro-inflammatory adipokines such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), interleukin-β (IL-β), Chemokine (C-C Motif) Ligand 2 (CCL2) which mediates macrophage phagocytic and inflammatory responses (**Figure 7**) [112, 113].

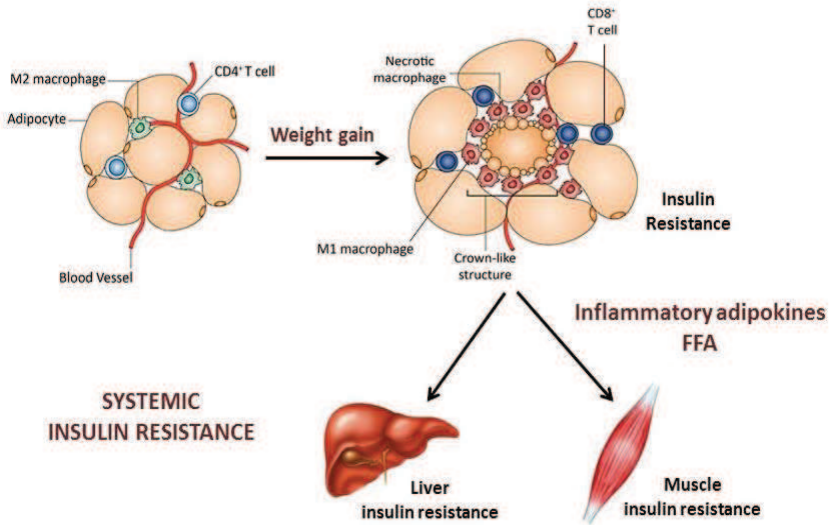


Figure 7. Adipose tissue expansion and its resultant inflammation and metabolic dysfunction. Weight gain leads to adipose tissue expansion and the infiltration of pro-inflammatory immune cells, M1 macrophages and $CD8^+$ T cell. Consequently, adipocytes produce inflammatory cytokines and secrete free fatty acids (FFA) antagonizing local insulin signaling in adipocytes an also in distant organs such as muscle and liver leading to systemic insulin resistance. Adapted from Ouchi et al. [111].

This imbalance in the expression of these pro-inflammatory adipokines leads to various metabolic abnormalities including low-grade inflammation, deregulation of fatty acid metabolism and storage and finally, insulin resistance. This failure of insulin sensitivity can occur locally and/or in distant organs such as muscle and liver (**Figure 7**) [103, 105, 114, 115].

Adipokines induce insulin resistance and low-grade inflammation through the activation of stress-related protein kinases, e.g c-Jun NH2 terminal kinase 1 (JNK-1) and inhibitor kappa kinase β (IKKβ). JNK-1 phosphorylates insulin receptor substrate-1 protein (IRS-1) inactivating insulin signaling and, IKKβ activation leads to the translocation of the nuclear factor κβ (NF-κβ) into the nucleus, resulting in an enhanced synthesis of inflammatory cytokines [116].

In skeletal muscle insulin predominantly induces glucose uptake by stimulating the translocation of glucose transporter type 4 (GLUT4) to the plasma membrane and in the liver, insulin inhibits gluconeogenesis. Furthermore, in adipose tissue insulin is a key regulator of circulating FFA, it decreases lipolysis and thereby reduces FFA efflux from adipocytes [117]. Accordingly, in the absent of competent insulin response in adipose tissue, excess lipolysis ensues leading to an increased release of free fatty acids in the circulation that could be taken by the liver. FFA lipotoxicity involves the suppression of insulin receptor activation and inflammation through the impairment of the phosphorylation of IRS-1. Moreover, this excessive lipid storage may directly contribute to organelle failure including mitochondrial dysfunction, and endoplasmic reticulum stress [109, 118].

Energy metabolism within the liver is tightly regulated. Two transcription factors, sterol regulatory element-binding protein 1 (SREBP-1) and carbohydrate-responsive element-binding protein (ChREBP) are intimately involved in hepatic glucose and lipid metabolism [118]. Dysfunction of the insulin receptor causes hyperglycemia and hyperinsulinemia. On the one hand, hyperinsulinemia leads to increase SREBP-1c expression resulting in increased *de novo* lipogenesis and decreased fatty acid oxidation. In the other hand, hyperglycemia induces ChREBP and leads to further increase in *de novo* lipogenesis. Decreased hepatic lipid transport may also occur via

altered synthesis of apolipoprotein B, leading to decreased VLDL production [114-116, 118, 119].

Although insulin resistance is considered the key factor in developing hepatic steatosis further research is needed to determine the cause and effect relationship between both, in other words, if NAFLD is a cause or a consequence of insulin resistance. In fact, excessive intrahepatic triglycerides content could be both a cause and a manifestation of insulin resistance, resulting from a sequence of events initiated by adipose tissue insulin resistance and propagated to other tissues [120, 121].

Increased intrahepatic fatty acid content provides a source of inflammation and oxidative stress, which may be responsible for the progression from NAFLD to NASH and predisposing to the risk for further severe progression. Mitochondria are the main cellular source of reactive oxygen species, which may trigger steatohepatitis and fibrosis by three different mechanisms: 1) lipid peroxidation and mitochondrial dysfunction, 2) the release of inflammatory cytokine and 3) the consequent lobular inflammation, hepatocyte necrosis, apoptosis and cell dropout. The final result is a necroinflammatory hepatitis that can lead to fibrosis, cirrhosis, hepatocellular carcinoma and liver failure [86, 89].

2.3 NAFLD and mitochondrial dysfunction

Mitochondria have a critical role in the regulation of global metabolic homeostasis. These organelles supply the cell with ATP through oxidative phosphorylation, synthesize key molecules and control calcium homeostasis, among other useful processes. However, mitochondria are also a source of free radicals. Thereby, it is not surprising that mitochondrial health is tightly regulated and mitochondrial dysfunction or damage can greatly perturb metabolic homeostasis impacting metabolic diseases [122].

Cells can manage nutrient supply increasing mitochondrial content. Moreover, mitochondria exhibit the ability to adapt to changing metabolic conditions and are able to increase fatty acid oxidation in an attempt to counteract fat accumulation.

However, persistent nutrient surplus can overwhelmed the mitochondrial system and cause its dysfunction. Energy excess overloads and hyperpolarizes mitochondria, leading to the accumulation of incompletely oxidized lipid products from the citric acid cycle (CAC), also known as tricarboxylic acid cycle (TCA) or Krebs cycle, causing fat accumulation and excessive production of reactive oxygen species (ROS) and, consequently, oxidative stress. These intermediates also activate stress cascade which disrupt the insulin signaling pathway leading to insulin resistance. In addition, excessive ROS production causes mutations to the mitochondrial genomic content, affecting the respiration system, mitochondrial dynamics, decreasing mitophagy leading to aberrant mitochondria and, ultimately cell death (**Figure 8**) [123].

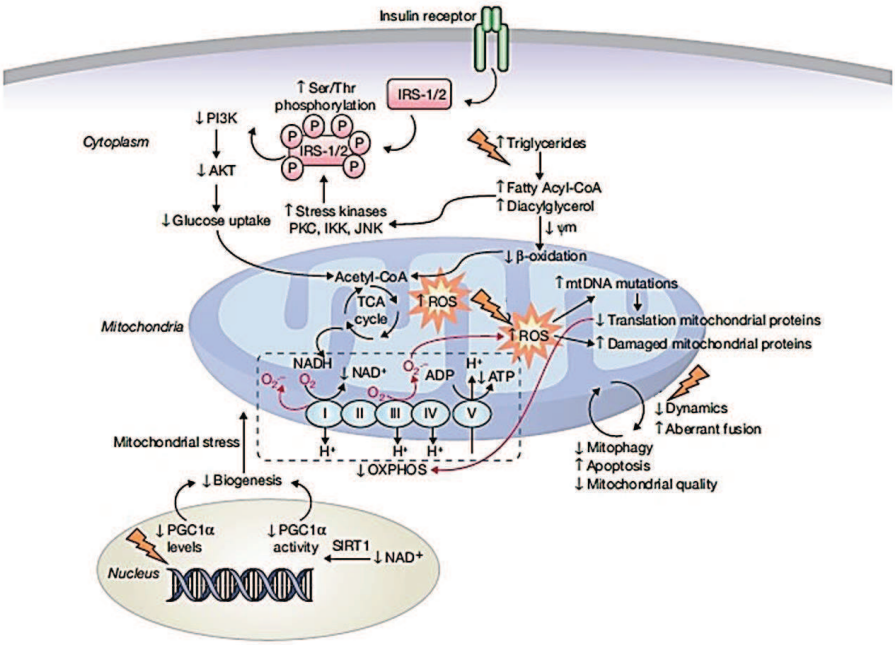


Figure 8. Mitochondrial dysfunction. Nutrient surplus promotes the accumulation of lipid metabolite intermediates that overload and hyperpolarize mitochondria. Then ROS build-up causing oxidative stress, disrupting insulin signaling pathway and impairing mitochondria biogenesis. Adapted from Riera et al. [123].

Mitochondrial architecture is tightly regulated by the dynamic, antagonistic and balanced opposing processes of fusion and fission. Mitochondrial fusion counterbalances functional defects and allows genetic compensation while mitochondrial fission allows the segregation of damaged mitochondria and their recycling through mitophagy, an autophagic process that selectively degrades

damaged mitochondria, ensuring mitochondrial turnover and cellular viability. Finally, insulin resistance also contributes to the development of an endless vicious cycle by impairing CAC, respiratory system and mitochondrial biogenesis inducing lipid accumulation in glucose-consuming tissues such as skeletal muscle and liver [122-127].

Thereby, we can conclude that mitochondria play a fundamental role in the development and progression of NAFLD. An increased lipid accumulation cause severe mitochondrial damage leading to the impairment of mitochondrial activity. These events initiate the first steps of NAFLD and, if they persist, hepatocyte recuperation could be compromised. Therefore, a careful and intensive investigation of molecular mechanism involving mitochondria metabolism and their role in NAFLD/NASH may contribute to the development of novel therapeutic strategies for the protection of these organelles and consequently, for the treatment of NAFLD.

A large number of transcription factors and co-regulators are involved in the regulation of cellular and mitochondrial metabolism. Therefore, molecular targets that can improve mitochondrial function have emerged over the past decade. One of the most characterized co-regulator of mitochondrial biogenesis and energy metabolism is the transcriptional peroxisome proliferator-activated receptor co-activator α (PGC1α) [123, 128]. A decrease of the expression of PGC1α has been implicated in skeletal muscle insulin resistance in humans and in animal models [129, 130]. In contrast, increasing PGC1α content preserves oxidative phosphorylation and inhibits insulin resistance and fat accumulation. PGC1α also minimizes the build-up of ROS through the transcriptional regulation of numerous ROS-detoxifying enzymes [131, 132]. AMP-activated protein kinase (AMPK) is another key factor. AMPK, one of the key energy sensors of the cell, activates PGC1α in conditions of low cellular energy, inhibiting anabolic processes and activating catabolic pathway producing energy such as fatty acid oxidation and respiration. AMPK orchestrates a complex catabolic response to increase mitochondrial biogenesis, enhances antioxidant defense and improve fatty-acid oxidation [133, 134]. For this reason, the development of strategies to directly manipulate AMPK to improve mitochondrial function is increasing and the use of metformin is an example [135, 136].

2.4 NAFLD management

Diagnosis

NAFLD is usually an asymptomatic disease and, consequently, it is often diagnosed accidentally following a routine blood tests or an imaging study done for other reasons. Clinicians should consider a diagnosis of NAFLD in patients with abnormal liver panel tests and the presence of one or more metabolic risk factors (increased BMI, elevated blood pressure, insulin resistance, etc.). It is important to exclude other common causes of liver injury such as alcohol or drug consumption, viral hepatitis, hemochromatosis as well as other coexisting etiologies for chronic liver disease [101].

While serum aspartate transaminase (AST) and alanine transaminase (ALT) levels may be abnormal in the presence of hepatic steatosis they are often not, even using more stringent cut-offs for the upper limit of normality. Consequently, they are considered as poor marker for the diagnosis of NAFLD due to their low specificity, sensitivity and prognostic value (50–80% of patients with hepatic steatosis have normal transaminase levels) [137-140]. For these reasons, other diagnostic methods are needed to confirm the suspected diagnosis of fatty liver.

Currently, liver biopsy still represents the gold standard for the diagnosis of NAFLD. However some physicians and patients are reluctant to carry out this invasive method. In addition to the sampling error, diagnosis is dependent on the subjectivity and experience of the pathologist and its cost and morbidity contribute to the search for additional modalities for diagnosis and staging of disease. In the last decade, many non-invasive methods have been developed to reduce the need for liver biopsy and to overcome its drawbacks [141-143].

The imaging technologies are of wide interest as a possible non-invasive method for evaluating and diagnosing liver steatosis and NASH. Ultrasonography still represents the most common method employed for qualitative assessment of hepatic steatosis. It is non-invasive, widely available, cheap and fast. However, it has several limitations: it is subjective, shows poor sensitivity for the detection of mild steatosis, cannot distinguish NASH from simple steatosis and cirrhosis can only be diagnosed in

advances cases. Computed tomography (CT) and magnetic resonance spectroscopy (MRS) seem to be more sensitive techniques for the quantification of liver steatosis. However CT can only assess moderate steatosis or more, cannot detect early cirrhosis or the degree of fibrosis and there is the drawback of radiation exposure. Moreover, MRS is still less widely available and much more expensive [144-147]. Finally, transient elastography (TE) (Fibroscan®) is another non-invasive imaging tool developed in the last decade. It is an accurate and reproducible test of liver fibrosis and possibly hepatic steatosis and has been validated in a wide spectrum of liver diseases. However one of the limitations of these procedures is its high cost and that, nowadays, is not widely available [148-150].

A growing number of potential biomarkers have been proposed for the diagnosis of NAFLD. Markers of the mechanisms that lead to liver injury and disease progression in NAFLD are potential targets.

Serum cytokeratin-18 (CK18), a major intermediate filament protein in hepatocytes related with hepatocyte apoptosis, is one of the most widely investigated biomarkers and has shown as the most promise in the diagnosis of NASH [151-153]. However, nowadays, this test is not routinely available in many centers and there is no established cut-off values [154-156]. Further biomarkers have been evaluated for the diagnosis of fatty liver and NASH including various cytokines (TNF-α, IL-6 and the chemokines CCL2 and RANTES), acute phase proteins as, for example, C-reactive proteins (CRP), and oxidative stress markers [157, 158]. However, only few have been independently validated. Hence, more extensive studies are needed to prove their overall clinical utility.

Finally, the emergent field of metabolomics is increasingly being applied towards the identification of biomarkers for disease diagnosis, prognosis and risk prediction. Metabolomics involves the quantification of a large number of low molecular weight compounds in plasma and tissue samples. Recent developments in high throughput analysis and robust statistical analysis have allowed investigators to detect changes in cellular and tissue metabolism related with some diseases such as Parkinson's and type 2 diabetes mellitus and more recently, NAFLD [159-166].

Treatment

Interventions for the treatment of NAFLD target excess body weight and the main causes of the pathophysiology of fatty liver: insulin resistance, inflammation and oxidative stress among others.

Obesity is one of the most important risk factor of NAFLD. Accordingly, weight loss is the cornerstone in the management of steatosis and NASH in most cases. Weight loss can be achieved through healthy habits, in terms of food intake and physical activity. For this reason they are the first-line approach to the prevention and treatment of NAFLD. It has been demonstrated that lifestyle modifications leading to weight reduction and/or increased physical activity consistently reduces fat accumulation in the liver [167, 168]. However, these habits are rarely maintained in a long-term. Therefore, there are some weight loss medications that can be used in some patients in conjunction of lifestyle modifications (**Table 2**). Some pilot studies demonstrated that orlistat, an enteric lipase inhibitor which reduces dietary fat absorption, improves liver histology reducing fatty acid infiltration, inflammation and fibrosis in obese patients with NAFLD or NASH [169-171]. However, very few studies have specially tested drug-induced weight loss in NAFLD context; further studies are needed to elucidate its clinical utility. In contrast, the evaluation of potential benefits of bariatric surgery for NAFLD among patients with severe obesity is the goal of many studies.

Bariatric surgery has an increasing role in the management of patients with obesity and metabolic syndrome and recently, appears to be a promising therapeutic approach for NAFLD. Patients experience a high, fast and sustained weight loss. In addition, some studies have shown that surgery-induced weight loss is also associated with improved hepatic histology including reduced steatosis, steatohepatitis and fibrosis by ameliorating some factors that contribute to the pathogenesis of NAFLD (improvement of insulin sensitivity and inflammation) [172-175]. However, the role of bariatric surgery as a primary treatment for NAFLD has to be systematically studied.

Successful weight loss after lifestyle modifications, anti-obesity medication or as result of bariatric surgery has been demonstrated to improve both metabolic parameters and liver histology. The beneficial effects are probably mediated by an enhanced of adipose tissue function, an improvement of insulin sensitivity and a decrease of inflammation and oxidation. All together could modify the course of NAFLD [176-181].

Another treatment approach in patients with NAFLD is the liver-directed pharmacotherapy. As insulin resistance plays an important role in the pathophysiology of NAFLD, therapies targeting obesity and insulin resistance were widely studied. Thereby, insulin sensitizing agents, such as metformin and thiazolidinediones (TDZs) became the most promising drugs in NAFLD management [182, 183].

Metformin is a biguanide widely used for the treatment of type 2 diabetes which action is mediated by the activation of AMPK, a key regulator of lipid and glucose metabolism. TDZs enhance insulin sensitivity activating peroxisome proliferator-activated receptor γ (PPARγ). PPARγ is highly expressed in adipose tissue where it controls adipocyte differentiation. Its activation plays an important role in increasing insulin sensitivity as well as in promoting FFA uptake into adipocytes reducing its delivery to the liver or other organs. Thereby, metformin and TDZs have been successfully tested and some studies have demonstrated their beneficial effects on liver function and liver histology [135, 184-187].

Increased oxidative stress and the consequent depletion of antioxidant molecules within the hepatocytes are regarded as the initiators of the progression from simple hepatic steatosis to NASH [188]. For this reason, an antioxidant supplementation could be a potential treatment for NASH. As an example, some studies have showed that vitamin E inhibits peroxidation, suppresses inflammatory cytokines, counteracts the lower levels of antioxidants enzymes and consequently, ameliorates liver steatosis and the progression of the disease [189, 190].

Hypertriglyceridemia is a main component of metabolic syndrome and is strongly associated with NAFLD. Different lipid-lowing agents such as fibrates and statins (PPARs agonists) have been tested for the treatment of NAFLD, given that PPARs regulate transcriptional genes responsible for fatty acid oxidation, and showed beneficial effects [191, 192].

Finally, given the emerging importance of inflammation in fatty liver, cytokines and their regulatory molecules can be another therapeutic target in near future. Anti-TNFα agents have beneficial effects in animal models of NASH and some pilot studies in patients reported a significant reduction in liver enzymes levels associated with improved steatosis, lobular inflammation and fibrosis stage [193, 194]. In this way, our studies have found that the continuous administration of polyphenols in animals model reduce weight gain, liver steatosis and insulin resistance probably by the combination of anti-inflammatory and anti-oxidant effects provided by these bioactive compounds [195-198].

Despite considerable research on pharmacotherapy for the treatment of NAFLD its efficacy and safety remains inconclusive, and nowadays there are no licensed single pharmacological agent. However, there are many late phase clinical trials in progress, so the situation will probably change in a near future. Moreover, further advances in the understanding of the pathogenesis of NAFLD will also help in the developing of reliable therapeutic strategies for this increasingly liver injury.

Finally, patients with end-stage of NAFLD (with decompensated cirrhosis or hepatocellular carcinoma) are candidates for liver transplantation which definitely is the only possible treatment [199].

3. Inflammation and metabolism: the role of Chemokine (C-C motif) Ligand 2 (CCL2)

Historically, immunity and energy metabolism have been considered to be distinct capabilities to the maintenance of homeostasis, supported by different cell-types and differentially regulated. However, during the last decades, a wealth of evidence has come out demonstrating that immunity and metabolism are closely interconnected [200-202].

The huge search for the discovery of the unifying mechanism responsible of the pathogenesis of obesity and its comorbidities has revealed a strong relationship between nutrient excess and derangements in immune system. Consequently, the classical view of inflammation, described as the principal coordinated response of the body to harmful stimuli (infection, tissue stress, injury, etc.) by many complex signals in distinct cells and organs [189], needs to be expanded to fully explain the inflammatory processes induced by adverse metabolic conditions and the accompanying deleterious effects.

Although the often short-term response of inflammation is a crucial defense mechanism, its long-term consequences, mainly caused by a continuous nutritional surplus, are not beneficial and leads to the development of metabolic diseases. All this resulted in a new concept known as “metainflammation” (metabolically triggered inflammation) to describe the low-grade chronic inflammation related to metabolic disorders [200, 203]. Consequently, the historical and simple point of view of obesity as lipid storage disease has been replaced by the concept that it is an inflammatory disease.

The first discovery of inflammation in obesity was carried out by Dr. Hotamisligil who observed increased levels of TNF-α in adipose tissue of obese mice compared with lean controls [204-206]. This discovery was then followed by many studies describing these inflammatory differences in obese and lean animals as well as in humans. Thus, it has been demonstrated that chemokines and their receptors play an important role in the pathogenesis of all metabolic disorders which make them and their receptors attractive as therapeutic targets.

3.1 Chemokine and chemokine receptor family

Chemokines or chemotactic cytokines are a group of small secreted proteins consisting of 60 to 100 amino acids of about 8-14 kDa, with a highly variable sequence homology (from <20% to 90%) and represent the largest family of cytokines. They direct the migration of leukocytes throughout the body under physiological and pathological conditions [207-210].

Chemokines were first identified in 1977. Since then, approximately 50 chemokines and 20 chemokines receptors have been identified [210, 211].

Chemokines are divided into four families based on the arrangement of the two conserved cysteine residues located in the N-terminal region (**Figure 9)**. The two largest families are the CXC or α family in which one amino acid separates the first two cysteines, and CC or β family, where these two cysteines residues are adjacent. The other families are the (X)C or γ family, having only one of the first two cysteines, and finally, CX3C or δ family , which is currently represented by a single member named fractalkine (CX3CL1), which has three amino acids between the two cysteines residues as well as a transmembrane mucin-like domain [210-212].

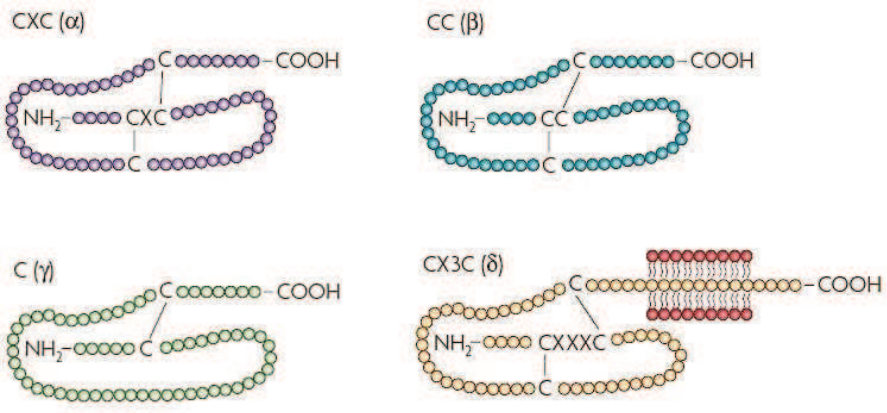


Figure 9. Classification of chemokines based on the position of the two highly conserved cysteines residues in N-terminus. Adapted from Rostene et al. [212].

The biological effects of chemokines are mediated by seven-transmembrane-domain receptors that represent a subset of the G-protein-coupled receptor superfamily (**Figure 10)**. The conventional signaling pathway attributed to chemokines receptors involves the mobilization of calcium from intracellular stores and the subsequently downstream cascades trigger conformational changes on leukocyte integrins that promote cell adhesion and extravasation. More specifically, for its activity the $\beta\gamma$ subunits are released and activate phosphoinositide-specific phospholipase C (PLC) isoenzymes leading to the formation of inositol-1,4,5-triphosphate (IP_3) and increasing the intracellular calcium concentration. The signals mediated by chemokine receptors are short and transient [207, 208, 213].

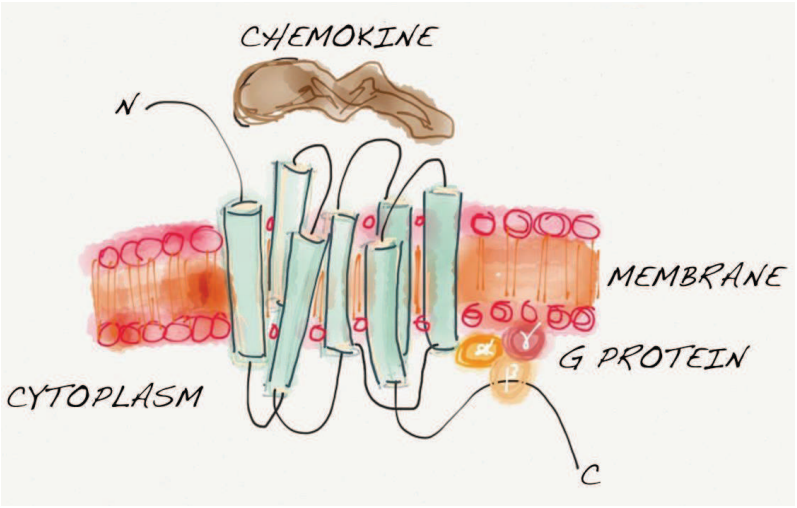


Figure 10. Chemokine receptor. They are found on the surface of certain cells and have a seven-transmembrane domain coupled to G-protein for signal transduction within the cell. From Camps et al. [213].

Besides these typical chemokine receptors, there are atypical chemokine receptors (Duffy antigen receptor for chemokines or DARC, D6, CCX-CKR) which bind their ligand with high affinity and specificity but they do not transduce the intracellular signal which leads to chemotactic and other cell responses after chemokine binding. In some cases, the ligand is transported across the cytoplasm and released on the other side of the cell while in other cases the chemokine is internalized and degraded. **Figure 11** contains the known human chemokines and their receptors [207, 208, 213, 214].

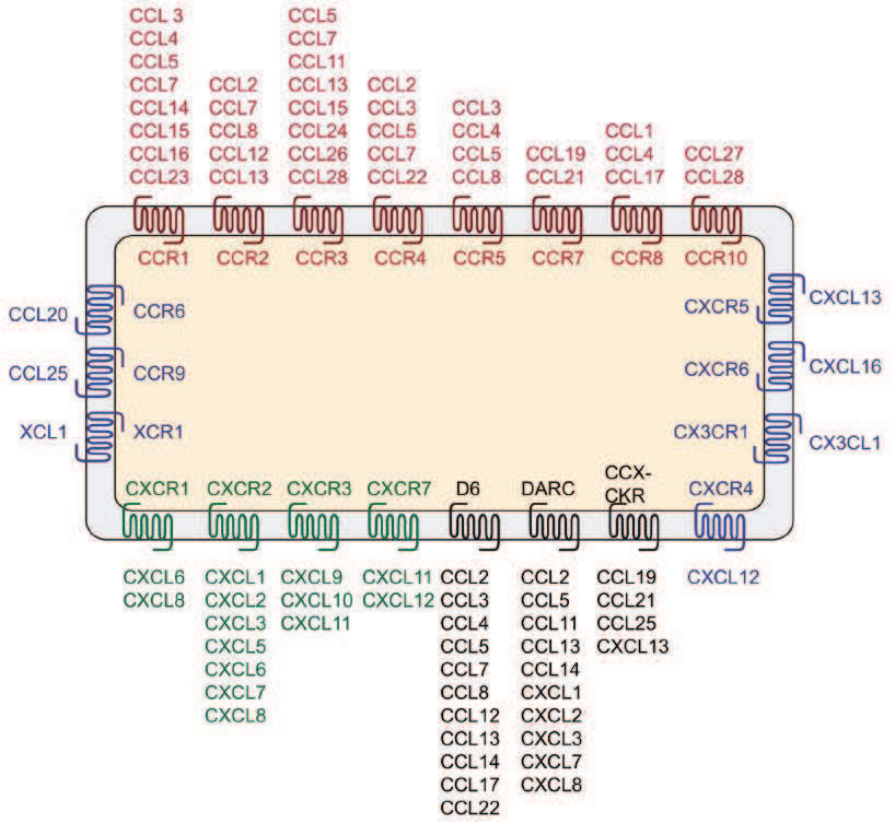


Figure 11. The chemokine superfamily consists of a large number of ligands and receptors. In red CC family, in green CXC family and in blue a minority of receptors which have only one ligand. Finally, atypical receptors and its possible ligands are represented in black. From Lazennec et al. [215].

The binding between ligands and their receptors seems to be promiscuous. Thus, a single chemokine can bind to several receptors and a single receptor may traduce signal for several chemokines. This idea could suggest the redundancy of these molecules in pathophysiology and it became a controversial issue. However, many studies suggest that each chemokine and receptor may have a special position on the orchestrated biological response and cannot be fully replaced by another ligand. In fact, various chemokines can induce different an even opposite biological effects although acting at the same receptor [209, 213].

Chemokines are also grouped into two main subfamilies; homeostatic and inflammatory chemokines based in the expression pattern and function. Homeostatic chemokines are generally constitutively expressed and are involved in

the recruitment of immune cells under normal conditions, for example, they direct the trafficking of lymphocytes to lymphoid tissues. On the other hand, inflammatory cytokines are upregulated by pro-inflammatory stimuli such as infection or trauma and participate in innate and adaptive immune response; they control the recruitment of leukocytes during inflammation and tissue injury. These classifications are not mutually exclusive, in other words, some inflammatory chemokines can have homeostatic functions and inversely, some homeostatic chemokines could be upregulated during pathogenic inflammation [207, 209, 210, 213, 216].

Once chemokine secretion is induced, the migration of cells expressing the appropriate chemokine receptors takes place by a chemokine gradient which allows cells to move in the direction of high local concentration of chemokines.

In addition to their major biological function, the regulation of cell trafficking, subsequent research has elucidated their involvement in other functions of the inflammatory processes such as fibrosis, tissue remodeling and angiogenesis, development, hematopoiesis, and homing [210, 211].

Finally, beside to their roles in the immune system, accumulating evidence suggests that, chemokines and their receptors play an important role in the pathophysiology of several diseases. Perturbations of chemokines and/or their receptors expression or function can lead to the persistence of an inflammatory reaction creating a key pathogenic event for the establishment of chronic inflammation which is the hallmark of many diseases such arthritis, asthma, HIV infection as well as obesity, NAFLD, insulin resistance, atherosclerosis and cancer [216-218].

For this reason, chemokines and their receptors have become an important target for searching novel biomarkers and specific therapeutic approaches. In this way, CCL2 and its receptor (CCR2), the most well studied chemokine-chemokine receptor systems, are potential targets for the treatment of various diseases.

3.2 Chemokine (C-C motif) Ligand 2 (CCL2)

Chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), is the first discovered and the most extensively studied human CC chemokine [219].

CCL2 was discovered in 1989 at the International Cancer Institute in Maryland (USA), identified from the conditioned media of human myelomonocytic cell line THP-1 based on its *in vitro* monocyte chemotactic activity [220, 221].

Structure

This protein is a member of the CC or β chemokine family and is typically secreted in two predominant forms with molecular weight of 9 and 13 kDa. These two isoforms are the result of a differential *O*-glycosylation with the disaccharide galactose-β-D-N-acetylgalactosamine, present only in 13kDa isoform. Even this differential glycosilation, they have the same capacity to induce monocyte migration [219, 222]. In addition, this protein seems to be identical to the murine JE gene product (mouse CCL2) [221, 222].

Regulation

CCL2 is encoded by *CCl2* gene which is located in chromosome 17 (17q11.2). It is expressed, either constitutive or after inflammatory stimuli, by different cell types including epithelial, endothelial, smooth muscle cells, fibroblasts and astrocyte but the major source of this protein are monocytes and macrophages and, more recently, adipocytes have been recognized as another important source of CCL2 [219, 221-223]. In addition, it has been demonstrated that CCL2 protein and mRNA are expressed in the majority of tissues, so there is a systemic production and, at the same time, the possibility to respond *in situ* to inflammatory stimuli [224, 225].

Its expression is regulated at transcriptional level by a variety of stimulatory mediators such as TNF-α, interferon gamma (INF-γ), platelet-derived growth factor (PDGF), interleukins IL-1 and IL-4, bacterial lipopolysaccharide and reactive oxygen species [219, 222]. The pro-inflammatory NF-κβ transcription factor is the key

mediator in the transcription of CCL2 due to the promoter region of *CCl2* gene contains two NF-κβ binding sites in the distal part [226-228].

Biological function

CCL2 is a potent chemoattractant which regulates the migration and infiltration of monocyte cells to sites of injury and inflammation. It is also important for T cell and natural killer cells differentiation [229]. Moreover, CCL2 participates in the first steps of inflammation process, for example, in adhesion (upregulating the expression of integrin on monocyte cell surface) and extravasation of monocytes through vascular endothelium to foci of active inflammation [219].

As mentioned before, this protein exerts its function through binding to CCR2, an heptahelical G-protein-coupled receptor embedded on the lipid bilayer of cells targeted for activation and migration. This receptor, once activated, trigger a signal transduction cascade that, as described previously, results in IP_3 formation, intracellular calcium release and protein kinase C (PKC) activation which finally, through NF-κβ transcription factor and Rho family proteins, regulates cell motion and mobility (**Figure 12**) [219, 230].

CCR2 is the receptor for all monocyte chemoattractant proteins: MCP-1, MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13) and MCP-5 (CCL12) [231]. It is expressed abundantly in monocytes, basophils, dendritic cells, natural killer cells and activated T lymphocytes. In addition, it could appear in two different forms (CCR2A I CCR2B) as a result of an alternatively splicing which only differ in carboxy-terminal region. The change in the sequence alters the location of the protein in the cell and CCR2B, the predominant isoform is mainly localized to the plasma membrane whereas the transcript variant A encodes the cytoplasmic isoform [232-235].

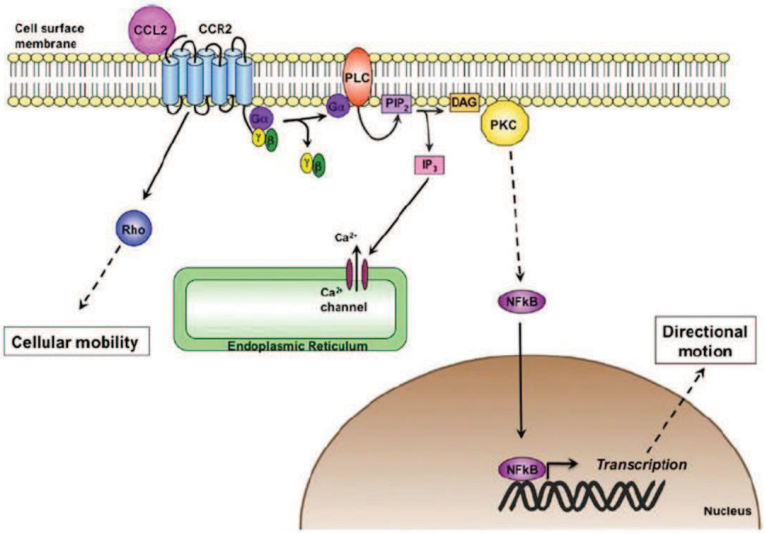


Figure 12. CCL2/CCR2 signaling pathway. DAG, diacylglycerol, IP_3, inositol triphosphate; PIP_2, phosphatidylinositol-biphosphate, PLC, phospholipase C. Adapted from Melgarejo et al. [219].

3.3 CCL2/CCR2 and disease

Recent studies have elucidated the role of chemokines and their receptors in several pathological processes. Chemokine receptor activation implicates the regulation of pleiotropic signaling pathways influencing a wide number of molecular and cellular processes. Hence, an inappropriate inflammatory response leads to numerous human diseases [230, 236].

The involvement of chemokines in disease has mainly been demonstrated using genetically modified mice, antibody or inhibitor-mediated neutralization and obviously, with epidemiological studies in humans [234].

CCL2 and its receptor CCR2 have been related to the pathogenesis of different diseases including vascular permeability and attraction of immune cells during metastasis, a number of different neurological disorders, autoimmune diseases and metabolic diseases such as obesity, insulin resistance, atherosclerosis and NAFLD becoming an interesting and highly studied target for novel therapeutic strategies [237].

CCL2/CCR2 in obesity

As mentioned before, obesity is associated with a chronic low-grade inflammation which implicates adipocyte dysfunction and endocrine activity of adipose tissue. In obesity, adipose tissue is characterized by macrophage infiltration and the release of multiple inflammatory mediators which lead to metabolic disturbance such as insulin resistance and ectopic lipid accumulation [113, 200, 238].

Evidence that CCL2 is related to obesity is provided by the observation of higher CCL2 plasma levels in obese patients than in lean controls [239, 240]. Similarly, high levels of circulating CCL2 also have been found in obese mice compared with wild-type littermates [241-243]. Furthermore, the different depots of adipose tissue, such as subcutaneous and visceral, show increased gene expression of CCL2 in obesity, both in animal models and in obese patients [244-246]. In contrast, weight loss decreases macrophage infiltration in adipose tissue and also the circulating CCL2 levels demonstrating that beneficial effects of weight loss are mediated by ameliorating the inflammatory status of adipose tissue [240, 247]. Similarly, different studies with CCL2 or CCR2 deficient mice, or with the use of CCR2 inhibitors in obese mice, have demonstrated that CCL2/CCR2 deficiency attenuates the development of obesity, adipose tissue macrophage infiltration, inflammation, and systemic insulin resistance [248-250]. Hence, CCL2 induces macrophage recruitment and adipose tissue inflammation as well as insulin resistance. Accordingly, the inhibition of this inflammatory pathway ameliorates metabolic disturbances [251, 252].

An increase in adipose tissue mass (in number and size of adipocytes) is the main feature of obesity. It has been demonstrated that CCL2 contributes to adipocyte differentiation and induces adipogenesis by the induction of a zinc-finger protein, MCP-1-induced protein (MCPIP) and the following signaling pathway which involves ROS, endoplasmic reticulum stress and autophagy. Moreover, CCL2 also contributes to the expansion and remodeling of adipose tissue [253-255].

CCL2/CCR2 in NAFLD

The adipose tissue inflammation caused by CCL2 macrophage recruitment and the subsequent insulin resistance results in increased lipolysis. The impaired lipid buffering leads to the exposure to an increased efflux of FFA of non-adipose tissues such as the liver. This metabolic stress can lead to an imbalance between FFA supply and hepatic FFA disposal pathways, causing lipid accumulation within hepatocytes [256].

Several studies have demonstrated that both, CCL2 circulating levels and gene expression are higher in patients with NAFLD [250, 257-259]. Moreover, serum CCL2 levels in NAFLD patients are positively correlated with the severity of liver damage, in other words, increasing levels from healthy control to simple steatosis reaching the highest concentration in patients with NASH [260-262].

The generation of oxidative stress-related molecules is responsible for the progression from simple steatosis to NASH. Some studies have demonstrated that ROS and some products of lipid peroxidation such as 4-hydroxynonenal and malondialdehyde are significantly lower when *Ccl2* is deleted. Moreover, the lack of CCL2 is associated with a reduced expression of several gens implicated in fibrogenesis [263-265].

Finally, CCL2/CCR2 deficiency or their pharmacological inhibition in the presence of chronic liver damage attenuates the development of NAFLD [266, 267].

Taken together, all these data indicate that CCL2 plays an important role in the pathogenesis of NAFLD [218, 256].

4. Autophagy: the cellular housekeeper

The literal translation of autophagy emanates from the Greek roots “auto” (self) and “phagy” (eating) “self-eating” and broadly refers to an intracellular catabolic pathway that targets cytosolic components to lysosomes to be degraded. This process responds to stresses with the ultimate purpose of self-preservation. The autophagic process is suited uniquely to the timely identification of surplus, unnecessary or dysfunctional proteins and organelles for the maintenance of cellular homeostasis and supplying substrates for energy generation [268, 269].

The term *autophagy* was first introduced in 1963 by De Duve et al. Over the past few years, the study of this field has witnessed a dramatic growth. This is attributed partly to the discovery of the key components of its cellular machinery through studies conducted in yeast and *Drosophila*, but more important, from studies in mammalian cells and tissues [269].

Three primary types of autophagy have been identified: macroautophagy, chaperon-mediated autophagy (CMA) and microautophagy, depending on its physiological function and the mode of cargo delivery to the lysosome (**Figure 13**).

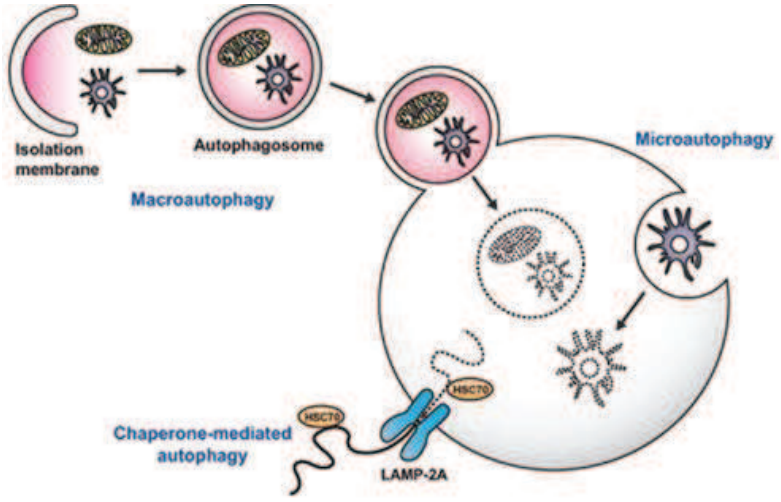


Figure 13. The three types of autophagy. In macroautophagy, an autophagosome is formed and it is fused with lysosomes to degrade the cellular constituents or pathogens. Microautophagy involves the uptake of cellular components within an invagination of the lysosomal membrane for enzymatic degradation. Finally, in chaperone-mediated autophagy, cytosolic proteins containing a pentapeptide motif bind to the chaperone Hsc70. This complex binds to the LAMP-2A receptor on the lysosome for internalization and degradation. From Oh et al. [270].

As a briefly mention, CMA involves selective translocation of the cytosolic proteins which are marked by a pentapeptide motif with a consensus sequence similar to KFERQ across the lysosomal membrane. Cytosolic chaperones aid in the target recognition and unfolding, and a multimer comprising the lysosomal protein LAMP-2a (lysosomal-associated membrane protein-2a) subunits is thought to be rate-limiting for target translocation into lysosomes [271, 272]. On the other hand, in microautophagy, a poorly understood phenomenon in mammalian cells, the cytosolic contents are engulfed by direct invagination of the lysosomal membranes into tubulovesicular structures [273, 274]. However, macroautophagy is considered to play the most important role in pathophysiology and has been well studied in recent years. For this reason, the term autophagy is usually referred to macroautophagy and this thesis is focused on it.

Macroautophagy is the major regulated catabolic mechanism that eukaryotic cells use to degrade proteins and organelles. This form of autophagy involves the sequestration of portions of the cytoplasm within a vesicle (autophagosome) which fuses to the lysosome (autolysosome) in which the captured material (cargo), together with the inner membrane, is degraded. The autophagosome lacks the acidic environment and the enzymes required for terminal digestion of the engulfed contents; thus, the fusion of the autophagosome with the lysosome supplies the acidic environment as well as a battery of hydrolases [275, 276].

Autophagosome formation is a complex and highly regulated process that requires more than 30 autophagy-related genes (*Atg*) and the resultant proteins. These proteins form functional complexes and can be grouped according to their functions in the key stages of this pathway. The main steps are: induction or initiation, nucleation, membrane elongation and enclosure and, finally, the fusion with lysosomes. As a summary, initiation is controlled by the ULK complex, followed by activation of the PI3-kinase complex leading to nucleation of the phagophore, the initial *de novo* formation of a double-membrane that encloses a portion of cytoplasm. The origin of the membranes involved in the formation of autophagosomes could be the endoplasmic reticulum, mitochondria, and golgi apparatus. However, it is still not clear which is the major contributor [275-278].

Membrane elongation is governed by two ubiquitin-like conjugation systems. The first is the conjugation of Atg12 to Atg5 mediated by two ligases, Atg7 and Atg10. Atg5 also associates with Atg16 to form the Atg12-Atg5-Atg16 complex. The second involves the cleavage of LC3/Atg8 by Atg4 leading to the soluble form LC3-I (microtubule-associated protein light chain 3-I), which is then conjugated to phosphatidylethanolamine (PE), via the participation of Atg7 and Atg3. This lipid conjugation forms the autophagic double-membrane associated LC3-II protein allowing the closure of the autophagic vacuole. Accordingly, LC3-II, is used as a marker of autophagosomes due to its important role in the autophagic process. Finally, autophagosomes fuse with lysosomes, where the breakdown of the autophagic cargo takes place [275-278]. **Figure 14** illustrates the molecular mechanism underlying autophagy.

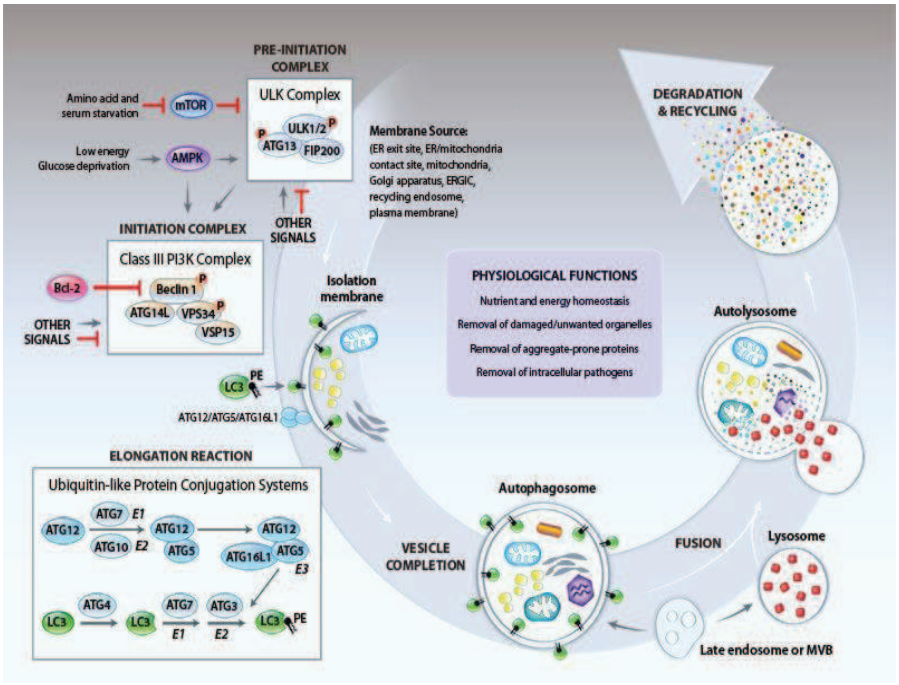


Figure 14. The macroautophagy pathway. Following inhibition of MTOR, the ULK complex is activated and initiates autophagosome formation. The class III PI3 kinase complex also regulates the autophagosome nucleation step. To expand the autophagosome membrane, two ubiquitin-like conjugation systems are required for conjugation of LC3 and Atg12 to phosphatidylethanolamine (PE) on the autophagosome membrane and Atg5, respectively. The complete autophagosome fuses with the lysosome to form the autolysosome, and cargo molecules engulfed by autophagosomes are degraded by lysosomal hydrolases and recycled back to the cytoplasm.

Regulation of macroautophagy occurs via mechanistic target of rapamycin (MTOR), a nutrient and energy-sensing kinase. A cell with a stable energy source has active MTOR signaling through its phosphorylation, leading to inhibition of autophagy interacting with autophagy effectors (ULK1, FIP200). Conversely, a nutrient-depleted cell has decreased or inhibited MTOR activity, thus autophagy is activated. Moreover, an additional level of cross-talk between MTOR and autophagy is provided by AMPK, the main sensor of intracellular energy. When cells become energy-deprived, AMPK efficiently turns-off the MTOR pathway and also positively regulates ULK complex to induce autophagy (**Figure 14)** [279-284].

Insufficient or dysfunctional autophagy accumulates abnormal or dysfunctional material. Thus, in agreement to this context, a number of diseases are associated with impaired autophagy such as neurodegenerative diseases, cancer, aging, inflammation, obesity and liver diseases [285-287]. For this reason, an improved understanding of autophagic process should provide substantial information for new therapeutic manipulations of this endogenous process.

MTOR, as a master regulator of cellular metabolism, has become an appealing pharmacologic target to manipulate autophagy. As implied in its name, rapamycin is the most available MTOR inhibitor that has been tested for clinical practice. Rapamycin induces autophagy and has demonstrated beneficial effects in cancer and some neurodegenerative diseases [288-290].

Moreover, as mentioned before, the activation of AMPK inhibits MTOR and consequently, enhances autophagy. Hence the administration of AMPK activators such as metformin stimulates autophagic process and could be considered as another possible therapeutic target [291, 292].

Further evaluations are needed to determine the effectiveness of MTOR inhibitors for inducing autophagy with minimal side effects to provide potential candidates for developing novel therapeutics for the management of human diseases.

4.1 Autophagy and NAFLD

Autophagy in the liver has been well studied. The features and functions of the liver make this organ very dependent on autophagy. Accordingly, impairment of autophagy has profound deleterious effects on liver function [293-295].

The autophagic engulfment of organelles such as mitochondria (mitophagy) or discrete molecules such as lipids (lipophagy) serves as integral machinery of hepatocellular homeostasis [296].

Lipophagy, the delivery of cellular lipid droplets to the lysosomes, helps to modulate the lipid stores more efficiently to respond to energy demand and to protect cells from lipid-mediated damage [297-299]. Therefore, a decrease of autophagy in the liver leads to lipid accumulation and subsequent development of NAFLD [300].

Several studies in cultured hepatocytes with impaired autophagy, generated by pharmacologic inhibition or by using RNA interference against *Atg*5 or *Atg*7, have revealed that inhibition of autophagy leads to higher accumulation of triglycerides respect to controls. These results were also obtained in hepatocyte-specific *Atg*7-deficient mice [301].

Moreover, high-fat diet fed mice exhibit an impairment of hepatic autophagy as demonstrated by decreased mobilization of lipid into the autophagic compartment. Lipid accumulation altered the membrane structure, and a resultant decrease in the efficiency of fusion between autophagosomes and lysosomes may explain this inhibitory effect. Moreover, the ability of excessive cellular lipid accumulation to impair autophagy provides another mechanism for the progression of simple steatosis to NASH and its complications [301, 302].

Studies conducted in humans also revealed the relationship between autophagy and NAFLD. A recent report demonstrated that autophagy was decreased in obese patients with steatosis and NASH [303].

It has been demonstrated that an activation of liver autophagy constitutes a promising therapeutic approach against steatosis and hepatic complications.

Accordingly, it has been reported that hepatic overexpression of Atg7 in high-fat diet fed mice or obese mice improved the condition of the fatty liver and insulin resistance [304]. Moreover, enhancers of autophagy such as rapamycin have been tested in obese mice and they ameliorated the presence of steatosis and insulin resistance [305].

For this reason, therapeutic efforts to decrease hepatic lipid stores by raising autophagic function could prevent the initiation of liver injury or the development of NASH complications, such as hepatocellular carcinoma.

4.2 Autophagy and inflammation

Growing evidence suggests that autophagy not only regulates cellular homeostasis but also plays an important role in protection against pathogens. In this line, autophagy has been linked with innate and adaptive immune response in host defense by regulation of cytokine production [306-308].

Furthermore, autophagy is implicated in cellular development and differentiation including adipogenesis, a key feature of obesity. It has been shown that inhibition of autophagy reduces accumulation of triglycerides within adipocytes, increases transcription factors involved in adipocyte differentiation and also increases mitochondrial function [299, 309-311].

In contrast, other data report that autophagy modulates adipose tissue inflammation controlling the production of cytokines. Some studies reveal that the inhibition of autophagy is related with the gene expression and the secretion of pro-inflammatory cytokines in both animals and murine adipose tissue. Accordingly, autophagy may act as a protective mechanism to limit chronic low-grade inflammation of adipose tissue related with obesity [311, 312].

For this reason it is required further exploration to elucidate if autophagy could be a potential regulatory mechanism that links inflammation and metabolic disorders. Understanding the mechanisms and pathways involved in autophagy could lead to future therapeutic strategies against inflammation and related conditions such as obesity, insulin resistance, atherosclerosis and liver disease.

5. Animal models in scientific research

Animal research plays a crucial role to better understand the pathophysiology of several human diseases and, consequently, contributes to the development of effective medical treatments.

Research animals provide scientists with complex living systems consisting of cells, tissues and organs. The most important features are their striking similarity to humans in anatomy, physiology and genetics. Over 95% of the mouse genome is similar to our own, and they are vulnerable of the same health problems, making its use particularly applicable to human diseases [313].

Practically, mice are a cost-effective and efficient tool to speed research and the development of drug therapies. Mice are small, have a short generation time and an accelerated lifespan, keeping the costs, space, and time required to perform research manageable [313].

In addition, our ability to directly manipulate its genome provides an incredible powerful tool to model specific diseases. Depending on the target in question, there are a number of models that can be applied. Perhaps, this is the most important advantage to using mouse for biomedical research [314]. Genes can be injected directly into the fertilized egg of mouse creating what is known as a transgenic animal. This approach allows us to create a new animal model which overexpresses CCL2, the chemokine in which we have focused all our efforts and which is involved in many pathological processes.

In the same way, scientists developed techniques that allowed them to target genes within mouse genome (so-called knockouts). These knockout mice are a resource to understand the genetic basis of different metabolic diseases such as obesity, atherosclerosis and fatty liver among others.

Thus, with these kind of animal studies scientist will expand their ability to model human diseases more accurately, as well as directly test their theories and novel therapeutic approaches.

HYPOTHESIS & AIMS

Hypothesis

Excessive energy intake alters metabolic homeostasis and leads to a state of low-grade inflammation which has an important role in the development of non-communicable diseases such as obesity and NAFLD. These metabolic disturbances might be detected in plasma revealing new biomarkers. Moreover, the assessment of the role of chronic inflammation in animal models fed an energy surplus could suggest novel therapeutic strategies for the management of these prevalent diseases.

Aims

- ✓ To better understand the pathogenesis of NAFLD in obesity.
- ✓ To identify a non-invasive metabolite biomarker of NAFLD.
- ✓ To determine the effects of a continuous and ubiquitous overexpression of CCL2 combined with excessive energy intake.
- ✓ To evaluate the absence of CCR2 receptor in this genetic and environmental background for the development of novel therapeutic strategies.

RESULTS

STUDY 1

Mapping of the circulating metabolome reveals α-ketoglutarate as a predictor of morbid obesity-associated non-alcoholic fatty liver disease

Int J Obes (Lond) 2015; 39(2):279-87

Abstract

Background: Obesity severely affects human health, and the accompanying non-alcoholic fatty liver disease (NAFLD) is associated with high morbidity and mortality. Rapid and non-invasive methods to detect this condition may substantially improve clinical care.

Methods: We used liquid and gas chromatography–quadruple time-of-flight–mass spectrometry (LC/GC-QTOF-MS) analysis in a non-targeted metabolomics approach on the plasma from morbidly obese patients undergoing bariatric surgery to gain a comprehensive measure of metabolite levels. On the basis of these findings, we developed a method (GC-QTOF-MS) for the accurate quantification of plasma α-ketoglutarate to explore its potential as a novel biomarker for the detection of NAFLD.

Results: Plasma biochemical differences were observed between patients with and without NAFLD indicating that the accumulation of lipids in hepatocytes decreased β-oxidation energy production, reduced liver function and altered glucose metabolism. The results obtained from the plasma analysis suggest pathophysiological insights that link lipid and glucose disturbances with α-ketoglutarate. Plasma α-ketoglutarate levels are significantly increased in obese patients compared with lean controls. Among obese patients, the measurement of this metabolite differentiates between those with or without NAFLD. Data from the liver were consistent with data from plasma. Clinical utility was assessed, and the results revealed that plasma α-ketoglutarate is a fair-to-good biomarker in patients (n=230). Other common laboratory liver tests used in routine application did not favourably compare.

Conclusion: Plasma α-ketoglutarate is superior to common liver function tests in obese patients as a surrogate biomarker of NAFLD. The measurement of this biomarker may potentiate the search for a therapeutic approach, may decrease the need for liver biopsy and may be useful in the assessment of disease progression.

Introduction

Obesity severely affects human health. The number of adults with morbid obesity (that is, a body mass index (BMI) $\geq$40 kg/m^2) is increasing, and the prevalence of obesity (>30% in some countries) is unacceptably high. Moreover, the incidence of obesity is increasing among children, and obesity-associated premature mortality rivals that of smoking [1–4]. Morbidly obese patients undergoing bariatric surgery share a common metabolic background and similar environmental factors. We assume that possible genetic differences among these particular obese patients are likely negligible. Non-alcoholic fatty liver disease (NAFLD) is no longer a benign condition and is now considered an important co-morbidity in these patients [5–7]. The prognosis is pessimistic because of the risk of progressive liver disease (for example, non-alcoholic steatohepatitis, fibrosis, cirrhosis, liver failure and hepatocarcinoma) [8]. There is a great need for NAFLD biomarker discovery because it is often difficult to determine when a liver biopsy is appropriate. A significant number of individuals are asymptomatic until their liver begins to fail, at which point conventional treatments are useless.

Current laboratory tests are insufficient and unreliable for the determination of NAFLD presence. Previous estimates using common liver function tests in the plasma, such as the determination of alanine aminotransferase levels, suggest that the prevalence of NAFLD should be nearly 100% in obese patients [9]. Evaluation by proton magnetic resonance spectroscopy is expensive and is currently being refined [10], and patients of this size did not typically fit into the apparatus. However, the use of liver biopsy revealed in our patients that a significant portion did not display fatty infiltration of hepatocytes. In this study, we aimed to better understand NAFLD pathogenesis in obesity and to identify a non-invasive metabolite biomarker.

We hypothesised that the excess in energy intake alters anabolic and catabolic functions, especially in the liver, which may be detected in a plasma metabolomic profile. We used non-targeted metabolomics to compare obese patients without steatosis with their affected counterparts to identify differences between these groups. We expected numerous and low differences among the measured

metabolites, which may explain the lack of previous metabolomics-based testing for these diseases [11]. However, post hoc analysis and further quantification resulted in the qualification and verification of the plasma α-ketoglutarate concentration as a good indicator of NAFLD in obese patients.

Material and methods

Participants

Our local ethics committee approved the study protocol, and written informed consent was obtained from the participants (EPINOLS/12-03-29/3proj6). Patients fulfilling the criteria established for morbid obesity (BMI ≥40 kg/m^2), for which bariatric surgery was indicated after numerous failed attempts to lose weight using non-surgical means, were recruited from the outpatient clinic between 2006 and 2012. We first conducted a non-targeted metabolomic analysis in a limited group of patients with or without steatosis (n=15 in each group). Steatohepatitis, fibrosis and hepatocyte injury in these patients were histologically ruled out. These samples were sent to Metabolon Inc. (Durham, NC, USA) for analyses. No differences in the quality of life were observed between the groups, and the selected patients did not consume alcohol or any prescribed medication that could alter liver function, including vitamin supplements. The results of these non-targeted preliminary analyses prompted us to qualify and verify plasma α-ketoglutarate as a candidate biomarker to identify NAFLD in obese patients. For this purpose, we recruited 230 patients in which the only inclusion criterion was application for bariatric surgery, thus including a wide variety of conditions likely to be encountered in clinical practise. Blood was obtained immediately before surgery and after clinical nutrition evaluation. Portions of the liver were obtained during the surgical procedure after patient consultation to minimise risks and to limit the variability based on location of biopsy [12]. Bio-banked samples (n=54) from a group of age- and sex-matched lean, healthy controls (BMI <24 kg/m^2) [13] were used to assess differences in plasma α-ketoglutarate levels between lean and obese patients. Dietary advice and standardised overnight fasting were implemented to ensure uniformity and consistency. Plasma aliquots were anticoagulated with EDTA and were frozen at −80°C within 2 h after collection.

Clinical data and analytic measurements

Relevant data were extracted from clinical records or were obtained using standardised guidelines and routine laboratory methods [14]. The BMI was calculated as the weight in kilograms divided by the height in metres squared. Histological alterations in the liver biopsies were evaluated in sections stained with haematoxylin and eosin. The degree of steatosis was evaluated using image analysis software and was expressed as percentages (AnalySIS image software system, Soft Imaging System, Munster, Germany). Patients were considered free of steatosis when values were ≤5%. Patients with steatosis were arbitrarily classified as mild: 6-30%; moderate: 31–60%; and severe: >61% [8, 15]. Portions of the liver biopsies were also used to measure α-ketoglutarate in tissues from patients with or without steatosis (n=6 in each group). The shape and size of mitochondria were determined in portions of the liver using standard transmission electronic microscopy (n=3 in each group).

Non-targeted metabolomic platform

Specimens. Samples outsourced to Metabolon's laboratory were extracted upon arrival. The instrument and overall process variability was 4% and 9%, respectively.

Chromatography. Chromatographic conditions have been previously described [16]. In brief, the liquid chromatography–mass spectrometry (LC–MS, LC–MS^2) platform was based on a Waters ACQUITY UPLC (Waters Technologies, Milford, MA, USA) and a Thermo-Finnigan (San Jose, CA, USA) LTQ mass spectrometer, which consisted of an electrospray ionisation source and a linear ion-trap mass analyser. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion. The samples for gas chromatography/mass spectrometry (MS) analysis were derivatised and analysed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionisation. Metabolites were identified using a reference library of ~2800 standard chemical entries that included retention times, mass (m/z) and MS or MS^2 spectra.

Quantitative measurement of plasma α-ketoglutarate using gas chromatography–quadrupole time-of-flight mass spectrometry analysis

*Sample pre-treatment***.** This method was developed in our laboratory. A surrogate standard was added to maximise technical precision during the injection and recovery during the extraction procedure. We used deuterated succinic acid (Isotec Stable Isotopes, Miamisburg, OH, USA) rather than deuterated α-ketoglutarate, which readily exchanges deuterium [17]. A solution of deuterated succinic acid (25µl, 2 mg/l) was added to aliquots of plasma that were thawed on ice at 4 °C (50 µl), deproteinised with 400 µl of methanol, mixed using a vortex (2 min) and centrifuged (15000 g, 15 min, 4 °C). The supernatant was dried and stored at −80 °C. Samples were derivatised using 30 µl of methoxyamine hydrochloride in pyridine (30 mg/ml) and incubated 1.5 h at 37 °C with agitation. Then, 30 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich, Steinheim, Germany) was added with shaking and further incubated in darkness for 1 h before analysis. We found this derivatisation step to be a reproducible and robust method, independent of the matrix and without appreciable losses in yield that minimised the loss of α-ketoacids to decarboxylation [18]. A calibration curve of α-ketoglutaric acid (Fluka, St Gallen, Switzerland) was prepared (0–82.5 µM) immediately before each assay.

Chromatographic analysis. Samples (1 µl) were automatically injected onto a 7890A gas chromatograph coupled to a 7200 quadrupole time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, USA) equipped with a J&W Scientific (Folsom, CA, USA) HP5-MS column (19091S-433). Helium was used as a carrier gas at a flow rate of 1.5 ml min^{-1} in constant-flow mode. The oven programme was set at an initial temperature of 70 °C that was increased to 190 °C at a rate of 12 °C/min followed by an increase to 325 °C at a rate of 20 °C/min and a final hold at 325 °C for 3.25 min. Ionisation was performed using electronic impact with an electron energy of 70 eV and an emission intensity of 35 µA. Raw data were processed using MassHunter B.05.00 (Agilent Technologies). Plasma α-ketoglutaric acid was quantified using a target ion and was identified using qualifier ions and the retention time. The molecular weight of the derivatised molecule, retention time, quantifier and qualifier ions, relative abundance, recovery, accuracy, precision and additional pertinent

results are provided in **Supplementary Figure S1**. The limit of detection was 0.001µM. Instrumental reproducibility was 2.2%. Standard curves for the analysis were reproducible and displayed R2 values of ≥0.99, which indicated linearity over the measured concentration range; we did not detect carryover.

Statistical analysis

Significantly altered metabolites, which were corrected for multiple testing, were defined using a P-value <0.05 and a predesigned false discovery rate [19]. We performed Welch's t-tests and/or Wilcoxon's rank sum tests for pairwise comparisons. A repeated measurement analysis of variance was used in some instances. We used multivariate statistics to improve the refining and distilling of complex raw data and for pattern recognition. Random Forests is a supervised classification technique based on an ensemble of decision trees [20]. This method provides an unbiased estimate of how well one can predict sample classes in a new data set (prediction accuracy) and a selection of variables that make the largest contributions to the classification. We also used linear discriminant analysis as a classical method of classification and principal component analysis as an unsupervised data analysis, which measures the innate variation in data sets. Ingenuity pathway analysis, which is a web-based functional analysis, was used to explore biomolecular interaction networks to identify the signalling and metabolic pathways and to compare the affected pathways. Fisher's exact test was used to calculate a P-value to determine the probability of the association between the metabolites and the canonical pathway. The network score was based on the hypergeometric distribution and was calculated using the right-tailed Fisher's exact test. Logistic regression analysis and receiver operator characteristic curves described and assessed the binary classification [11, 21]. The employed statistical software included the program 'R' (http://cran.r-project.org) and the SPSS 18.0 package (IBM, Madrid, Spain).

Results

Plasma metabolites and obesity-related liver steatosis

Raw data. The baseline characteristics of the selected patients, raw data and an exhaustive list of the measured metabolites in untargeted metabolomic analyses are shown in **Supplementary Tables S1** and **S2**. Most patients were female (73%) and BMI values ranged between 43.1 and 52.1 kg/m^2. Patients with steatosis showed significantly higher plasma cholesterol and triglyceride concentrations than those without steatosis. Common liver function tests revealed higher values in patients with steatosis, but significance was only reached for γ-glutamyl transpeptidase (**Supplementary Table S1**). We identified 316 metabolites of which 38 were significantly different between groups. Finally, 19 metabolites with the highest statistical difference were chosen using more stringent conditions for further consideration (**Supplementary Figure S2**). The relative abundance of perturbations in amino acids and lipid metabolism are shown in **Figure 1a**.

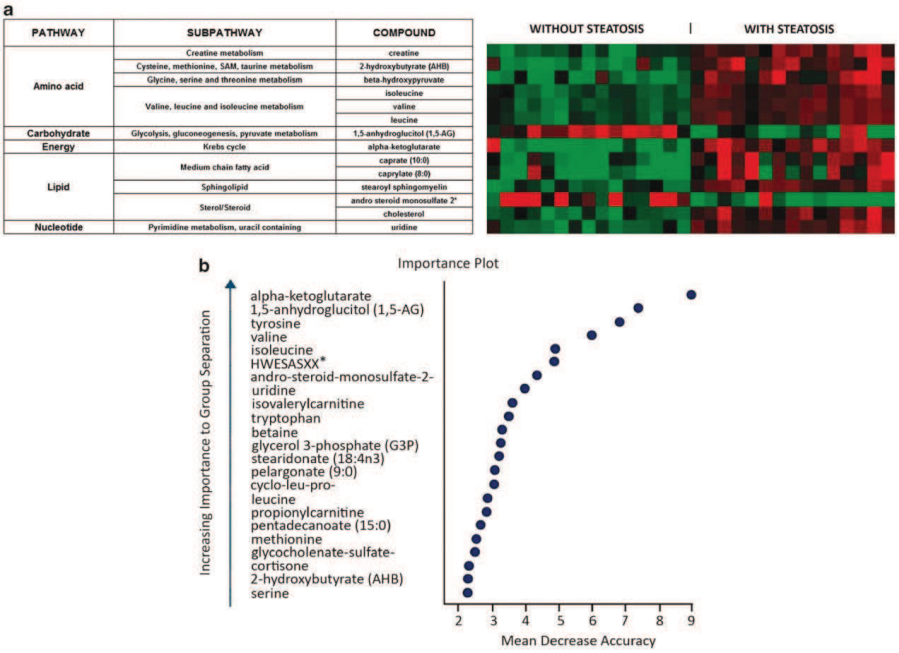


Figure 1. (**a**) Heat map of the relative plasma concentration of selected metabolites that may distinguish obese patients with or without steatosis (green, lower; red, greater). (**b**) Random (decision) Forest analysis was used as implemented in the R package software as a framework to create a large number of particular models built around the presence or absence of steatosis.

Interpretation. The citric acid cycle is the most important metabolic pathway for the energy supply and connects most metabolic pathways. The following alterations support the idea of defective liver function: 1) the levels of all three branched-chain amino acids (leucine, isoleucine and valine) were significantly increased in patients with steatosis; and 2) the levels of branched-chain keto acids (3-methyl-2-oxobutyrate, 3-methyl-2-oxovalerate and 4-methyl-2-oxopentanoate) were slightly elevated in the plasma from patients with steatosis.

Steatosis may also sequester fatty acids from β-oxidation in liver cells using an alternative energy source, as shown by significant decreased levels of 3-hydroxybutyrate and a significant increase in the concentration of plasma α-ketoglutarate and succinylcarnitine in patients with steatosis. The mechanisms of lipolysis and gluconeogenesis appear to be affected (**Supplementary Figure S3**).

Glucose metabolism is also altered in patients with steatosis. The significant decrease in the concentration of plasma 1,5-anhydroglucitol in these patients probably indicates long-term higher hyperglycaemia. In addition, hyperglycaemia-induced oxidative stress is likely in patients with steatosis, as indicated by the increased plasma levels of bradykinin and des-Arg9-bradykinin. The plasma level of the dicarboxylic acid 2-hydroxybutyrate (α–hydroxybutyrate), which is an early marker for impaired glucose regulation, was also significantly increased.

Plasma glycocholate and taurocholate concentrations were higher in patients with steatosis, which indicates that the damaged livers were not functioning properly in the uptake of these compounds. The excretion of steroids is also limited in liver steatosis because these compounds are not effectively sulphated, which was indicated by significantly lower plasma levels of sulphated steroids (that is, 4-androsten-3β, 17-β-diol disulphate 1, 4-androsten-3β, 17-β-diol disulphate 2, 5α-androstan-3β, 17β-diol disulphate, 5α-pregnan-3β, 20α-diol disulphate, pregnen-diol disulphate, pregnen steroid monosulphate, andro steroid monosulphate 2 and 21-hydroxypregnenolone disulphate).

Pattern recognition

Random Forest analysis resulted in a predictive accuracy of >80% to distinguish patients with or without steatosis and revealed plasma α-ketoglutarate as the primary differentiator in a ranked list of metabolites in order of their importance in the classification scheme (**Figure 1b**). The application of linear discriminant analysis and principal component analysis yielded similar results for group clustering and pattern recognition (**Figure 2**), and logistic regression and receiver operator characteristic analyses produced a list of possible biomarkers.

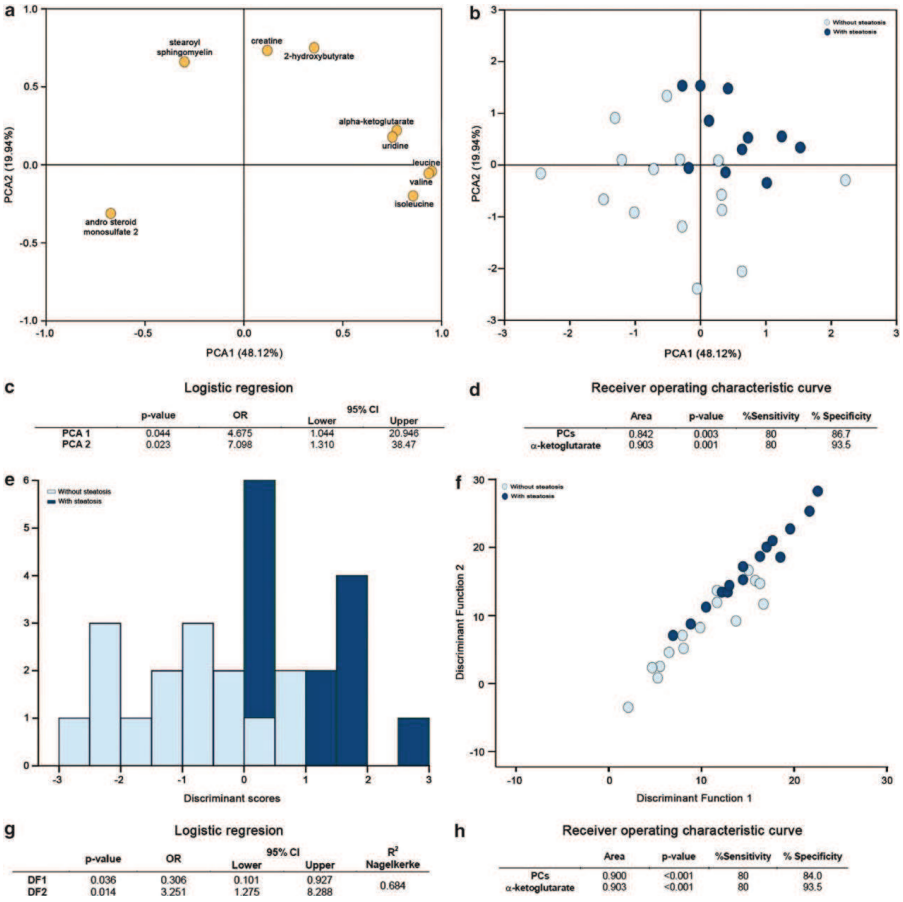


Figure 2. Model plots constructed using principal component analysis (**a** and **b**) and linear discrimination analysis (**e** and **f**). Logistic regression analysis and receiver operator characteristic curves indicated the presence of a pattern for the selection of candidate biomarkers and group clustering. As shown in the calculations (**c**, **d**, **g** and **h**), plasma α-ketoglutarate was the most qualified for this purpose.

Metabolite interaction networks

We uploaded the metabolite lists (with kyoto encyclopedia of genes and genomes IDs) onto an ingenuity pathway analysis server to identify the biological pathways and functions of the biomolecules of interest. The top-associated network functions were limited to scores of >24.

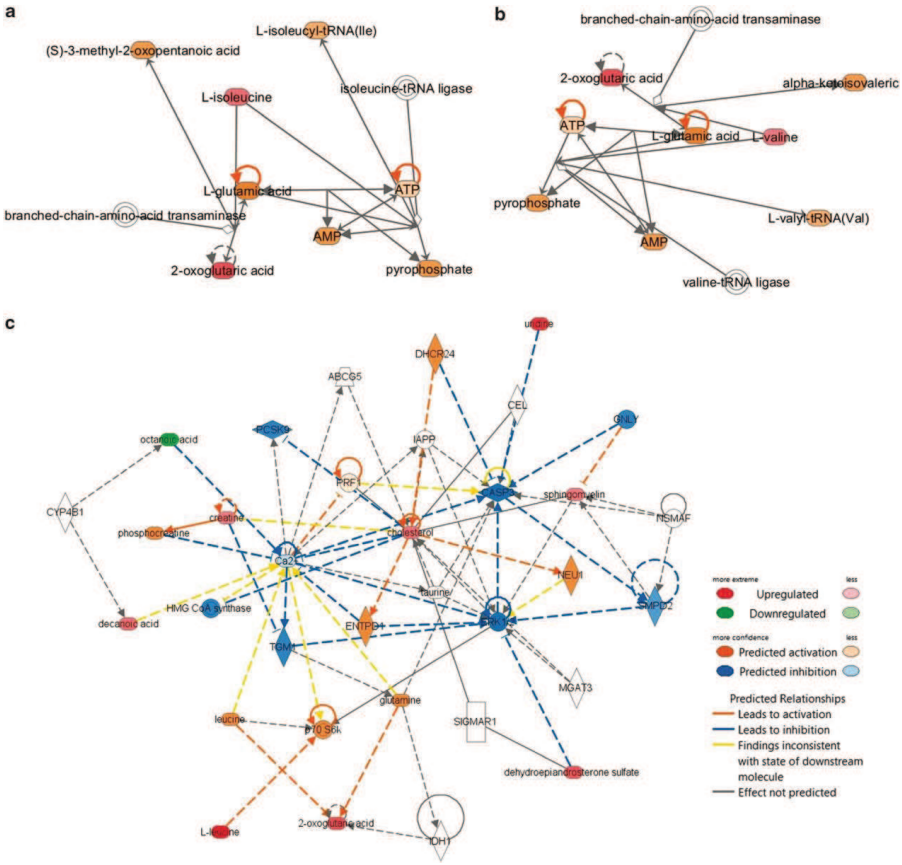


Figure 3. Interaction and regulatory networks associated with steatosis in morbidly obese patients as identified using the ingenuity pathway analysis. Plots are depicted for the top-associated network functions (score >24, **a** and **b**) and top canonical pathway (**c**).

We highlighted networks that were similar and that shared identical functions, that is, lipid metabolism, amino-acid metabolism, molecular transport and small molecule biochemistry. Only tRNA charging (P=0.0007) and isoleucine degradation (P=0.0009) were significantly different among the top canonical pathways. Interactions and regulatory networks are depicted in **Figure 3**, which integrates most of the altered

metabolites based on their biochemical relationships in obese patients with steatosis.

These plots indicate that L-glutamic acid upregulates α-ketoglutarate (2-oxoglutaric acid) and suggest a role of branched-chain amino-acid transaminase. Predicted disturbances, which probably result in α-ketoglutarate accumulation in the plasma, are depicted in **Supplementary Figure S4**.

These results indicate a role for mitochondrial dysfunction. Our preliminary findings also indicate that in patients without steatosis the mean diameter of the mitochondria was slightly lower, the matrix was more electron dense and the crests were more visible compared with patients with steatosis. More importantly, toroidal mitochondria were abundant and only observed in non-steatotic patients and there was a significantly higher accumulation of autophagosomes in patients with steatosis. It is therefore plausible that the detection of perturbations in the citric acid cycle (CAC) may facilitate the evaluation of mitochondrial functions in physiology and disease [22].

Plasma α-ketoglutarate as a biomarker

The obtained results identified and qualified plasma α-ketoglutarate as a diagnostic biomarker [23]. We calculated, with 95% confidence, that the true area under the curve of the reported receiver operator characteristic curve for plasma α-ketoglutarate ranged from 0.90 to 0.96 with a specificity of 0.93 at a fixed sensitivity of 0.8 (**Figure 2**). However, these results were calculated using a low number of carefully selected patients. To decrease uncertainty or margin of error in the relevance of these measurements, we extended the analysis to a broad range of morbidly obese patients (**Table 1**) in which the degree of steatosis was widely distributed.

Table 1. Selected clinical characteristics and laboratory variables in obese patients segregated by the degree of steatosis

	Without steatosis (n = 76)	*Mild steatosis (n = 86)*	*Moderate steatosis (n = 52)*	*Severe steatosis (n = 16)*
Clinical characteristics				
Male, *n* (%)	8 (10.5)	12 (13.9)	10 (19.2)	4(25.0)
BMI, kg m^{-2}	47.0 (44.1–51.0)	45.9 (43.2–49.5)	48.1 (44.1–51.1)	44.5 (42.9–47.8)
Laboratory variables[a]				
Total cholesterol, mmol l^{-1}	4.0 (3.4–4.9)	4.5 (3.9–5.4)	4.9 (4.2–5.5)[b]	4.2 (3.5–5.6)
HDL cholesterol, mmol l^{-1}	0.9 (0.7–1.1)	0.9 (0.7–1.0)	0.9 (0.8–3.4)	0.8 (0.5–1.1)
LDL cholesterol, mmol l^{-1}	2.4 (1.9–3.0)	2.7 (2.6–3.3)	3.0 (2.3–3.4)	2.6 (1.9–3.0)
Triglycerides, mmol l^{-1}	1.6 (1.2–1.9)	2.2 (1.6–2.7)[c]	2.0 (1.7–3.0)[b]	2.5 (1.6–3.0)
NEFAs, mEq l^{-1}	1.1 (1.0–1.4)	1.2 (1.0–1.5)	1.1 (0.8–1.6)	1.0 (0.9–1.7)
Glucose, mmol l^{-1}	7.1 (5.9–8.4)	7.4 (6.2–10.0)	7.9 (7.0–9.8)[b]	7.6 (6.7–13.4)
Insulin, pmol l^{-1}	74.6 (41.9–117.6)	79.3 (42.5–122.3)	82.7 (56.8–137.3)	91.5 (40.8–172.2)
HOMA-IR	3.3 (1.9–6.1)	3.9 (2.6–6.1)	3.9 (2.7–8.0)	3.61 (2.02–11.6)
AST, μKat l^{-1}	0.5 (0.3–0.8)	0.5 (0.4–0.7)	0.9 (0.6–1.1)[b,d]	1.1 (0.6–2.0)[e,f]
ALT, μKat l^{-1}	0.5 (0.4–0.6)	0.4 (0.4–0.7)	0.9 (0.5–1.2)[b,d]	0.6 (0.4–1.9)
GGT, μKat l^{-1}	0.2 (0.2–0.3)	0.3 (0.2–0.8)	0.4 (0.3–0.6)[b]	0.4 (0.3–0.8)[e]
LDH, μKat l^{-1}	2.5 (2.2–2.9)	2.7 (2.3–3.3)	3.2 (2.6–4.0)[b,d]	4.0 (2.8–6.7)[e,f]
Total bilirubin, mmol l^{-1}	7.0 (5.0–10.0)	8.0 (5.3–11.8)	8.5 (4.5–11.0)	9.5 (5.8–20.8)
Leptin, ng ml^{-1}	68.5 (55.4–97.5)	73.7 (51.9–97.5)	83.2 (59.5–114.1)	87.8 (75.2–185.4)
Adiponectin, μg ml^{-1}	3.6 (2.4–4.3)	2.6 (1.8–4.2)	2.4 (1.8–3.9)	2.2 (1.6–3.4)
CRP, mg l^{-1}	6.6 (5.0–10.9)	9.2 (6.7–13.2)	12.7 (8.1–18.5)[b]	11.4 (5.9–14.4)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, γ-glutamyl transpeptidase; HOMA-IR, homoeostasis model assessment-estimated insulin resistance; LDH, lactate dehydrogenase; NEFAs, non-esterified fatty acids. Significant differences (at least $P < 0.05$) in comparisons. [a]Values are given as median (interquartile range). [b]Without steatosis vs moderate steatosis. [c]Without steatosis vs mild steatosis. [d]Mild steatosis vs moderate steatosis. [e]Without steatosis vs severe steatosis. [f]Mild steatosis vs severe steatosis.

Notably, steatosis was predominantly mild, and the other liver alterations were primarily benign (**Supplementary Figure S5**). Approximately 25% of our patients were insulin sensitive, but this condition was unrelated to the presence of NAFLD. Plasma α-ketoglutarate was slightly but significantly associated with homoeostasis model assessment values (ρ=0.25, P=0.01).

We calculated that at least 115 cases would be required to determine whether plasma α-ketoglutarate outperforms other commonly used biomarkers using an inferential approach [24]. These minimum requirements were increased to 230 cases to ensure validity in a target group in which positive and negative outcomes are not equally distributed and in which the addition of fibrosis and/or inflammation may complicate the interpretation.

A significant association between circulating triglycerides and steatosis was observed in these patients (ρ=0.42, P<0.001; see also **Table 1**), which is similar to the association observed with plasma α-ketoglutarate levels (ρ=0.49, P<0.001). Lean controls exhibited significantly (P<0.001) lower (1.1 µM (0.82–1.37)) plasma α-ketoglutarate levels than obese patients (7.5 µM (5.5–10.8) without overlap (median (interquartile range)). The values were also significantly higher in obese patients with steatosis than in those without steatosis, indicating the potential to discriminate between the different stages in the progression of these conditions (**Figure 4**). These differences were also observed in liver tissue; in samples without steatosis, α-ketoglutarate concentration was significantly (P=0.017) lower (22.1 µM per 100mg dry weight (11.3–31.3)) than in those with steatosis (57.8 µM per 100 mg dry weight (32.8–63.1)).

Among the measured variables, only the plasma α-ketoglutarate concentration exhibited significant agreement with the degree of steatosis, which may differentiate between mild, moderate and severe steatosis. Although comparisons were significant, data are not shown for severe steatosis because the number of patients was considered too low to yield relevance. The area under the curve of the receiver operator characteristic curves exhibited a fair-to-good clinical utility (**Figure 4**).

Other common laboratory liver function tests failed to discriminate between patients with or without NAFLD. Interestingly, the clinically considered 'gold standard', alanine aminotransferase, exhibited the worst performance. In contrast, γ-glutamyl transpeptidase performed relatively well (**Supplementary Figures S6** and **S7**).

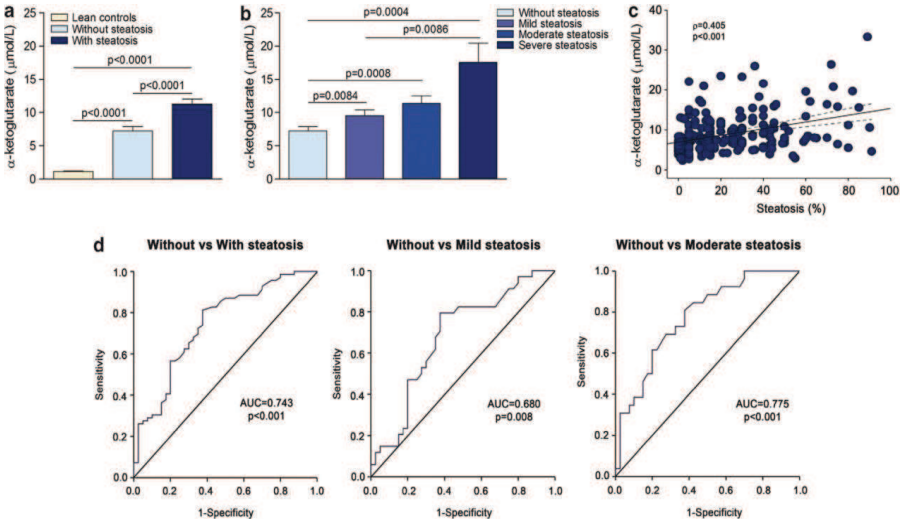
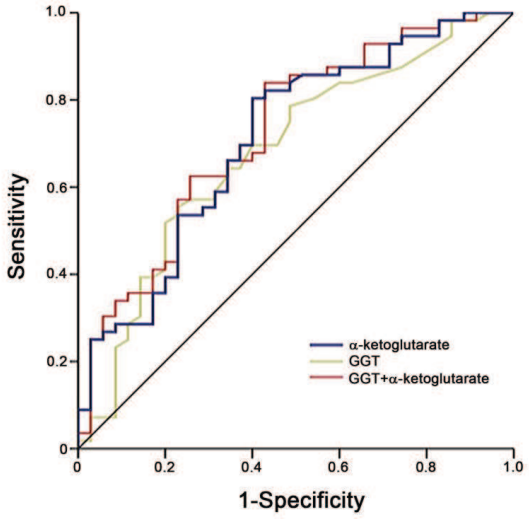


Figure 4. The mean plasma α-ketoglutarate concentration was significantly lower in lean controls and patients without steatosis (**a**) and displayed significant agreement with the degree of steatosis (**b** and **c**). The area under the curve of the receiver operator characteristic curves were significant, and clinical utility was approximately fair to good; for this purpose, the number of patients with severe steatosis was considered too low to yield relevance (**d**).

We subsequently constructed multivariate models that combined the values of the available biomarkers, but the performance was lower than plasma α- ketoglutarate alone. Conversely, the addition of plasma α-ketoglutarate to any of these models improved the performance (**Figure 5**). The optimal clinically useful threshold (that is, the plasma concentration) rather than the mathematically optimal threshold is difficult to assign.

The choice is highly dependent on the intended use. Ideally, all patients with steatosis should be correctly classified, but this may come at the cost that some patients may be incorrectly classified as free of steatosis. For example, 8 or 4 μM (which represent two mathematically possible candidates) yield a similar positive predictive value (>80%), but 8 μM provides high specificity (>85%) and 4 μM provides high sensitivity (>97%).



Variable	Area	Error	P-value	95% CI		Sensitivity (%)	Specificity (%)
				Lower	Upper		
α-Ketoglutarate	0.743	0.050	**<0.001**	0.645	0.840	80.0	62.5
GGT	0.685	0.058	**0.003**	0.571	0.799	80.0	47.5
GGT+α-Ketoglutarate	0.727	0.055	**<0.001**	0.619	0.834	80.0	57.0

Figure 5. Multimetabolite biomarker models should provide more information than a single biochemical measurement, as assessed using receiver operator characteristic curves. However, predictive scores from multivariate models that combined several variables were not superior to plasma α–ketoglutarate alone, as exemplified by the combination with plasma γ-glutamyl transpeptidase (GGT), which displayed the best performance of commonly used laboratory biomarkers.

Discussion

There is currently no clinically useful plasma surrogate for the assessment of hepatic steatosis in obesity. Multivariate metabolomics analyses provide meaningful information owing to the simultaneous global assessment of hundreds of endogenous metabolites in a biological sample. To perform studies in this context, it is important to ensure the maintenance of pre-analytical aspects. Similar nutritional status and similar food intake are necessary because dietary factors have an important role as a causative factor of NAFLD [25–27]. As we predicted, inflammation does not appear to be a prominent factor, [28, 29] but neither obesity nor NAFLD are simple, monogenic disorders. Therefore, we chose clinically controlled bariatric patients who exhibited a similarly high BMI.

The accumulation of lipids in hepatocytes induced a relative energy deficit, reduced liver function and disturbed insulin resistance, which is apparently accompanied by an acquired and reversible mitochondrial dysfunction [30]. Our data also partially support the importance of a newly described mechanism (lipophagy) that links lipolysis and regulation of intracellular lipid stores [31]. Notably, the deficit in sulphonation that we have observed in NAFLD patients may be clinically relevant. This is because phase II drug-metabolising enzymes maintain cellular homoeostasis via the metabolism of several endogenous molecules that may facilitate metabolic disorders and may result in the improper management of xenobiotics and endobiotics [32, 33]. Therefore, the search for NAFLD diagnostics in obesity should focus on mechanisms of energy homoeostasis, mitochondrial biogenesis, fatty acid oxidation and glucose metabolism, which may require new strategies [34]. The results obtained from the plasma analysis are consistent with those obtained in the livers of different animal models and human studies, which demonstrate that steatosis is the cause or consequence of disturbances in hepatic lipid and glucose metabolism that decreases the ability to obtain energy [8, 25, 35, 36]. The metabolic imbalance caused by obesity and NAFLD affects mitochondrial metabolism and metabolic pathways, which are important for recycling α-ketoglutarate into and out of the mitochondrion and allow for the continuous production of intracellular messengers [37]. Finally, it has been recently described in a model of mitochondrial dysfunction (PINK1 deficiency) that increased expression of α-ketoglutarate is the most prominent and early effect, probably representing a compensatory mechanism [38]. Monitoring mitochondrial function via the quantitative evaluation of mitochondrial metabolite abundances may be an important new avenue of research in obesity-associated NAFLD. This is mainly because CAC metabolites have a central role in catabolic and anabolic functions (that is, it is amphibolic in nature). As in other non-communicable diseases, it is most likely adequate to adopt the view that steatosis is governed by a pivotal regulatory role of metabolic reprogramming in cell fate decisions [39, 40].

Plasma α-ketoglutarate levels may distinguish lean controls from obese patients with a "predictive accuracy" of 100% and predict obese patients with or without NAFLD better than commonly used biomarkers. This result supports the potential clinical utility of plasma α-ketoglutarate levels. However, additional validation in other patients from an identical target population is required. Preliminary results in ongoing validation studies suggest that this novel biomarker deserves further evaluation and development and that this biomarker should be added to clinical practise. In addition, pilot studies indicate the usefulness of performing serial measurements in the same patient, which do not require high specificity or sensitivity, to monitor disease progression and/or the response to treatment. The major limitation of our results is the confinement to morbid obesity. We believe this is a blood test that provides a solution for a major unmet clinical need but implementation of a new biomarker is difficult and requires validation. Regardless of the attractiveness of plasma α-ketoglutarate as a biomarker, the high costs of validation hamper the introduction of new biomarkers and therefore commercial considerations may ultimately determine the clinical use [41]. However, commonly used laboratory tests do not appropriately guide clinical decisions. Imaging studies are also not possible for patients of this size, and patients do not readily accept more invasive studies.

We also confirmed the potential of metabolomics to influence patient care. Metabolomics is designed to delineate biological processes, and the selection of metabolites for development as clinical biomarkers should be performed a priori rather than post hoc. Our results indicate that this approach is not necessarily true if compound quantification follows metabolomics, which is a strategy that is currently feasible and is not time consuming. This study successfully utilised a metabolomics approach to provide new insights into the consequences of NAFLD in obese patients and supports the possible translational relevance of plasma α-ketoglutarate as a biomarker of a condition that carries an enormous burden of disease.

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STUDY 2

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation

Mediators Inflamm. 2013; 2013:953841

Abstract

Excessive energy management leads to low-grade, chronic inflammation, which is a significant factor predicting non-communicable diseases. In turn, inflammation, oxidation, and metabolism are associated with the course of these diseases; mitochondrial dysfunction seems to be at the crossroads of mutual relationships. The migration of immune cells during inflammation is governed by the interaction between chemokines and chemokine receptors. Chemokines, especially C-C-chemokine ligand 2 (CCL2), have a variety of additional functions that are involved in the maintenance of normal metabolism. It is our hypothesis that a ubiquitous and continuous secretion of CCL2 may represent an animal model of low-grade chronic inflammation that, in the presence of an energy surplus, could help to ascertain the afore-mentioned relationships and/or to search for specific therapeutic approaches. Here, we present preliminary data on a mouse model created by using targeted gene knock-in technology to integrate an additional copy of the *CCl2* gene in the Gt(ROSA)26Sor locus of the mouse genome via homologous recombination in embryonic stem cells. Short- term dietary manipulations were assessed and the findings include metabolic disturbances, premature death, and the manipulation of macrophage plasticity and autophagy. These results raise a number of mechanistic questions for future study.

Introduction

Excessive energy intake is a part of the current human lifestyle that leads to a state of chronic systemic low-grade inflammation, which is thought to play a role in the development of atherosclerosis, cancer, and other non-communicable diseases. At the same time, it is also plausible that the long- term consequences of prolonged inflammation exacerbate the deleterious effects of continuous nutrient surplus [1–3]. The immune system and metabolism are closely interconnected [4, 5]. During inflammation, the whole body is under metabolic stress, and energy excess management could compromise the relationships among metabolism, oxidation, and inflammation. We reasoned that searching for an adequate animal model [6] might allow us to better understand disease pathogenesis.

Chemokines are promising candidates for the design of such a model. Some of the functions of chemokines are associated with the migration of immune cells, and chemokines are important for the correct functioning of metabolism. In humans, C-C chemokine ligand 2 (CCL2; formerly referred as MCP-1 or monocyte chemoattractant protein-1) could be a marker of inflammation; it is overexpressed in non-communicable diseases and is involved in a variety of metabolic functions [7]. Actually, CCL2 modifies lipid and glucose metabolism and contributes to insulin resistance and hepatic steatosis [8–11]. Of note, circulating chemokines cause and maintain metabolic disturbances that may be reversed by anti-inflammatory drugs, and the role of chemokines is likely a causal and predisposing factor [12, 13]. Rather than local overexpression [14–17], it is now recognized that CCL2 protein and mRNA are expressed in the vast majority of tissues, suggesting both a systemic production and the ability to respond in situ to inflammatory stimuli [18, 19].

Therefore, we hypothesized that challenging an animal model that systemically overexpresses CCL2 with diets rich in fat and cholesterol could help to assess the role of chronic inflammation in response to excessive energy intake. We then proceeded to integrate a copy of the *Ccl2* gene in the Gt(ROSA)26Sor (commonly referred to as ROSA26) locus of the mouse genome via homologous recombination in embryonic stem cells (ES) to generate targeted transgenic mice [20-22] that overexpress CCL2 in all tissues. Preliminary data are promising and suggest a number of mechanistic questions for future study.

Material and methods

Animal Handling

All procedures and experimental protocols were examined and approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili. Basic protocols for tissue collection, diets, allocation concealment and metabolic assessment of the mice have been already described in detail [6, 18, 23]. Strains were backcrossed >10 generations to C57BL/6J mice and maintained homozygously. Littermates without mutations were used as controls (WT). We also provide data from knockouts (KO) of CCL2 (conveniently backcrossed), which were

purchased from the Jackson Laboratory (Sacramento, CA). Dietary experiments began at 10 weeks of age, when all strains display similar phenotypes. To avoid possible effects of immature adipocyte modelling, most results were obtained in different groups after 6 or 14 weeks of treatment (16 and 24 weeks old, resp.). To explore dietary effects, mice from each group were fed either chow (Teklad rodent diet; Harlan, Barcelona, Spain) or a high-fat diet (FuttermittelfürMaüse; SSniff spezial diäten, Soest, Deutschland) and caged indefinitely under supervision. The breeding of all experimental populations was performed in our own facilities, and the progenies were maintained under close surveillance. The animals were not kept under germ-free conditions.

Targeted Transgenic (TG) Mice

The transgenic model was generated via a gene targeted inducible knock-in (KI), that is, a line with a duplicated gene, approach using standard methods and proprietary technology from Ozgene (Bentley, WA, Australia). The mRNA sequence corresponding to the mouse *Ccl2* gene (NM_011333 and ENSMUSG00000035385) is located on chromosome 11. The gene has 3 exons spread over approximately 3 Kb. The gene fragment was obtained from C57BL/6 genomic DNA (PCR primers AGCAAGATGATCCCAATGAGTAGGC and GAGGTGGTTGTGGAAAAGGTAGTGG) to be inserted by gene targeting into the ROSA26 locus. Upstream regulatory elements are important in the transcriptional regulation of *Ccl2* gene. Human ubiquitin promoter (Ubic) was chosen for the transgene to produce a high-level of expression. A loxP-flanked STOP cassette prevents the transcription of the gene following the UbiC promoter (See **Figure 1** and **Supplementary Materials S1** and **S2** available online at http://dx.doi.org/10.1155/2013/953841). The STOP cassette can be removed using Cre recombinase. PGK-Neo-SD-IS, a selection cassette, is inserted downstream of the *Ccl2* gene to enrich homologous recombination events. The ROSA26 locus is conserved between mice and humans. The location is autosomal (chromosome 6) and is actively transcribed in most tissues (**Figure 1**). Moreover, epigenetic inactivation is unlikely [21, 24–26].

The combination of gene targeting and ES cell technology exploiting homologous recombination provides advantages over other techniques [27–31] (**Supplementary Material S3**). Mice are available upon request.

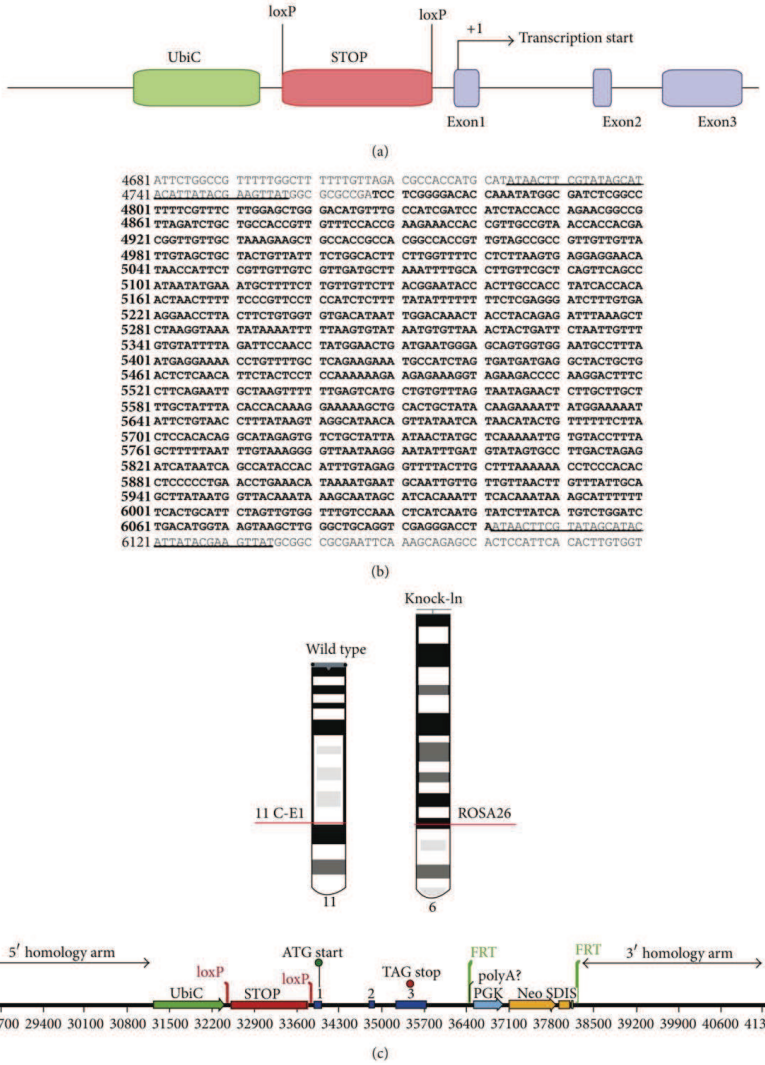


Figure 1. A STOP sequence flanked by loxP sites was inserted between the Ubiquitin promoter and the mouse Ccl2 gene (a). The sequences of both the STOP cassette (bold) and the loxP sites (underlined) are shown later (b). The wild-type allele for *Ccl2* gene is located in the region 11 C-E1 of chromosome 11 and the transgenic vector (bottom) is inserted in the ROSA26 locus of chromosome 6 (c). The procedure is designed to avoid chromosomal instabilities.

Immunopathology Studies and Assessment of Liver Steatosis

Portions of organs and tissues were either frozen in nitrogen or fixed in 4% phosphate-buffered formalin for 24 h at room temperature, washed twice with water, stored in 70% ethanol at 4 ºC, and embedded in paraffin for histological analyses. Primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and Serotec (Oxford, UK) [18, 32]. Detection was performed with the ABC peroxidase system (Vector, Burlingame, CA) using DAB (Dako, Glostrup, Denmark) as the substrate. To assess specificity, primary antibodies were omitted in the controls. Liver steatosis was assessed as previously described [6].

Laboratory Measurements

We measured murine CCL2 in plasma, serum, and tissues by ELISA (Peprotech, London, UK), according to the instructions of the manufacturer. Recombinant human CCL2 antigen was used as the calibrator for assay standardisation, and we found weak cross-reactivity with other chemokines, especially CCL7. The intraassay coefficients of variation were <3.2%, and the interassay of variation was <9.1%. Other biochemical measurements were performed in automated analysers using commercially available reagents as described [6, 33]. Selected tissues were homogenised using the Precellys 24 system (Izasa, Barcelona, Spain) with prefilled bead tubes in the buffer of choice. Fractions of the homogenised liver were immunoblotted as described [34], using antibodies and reagents from Santa Cruz Biotechnology (Heidelberg, Germany).

Transmission Electron Microscopy

Small pieces of the liver were immediately fixed in a 2% glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.4. Samples were then post-fixed in 1% osmium tetroxide (OsO_4) for 2 h and dehydrated in sequential steps of acetone prior to impregnation in increasing concentrations of the resin in acetone over a 24 h period. Semithin sections (500nm) were stained with 1% toluidine blue. Ultrathin sections (70 nm) were subsequently cut using a diamond knife, double-stained with uranyl

acetate and lead citrate, and examined using a transmission electron microscope (Hitachi, Tokyo, Japan).

Characterisation of Mouse Bone Marrow-Derived Macrophages

The methods were performed as previously described [35]. Bone marrow cells were isolated by removing leg bones from WT and TG mice (aged 10 weeks) and were cultured for 24 hours. Floating cells were removed, and the remaining attached cells were analysed. Cells were further cultured in DMEM supplemented with 10% inactivated foetal calf serum, 50 μM beta-mercaptoethanol, and 1000 U/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF) or 25 ng/mL human macrophage colony- stimulating factor (M-CSF) (ImmunoTools, Friesoythe, Germany) to provide polarised activation of cells into M1 and M2 as a simplified descriptor of their functional plasticity. To assess the effect of activation, macrophages were treated with 100 ng/mL *E. coli* 055:B5 lipopolysaccharide (LPS) for 24 hours and were compared with the respective untreated controls. After this treatment, supernatants from M1 (GM-CSF) and M2 (M-CSF) macrophages were tested for the presence of CCL2, tumour necrosis factor-α (TNFα), and interleukin 10 (IL-10) using ELISA (BioLegend, Inc., Madrid, Spain). Total RNA was extracted using the RNeasy kit (Qiagen, Barcelona, Spain) and was retrotranscribed using the Reverse Transcription System kit (Applied Biosystems; Invitrogen, Barcelona, Spain). Oligonucleotides for selected genes were designed according to the Roche so ware for quantitative real-time PCR (Universal Probe Roche library), which was performed using a LightCycler 480 (Roche Diagnostics, Barcelona, Spain). The assays were performed in triplicate, and the results normalised according to the expression level of TATA-binding protein mRNA. C-C chemokine receptor type 2 (CCR2 or CD192), TNFα, inhibin beta A (INHBA), inducible nitric oxide synthase (iNOS), C-C chemokine receptor type 7 (CCR7), and Egl nine homolog 3 (EGLN3) were chosen as M1 markers. Arginase (ARG), EMR1/F4/80, insulin growth factor-1 (IGF1), IL-10, the mannose receptor CD206, and growth arrest-specific 6 (GAS6) were chosen as M2 markers.

Statistical Analyses

The normality of the distributions was assessed using the Kolmogorov-Smirnov method. Variables were compared using Mann-Whitney tests or Kruskal-Wallis one-way analysis adjusted for multiple testing. Unless otherwise indicated, the values in the figures represent the mean and SEM obtained in groups of 8 mice. The χ^2 test was used to compare categorical variables. For all measurements, we used either SPSS (SSPS Inc., Chicago, IL) or GraphPad Prism software (http://www.graphpad.com/scientic-soware/prism/).

Results

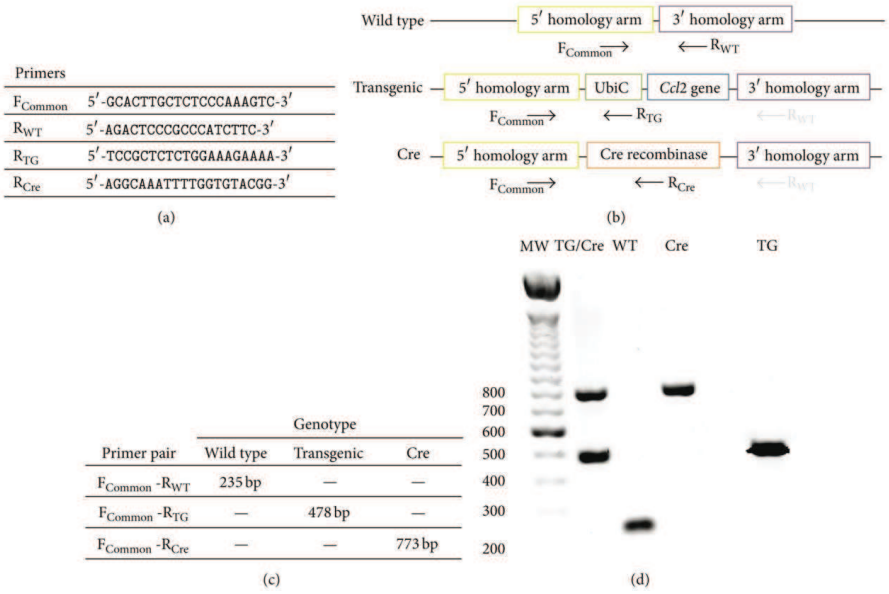
Targeted Transgenic Mice Do Not Display Physical Abnormalities

The resulting mice for the targeted mutation are viable, fertile, and normal in size and weight. The animals do not display apparent behavioural or reproductive defects. The transgene insertion of a single copy occurs at a defined site, which allows for easy genotyping (**Figure 2**) and eliminates possible instabilities, independent segregation during breeding, and unpredictable positions in the chromosomes. An additional advantage of this strategy is the Cre/lox recombination system that facilitates tissue-specific overexpression. The Ubic is conditioned by an Lox-Stop-Lox (LSL) element that is activated by Cre-mediated excision using the appropriate, tissue-specific Cre strain.

Transgenic Mice Overexpress CCL2 in Selected Tissues, and Circulating Protein Is Increased with respect to Controls

Consistently, transgenic mice displayed more CCL2 protein in all tissues examined with respect to WT animals. The differences increased with age, and there were minor relative differences among tissues (**Figure 3**). We confirm that CCL2 was immunologically detected in all selected tissues of the transgenic mice. The CCL2 mRNA expression in the transgenic mice was also higher in different types of cells with respect to WT mice. The amount of CCL2 was higher after the designed period of exposure to a diet with a high fat content. Of note, the serum and plasma CCL2 were also higher in transgenic mice than in WT mice, which is most likely caused by CCL2 secretion by multiple tissues. In accordance with previous observations, the

plasma concentrations differed from the serum concentration. The differences are likely caused by coagulation and handling, but the differences were not statistically significant in transgenic mice. Notably, CCL2 was also detected in KO mice, but with less intensity. This is most likely due to quantitatively minor cross reactivity, as described in the methods.



Primer pair	Genotype: Wild type	Transgenic	Cre
F_{Common} -R_{WT}	235 bp	—	—
F_{Common} -R_{TG}	—	478 bp	—
F_{Common} -R_{Cre}	—	—	773 bp

(c)

Figure 2. Simplified strategy for genotyping that includes the sequence of each primer (a), the reaction proposed for each primer (b), and the expected PCR products for each strain (c). The method is designed for the concomitant use of all primers and a representative gel is shown in (d).

Dietary Factors Influence Body Weight and Adipocyte Size

When mice were fed a regular chow diet, we did not observe significant differences in body weight increase among groups. The cumulative food intake was identical for the three strains examined. In contrast, when fed a high fat diet, both transgenic animals and WT animals developed obesity. Of note, the C57BL/6J male mouse is a commonly used model of diet-induced obesity [36]. The effect of CCL2 overexpression was apparent immediately after the ingestion of the high calorie diet, and the weight increased more rapidly than in WT mice. The absence of CCL2, however, protected the KO mice from excessive weight gain. The lack of significant differences in the food intake excluded any effect of CCL2 on appetite (**Figure 4**).

Overexpression of CCL2 also increased the size of the adipocytes. Data are presented for epididymal adipose tissue (**Figure 5**), but the effect was similar in other adipose tissues. The adipocyte size was significantly higher in CCL2 transgenic animals compared with WT and KO animals fed with both diets, but the difference was higher when mice were fed a diet with a high caloric content. When different types of adipose tissue were weighed, we found that the mice fed a chow diet showed no significant differences between the strains, with the possible exception of inguinal tissue. Conversely, the addition of fat to the diet resulted in a significant increase in the weight of white adipose tissue from other depots in mice with CCL2 overexpression. Notably, there was no effect on the weight of brown adipose tissue (**Figure 6** and **Supplementary Material S4**). However, these differences among groups in adipose tissues weight disappeared when mice were fed with a high fat diet for 14 weeks. These results are probably indicating an already reported effect of adipose tissue remodelling on the consequences of high-fat dietary intake [37] (**Supplementary Material S5**).

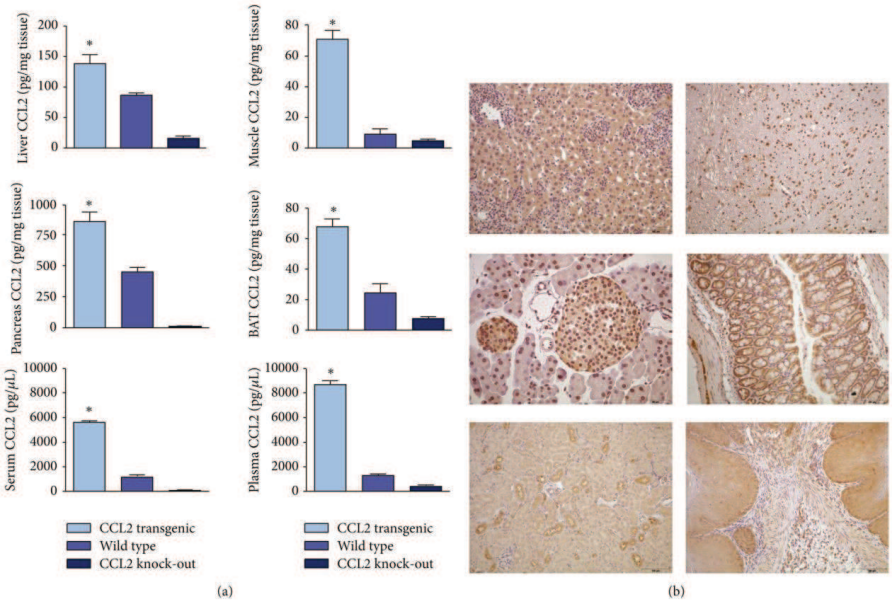


Figure 3. Overexpression of CCL2 with respect to wild type and knockout was observed in all selected tissues (extracts) in the transgenic mice as measured by ELISA. Differences were also observed in plasma and serum and there was cross-reactivity with similar chemokines that could explain the detection of CCL2 in KO mice (a). CCL2 was also detected by immunochemistry in different types of cells (b). $^{*}P<0.005$; Micrographs in the left column are representative for liver, pancreas, and kidney. Those in the right column were for brain, intestine, and stomach.

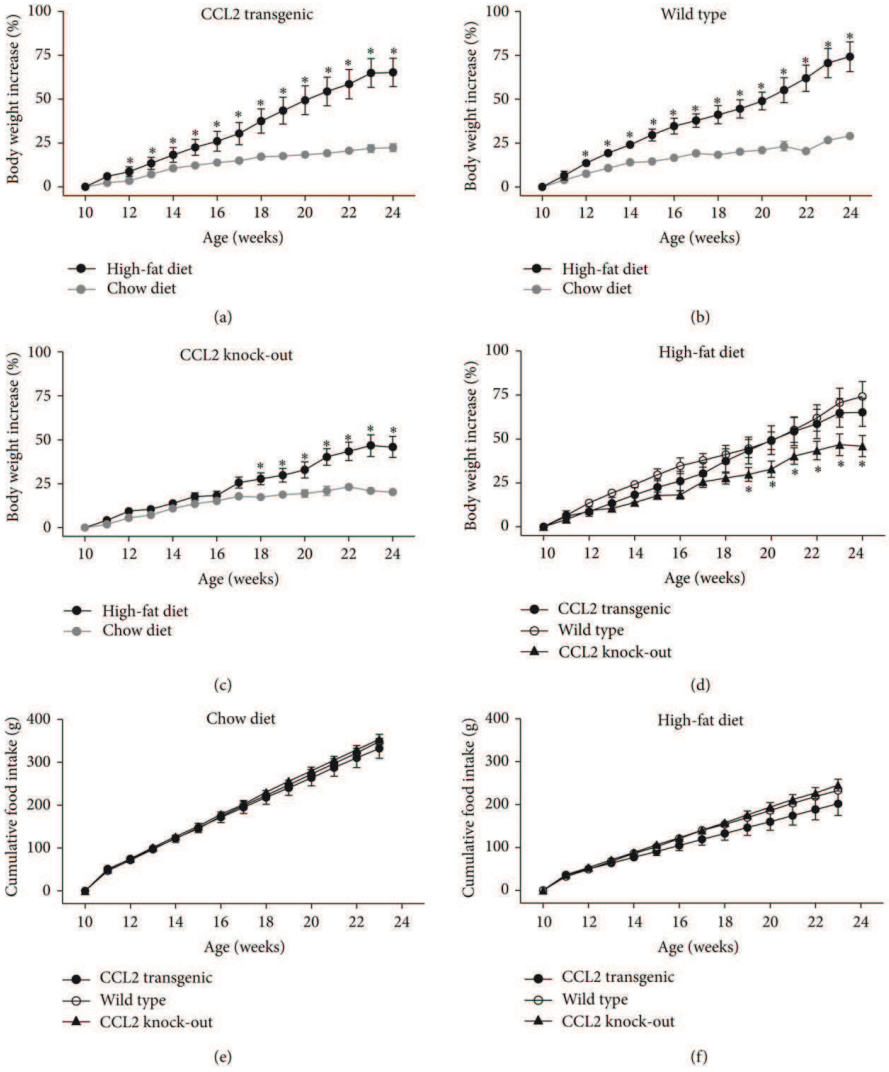


Figure 4. The effect of high-fat diet in body weight increase was evident in transgenic and wild-type mice ((a), (b)), but the different increase was immediate after dietary manipulation in transgenic. This effect was negligible in knockout mice (c). The combination of these effects with high-fat diet (d) shows similar results to facilitate comparison. These findings are not due to differences in the cumulative food intake ((e), (f)) indicating that CCL2 probably has no effect on appetite. $^{*}P<0.05$.

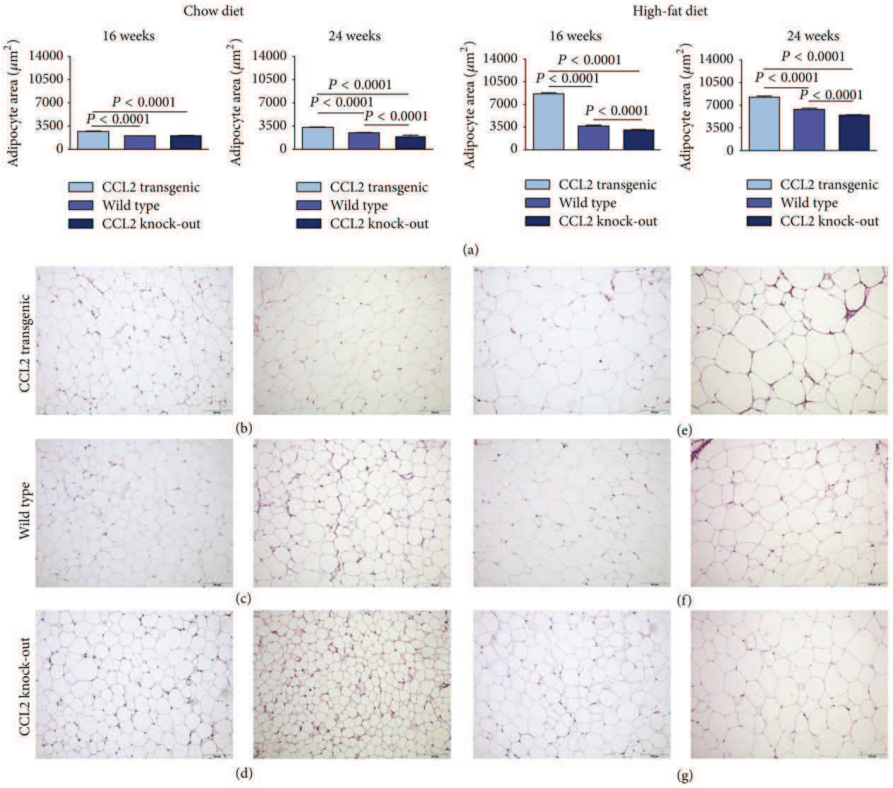


Figure 5. The size of the adipocytes was significantly higher in transgenic mice than in wild-type and knockout mice and the effect was observed with both dietary interventions regardless of the duration of the dietary treatment (6 or 14 weeks) (a) but it was more intense when mice were fed a high-fat diet. For clarity, values are indicated only for adipocytes in epididymal white adipose tissue. Representative micrographs are shown for transgenic, wild-type, and knockout animals ((b), (c) and (d), resp.) when fed a chow diet and for the corresponding animals fed a high-fat diet ((e), (f), (g)) at 16 and 24 weeks' old.

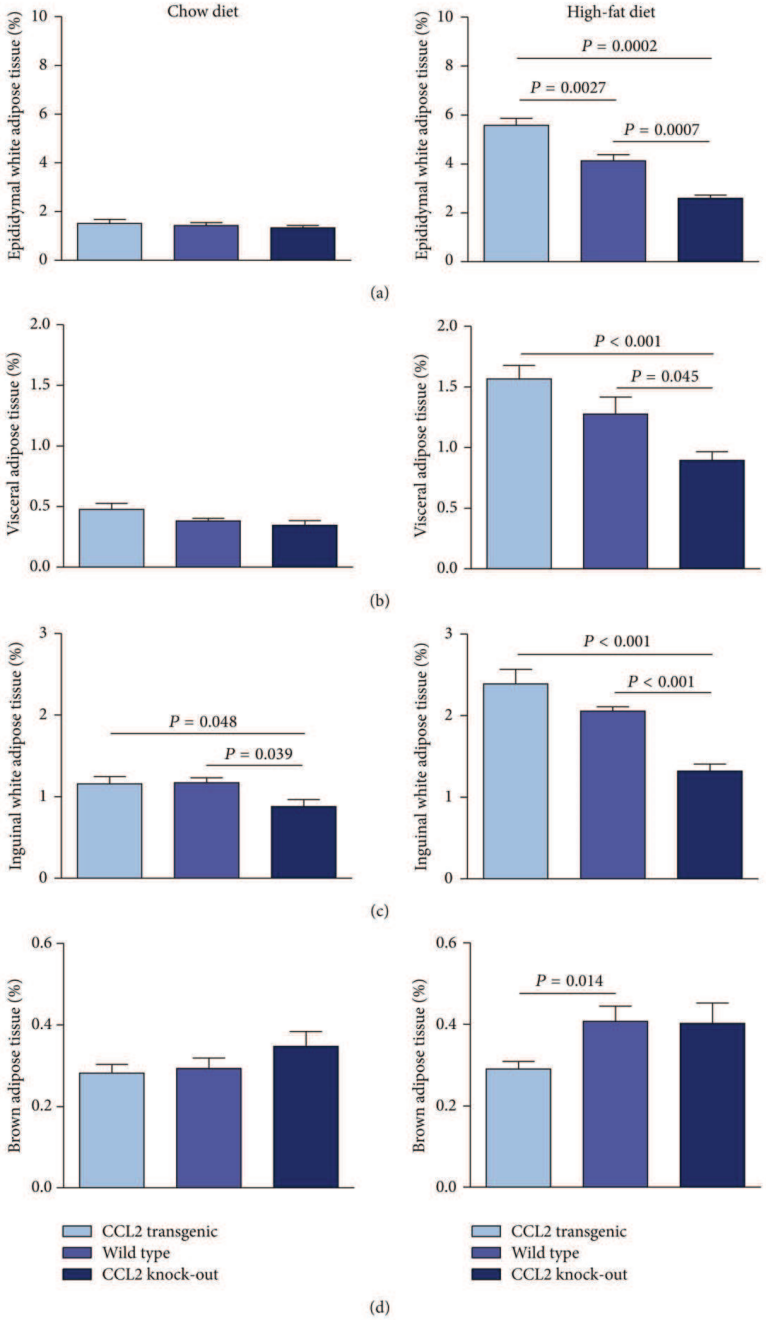


Figure 6. The effect of CCL2 expression in the weight of adipose tissue ((a)–(d)) of animals fed either chow (left column) or high-fat diet (right column). Of note, differences among strains were more evident during energy surplus and no change was observed in brown adipose tissue.

Diet-Induced Disturbances in Glucose and Lipid Metabolism

Glucose tolerance tests (a proxy for insulin resistance) were unaffected in strains fed the chow diet during the experimental period of 6 weeks. However, WT littermates, KO, and transgenic mice displayed abnormal values when fed a high-fat diet, con firming the effect of diet in the pathogenesis of insulin resistance and suggesting that this short-term intervention is not adequate to investigate a possible disturbances. Moreover, there were no differences among the strains in the plasma glucose levels after 6 hours of fasting, and after 3 hours in the fasting state, we found that the plasma glucose baseline concentrations were significantly higher in CCL2 overexpressing mice with respect to CCL2 deficient animals. This effect was more evident in the transgenic mice (**Supplementary Material S6**) but differences in plasma glucose disappeared after 14 weeks of dietary treatment suggesting immature adipose tissue remodelling [38]. When these tests were performed in animals fed a high-fat diet for a longer experimental period of 14 weeks in which adipose tissue is already well modelled, the lack of differences in insulin tolerance was maintained, probably indicating that the effect of CCL2 overexpression in the pathogenesis of insulin resistance is negligible. However, results in the absence of CCL2 indicate that this chemokine may modify glucose metabolism and therefore we cannot discard the effects under a more intense metabolic stress [9]. Variations in plasma cholesterol and triglycerides concentrations were minimal among the strains at 16 weeks old. A high-fat diet significantly increased the amount of circulating cholesterol, an effect that was higher in CCL2 overexpressing mice. Conversely, there were unexpected and most likely not representative, changes in the plasma triglycerides concentration of these mice as a consequence of dietary manipulations (data not shown).

The Influence of CCL2 and Dietary Manipulations in the Liver

When fed the chow diet, mice did not display significant differences among strains in the appearance of their liver tissue. The steatosis scores did not detect significant differences among strains, although some minor variations were detected (**Figure 7**) that did not correlate with the hepatic lipid content (data not shown). When mice were fed a high-fat diet, we found a certain amount of lipid accumulation in WT

mice, but this lipid accumulation was significantly more evident in transgenic mice. Conversely, there was no accumulation of lipids in KO mice (**Figure 7**). Therefore, the effect of CCL2 under these conditions is directly related to the amount of tissue CCL2 disposal; the absence of CCL2 prevents liver steatosis, and overexpression of CCL2 predisposes the liver to steatosis. We also found that the expression of fatty acid synthase in the liver increased significantly in all strains when fed a high-fat diet, but there were no significant differences in the comparisons between transgenic and KO mice. We also explored the activating phosphorylation of AMP-activated protein kinase (AMPK), and values did not change as a result of high-fat diet in transgenic mice and were significantly higher in KO mice compared with transgenic mice (**Supplementary Material S7**). When the livers were examined for the presence of F4/80 antigen, a widely accepted marker of macrophages, we found that both dietary fat and overexpression of CCL2 modify the size, number and morphology of liver macrophages (**Figure 8** and **Supplementary Material S8**). Of note, F4/80 stained cells were more frequent in KO mice, a finding that merits further study because these results could represent a change in function and could be responsible for the differential effects of CCL2 in liver steatosis. We then explored the influence of both CCL2 and diet in mitochondrial biogenesis. Based on the appearance of the matrix, the mitochondria are healthier in mice fed a chow diet than in those fed a high-fat diet. The matrix was also consistently less electron-dense in transgenic mice. We also found altered fusion dynamics. In transgenic mice fed a chow diet, the process was unbalanced towards mitochondrial fusion, but the dietary manipulation significantly elicited a shift towards fission. The changes were similar in WT mice, but the effect of diet was quantitatively less evident than in transgenic mice. In KO mice, however, there were more mitochondria per cell, and fusion and fission were correctly balanced and apparently not altered by differences in diet. These findings strongly support further mechanistic studies, which may link the expression of CCL2 with mitochondrial biogenesis, inflammation, and energy management. According to our results, these putative mechanisms are related to the autophagic response, which was clearly enhanced in transgenic mice. Conversely, most liver cells in WT and KO displayed no evidence of autophagy (**Figure 9**).

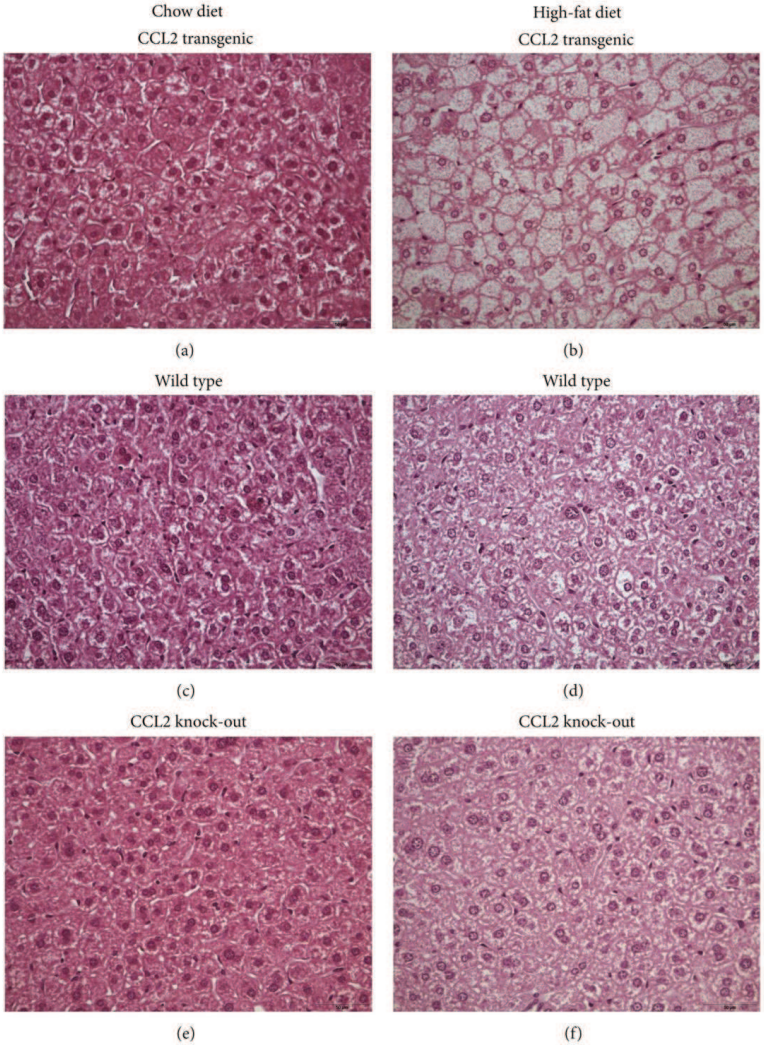


Figure 7. We found no significant differences among strains in the appearance of liver tissue when mice were fed a chow diet (left column (a), (c), (e); transgenic, wild-type, and knockout mice resp.). Representative micrographs show in the right column that a high fat diet produces steatosis in transgenic mice (b), dispersed lipid droplets in the liver of wild type mice (d), and no change in knockout mice (f).

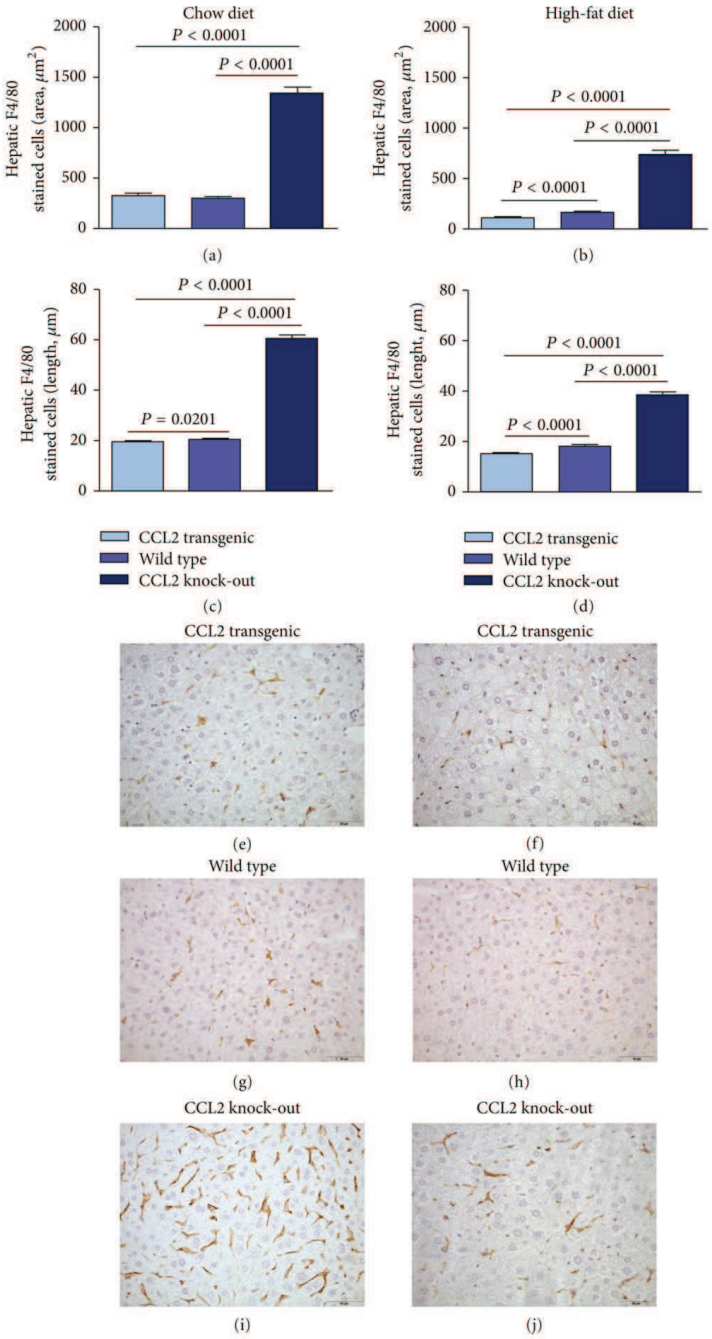


Figure 8. Dietary fat (right column) and CCL2 expression modify the size, number, and morphology of liver macrophages with respect to those fed a chow diet (left column) as assessed with F4/80 staining. Values for stained area and length of macrophages ((a)–(d)) are illustrated with representative microphotographs from transgenic ((e), (f)), WT ((g), (h)) and KO mice ((i), (j)).

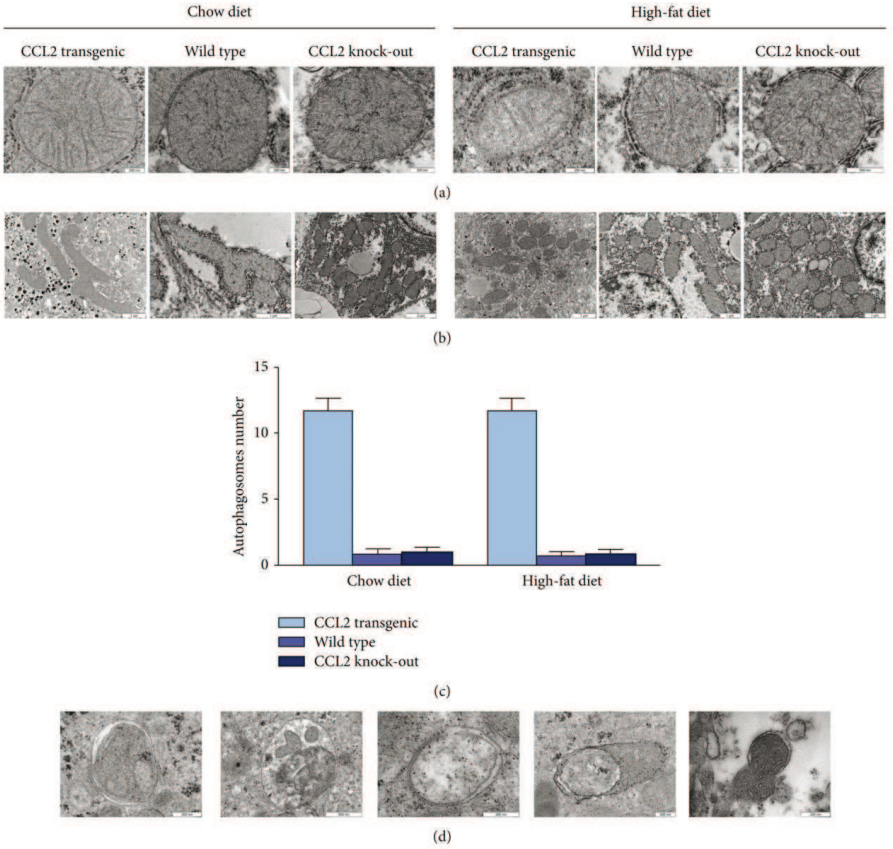


Figure 9. The appearance of mitochondria was affected by the dietary manipulation and the expression of CCL2 as shown in representative microphotographs (a) and these changes were accompanied by a significant effect in fusion-fission balance (b). The number of autophagosomes per cell was counted and was significantly higher in transgenic mice. Further, these were rare in both WT and KO and independent of diet (c). The heterogeneous nature of autophagic elements is illustrated in (d) (photographs obtained in transgenic mice).

Transgenic Mice at Overexpress CCL2 Die Prematurely When Fed High-Fat Diet

The transgenic mice fed a high-fat diet died prematurely between 10 and 14 months. The mice progressively decreased activity, reduced food intake and the appearance of frailty became evident. There was also a casualty in the transgenic mice fed chow diet, but it was sudden, unexpected, and without a prior decrease in weight or activity. Among the casualties, one was also observed in the WT group fed a high-fat diet (**Supplementary Material S9**). A full autopsy was performed, and the cause of death was uncertain. There was neither cancer nor arteriosclerosis in these animals, but there were some cutaneous, superficial, and localised lesions in the skin

accompanied with local loss of hair. There was also no evidence of sepsis. The only remarkable findings were limited to the spleen and the liver. The size and weight of the spleen was consistently higher in the transgenic mice fed high-fat diet. The presence of splenomegaly in these transgenic mice was consistent with the presence of giant cells that were identified as megakaryocytes (Factor VIII positive staining) and other proliferative signs. The weight of the liver was also higher in the transgenic mice, which is most likely due to the higher presence of steatosis. In the liver, there were signs of regenerative cells and increased apoptosis. Ongoing studies with higher sample sizes and the inclusion of females have been designed to further ascertain this point.

Bone Marrow Macrophages of Transgenic Mice: Expression of Selected Cytokines and mRNA

The CCL2 mRNA expression in the bone marrow macrophages was higher in transgenic than in WT mice, irrespective of stimulation with either GM-CSF (M1, pro-inflammatory) or M-CSF (M2, phagocytic). The mRNA expression of the selected M2 markers was similar, with either low or undetectable expression in the GM-CSF macrophages without differences between transgenic and WT mice. The expression of the selected M1 markers was practically identical in the GM-CSF macrophages from TG and WT mice, with the notable exception of CCR7. Surprisingly, the expression of this chemokine receptor was significantly lower in TG mice, indicating lower pro-inflammatory activity. The expression of the M1 markers in M-CSF macrophages showed a unique and significant decrease in CCR2 mRNA expression; however, some M2 markers, including CD206, GAS6, and IGF1, were also underexpressed. IL-10 expression also decreased, but the differences were not statistically significant. The results suggest that CCL2 overexpression may alter macrophage polarisation. Consequently, the secretion of selected cytokines was examined in macrophages that were treated with LPS and were compared with the relevant controls. The CCL2 secretion was higher in TG mice with both treatments compared with the WT mice and was 2–4 fold higher (2–4-fold change) in M-CSF macrophages. The IL-10 secretion was clearly detectable only in LPS-treated animals. The concentration in the supernatant was higher in TG than in WT mice, and the

differences were statistically significant in GM-CSF macrophages. Finally, TNFα secretion was ostensibly higher in LPS-treated animals and significantly higher in TG mice with respect to the relevant controls (**Figures 10** and **11**).

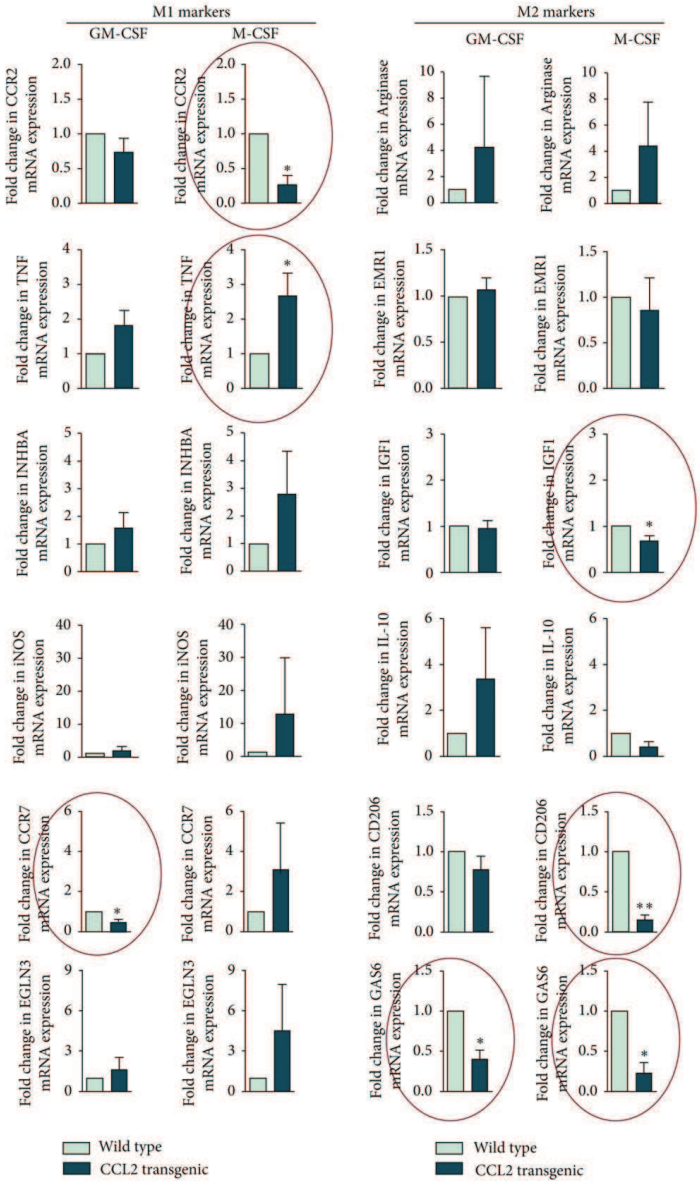


Figure 10. Relative mRNA expression in transgenic mice with respect to WT mice of selected markers for M1 and M2 macrophages in cells treated in vitro with either GC-MSF or M-CSF. Acronyms used were C-C chemokine receptor type 2 (CCR2), TNFα, Inhibin, beta A (INHBA), inducible nitric oxide synthase (iNOS), C-C chemokine receptor type 7 (CCR7), Egl nine homolog 3 (EGLN3), Arginase (ARG), EGF module-containing mucin-like hormone receptor EMR1 (F4/80), insulin growth factor-1 (IGF1), IL-10, the mannose receptor CD206, and Growth arrest-specific 6 (GAS6).

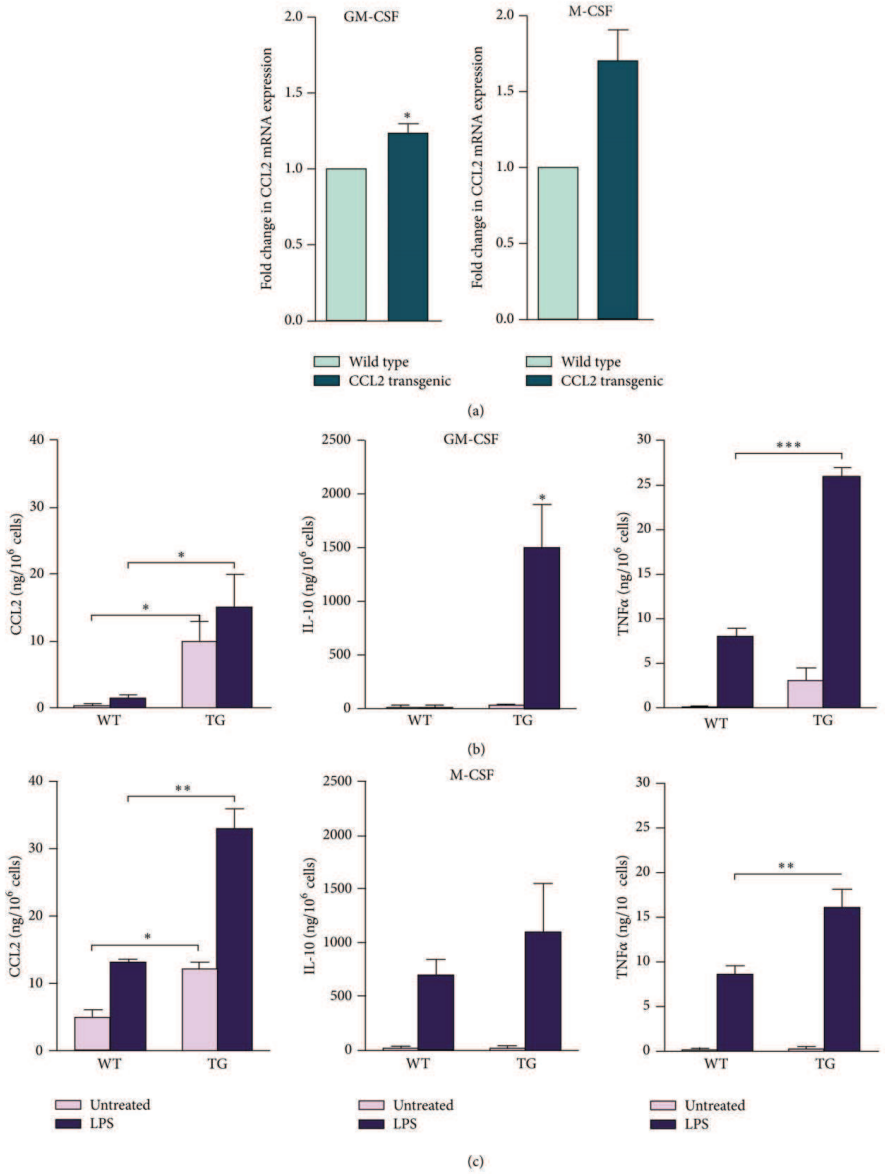


Figure 11. The relative CCL2 mRNA expression (a) and the secretion in supernatants of selected cytokines in bone marrow-derived macrophages of transgenic and WT cells treated in vitro with either GC-MSF (b) or M-CSF (c).

Discussion

The transgenic mice developed in this study systemically overexpress CCL2. These animals were created to assess the combined effect of the recruitment of circulating monocytes in all tissues and the response to the stimuli of high dietary fat and energy ingestion. The hypothesis was that the continuous overexpression of this chemokine could promote or worsen common pathological conditions, and as animal model could be useful for assessing the pathogenic mechanisms and therapeutic approaches [39].

Fertility, growth, and physical appearance were identical to the controls. CCL2 overexpression did not result in abnormalities in the mice that were fed a regular chow diet. However, adding fat to the diet during a short period of time caused differences in body weight, adipocyte size, disturbances in glucose and lipid metabolism, premature death, and liver alterations that included a higher predisposition to fatty liver disease and significant changes in mitochondrial biogenesis and autophagy. Additionally, we explored bone marrow macrophages under different in vitro conditions, and we found that CCL2 overexpression affects functional plasticity.

In previous studies, CCL2 has been considered a chemokine secreted by adipose tissue (adipokine), but systemic CCL2 overexpression regulates white adipose tissue (WAT) mass and size without apparent effects in brown adipose tissue (BAT). WAT serves primarily as lipid storage, and BAT is used for heat generation. The balance between the two adipose tissues affects the whole-body energy homeostasis, and the development and severity of obesity [40]. A higher production of CCL2 is not only a consequence of obesity but is most likely an exacerbating factor of diet-induced alterations. The roles of CCL2 in the aetiology of obesity and diabetes, the regulatory mechanisms, and the effect of therapies that inhibit CCL2 production have been recently reviewed [4, 41]. We have also found differences between fat cells in different adipose tissue depots and the heterogeneity of adipocytes within the same depots. Further examination of this issue is necessary because a different pattern of gene expression could explain the differential development of various types of

adipose tissue [42, 43]. Moreover, this is closely associated with the pattern of fat distribution, the extent of obesity, and consequently the impact of different fat depots on the severity of metabolic complications [44, 45].

The size and number of hepatic macrophages significantly differs between transgenic and KO mice when detected with antibodies directed against F4/80. Curiously, this is an extracellular antigen of unknown function that belongs to a subgroup of the G-protein-coupled receptors [46]. The changes in macrophages morphology could represent concomitant changes in function and whether the macrophages are resident or recruited. This is further substantiated by the fact that these transgenic mice were prone to develop fatty liver disease and the KO mice were protected. The role of increased CCL2 is not yet understood, but the recruitment of macrophages seems to be important in different animal models. In KO mice there is an increased expression of peroxisome proliferator-activated receptors accompanied by the induction of fatty acid metabolism-related genes and the inhibition of pro-inflammatory cytokine production [47–49]. We confirmed that the effect of fat in the pathogenesis of fatty liver disease [49, 50] is influenced by the amount of available CCL2 and that the linkage between chemokines and hepatic lipid metabolism is plausible.

The characterisation of bone marrow-derived cells in the transgenic mice indicates that CCL2 overexpression affects the transition in the secretory function of macrophages (or the M1-M2 paradigm as a simplified descriptor of functional plasticity). This is illustrated by differences in GM-CSF and M-CSF, which are cytokines that differentiate macrophages in vitro with distinct morphology and inflammatory function [51, 52]. The modulation of the phenotypic and functional differences in macrophage polarisation by CCL2 overexpression denotes a shift towards lower pro-inflammatory activity [53]. CCL2 decreased the expression of CCR7 in M1 and decreased the expression of CCR2, IGF1, CD206, IL-10, and GAS6 in M2. In cells under LPS treatment, however, CCL2 overexpression increased the secretion of IL-10 and TNFα with respect to WT controls. These changes could represent a quantitatively determinant factor in the development of macrophage-induced metabolic alterations. It should be highlighted that a high percentage of

total body resident macrophages are present in the liver and that adipose tissue is a major site for the accumulation of recruited macrophages [54, 55].

Notably, CCL2 is involved, directly and/or through the induced metabolic alterations, in mitochondrial biogenesis and autophagy. We add CCL2 to the growing list of nonessential regulators of mechanisms that divide and fuse mitochondria [56]. The balance between rejuvenation and elimination of damaged mitochondria via autophagy is affected by both the presence of CCL2 overexpression and the increased availability of energy. The antagonistic and balanced activities of the fusion and fission machineries are constantly providing responses to inflammation to tightly regulate homeostasis of the organism [57, 58]. This is expected because mitochondrial diseases are associated with metabolic alterations. Apparently, there is a shift towards fusion in CCL2 overexpression to maximise ATP synthesis. Contrarily, morphological findings in CCL2 deficient mice, which are independent of high-fat diet, suggest a perfect balance [59, 60]. A certain unbalance is expected in inflammatory conditions and other energy-dependent disturbances via mitochondrial dysfunction [61, 62]. This is important because mitochondria and the access to energy (calorie restriction or increased dietary fat) play a pivotal role mediated by the mechanistic target of Rapamycin (MTOR) in deciding whether liver cells live or die [63].

In transgenic mice, autophagy was increased with respect to WT and KO mice, which is particularly important because autophagy affects immune responses as a result of degradative, biogenetic, and secretory activities that respond to various inputs via MTOR [64, 65]. Autophagy might control the infection of certain pathogens but also prevents excessive inflammatory reactions in the host [66]. As shown in autophagy-deficient macrophages, autophagy removes a number of pro-inflammatory stimuli [67–69]. Therefore, increased liver autophagy during CCL2 overexpression could be interpreted as an e ort from the host to avoid the deleterious action of continuous inflammation. Links between autophagy and inflammation have also been found in immune functions affecting several diseases, opening a new dimension in the understanding of the multifactorial basis of non-communicable diseases. For example, increasing macrophage autophagy protects patients with advanced

atherosclerosis [70]. It has also been reported that CCL2 controls the extent of autophagy in human prostate cancer [71], and autophagy is pivotal for the survival and differentiation of monocytes [72].

Finally, CCL2 overexpression resulted in premature death when combined with a high-energy intake. These findings require more extensive examination, and the cause of death remains obscure. Mice progressively lost interest in the environment, reduced activity, and their intake of food decreased. No chronic disease was evident, and there were no signs of sepsis or major infection. It is tempting to consider the possibility of premature aging, and future investigations will include the characterisation of a senescence-associated secretory phenotype, particularly in pro-inflammatory cytokine enrichment [73] and the pro-inflammatory phenotype that accompanies aging [74, 75].

Conclusions, perspectives, and limitations

This animal model raises a number of questions about the prevalent diseases responsible for limiting the quality of modern life. Additionally, this model provides a link between inflammation and metabolism and suggests targets for the management of diseases in which there is a clear CCL2 over- expression. Specifically, this model can help to uncover the role of CCL2 in mitochondrial dysfunction, autophagy, and functionality of macrophages and aging in combination with excessive energy intake. Information gained could be useful for designing new mechanism-based therapeutic strategies. None of the described effects appear in mice that are fed a regular diet, and this fact highlights the importance of calorie restriction for health. Therefore, the nutrient-sensing MTOR pathway seems to be crucial for the management of non-communicable diseases. Consequently, drugs modulating MTOR are obvious candidates for assessment. For example, experiments on cancer, aging, and viral infections strongly suggest that this is the case for metformin [76-78]. This anti-diabetic drug activates AMPK and inhibits MTOR with potent anti-inflammatory actions. The usefulness of rapamycin, an MTOR inhibitor, and similar drugs in cancer prevention has been assayed [79]. Aspirin decreases inflammation, inhibits the MTOR pathway, decreases cancer incidence, and may reduce the burden of atherosclerosis [13, 80]. Lastly, although studies are scarce,

angiotensin-II-blockers and beta-blockers, widely used in hypertensive patients, can also prevent the activation of the MTOR pathway and the incidence of chronic diseases [81]. The potential indications for these drugs are mostly related to chronic diseases in which inflammation plays a crucial role. This animal model could be used to further select candidates and suggests a number of mechanistic questions for future study. Particularly, we consider this model as a valuable contribution to our evolving comprehension of the interphase between autophagy and inflammation. However, we acknowledge that care must be taken in analysing the results of studies performed in animal models and that further research e ort is necessary to fully characterize our observations. To name a few, possible effects of sex should be studied and metabolic alterations should be confirmed with the use of metabolic cages and more specific methods to detect significant differences. Particularly, CCL2 may have a higher influence if there is a relative contribution from different type of cells, particularly from immune cells [72].

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STUDY 3

CCL2 and metabolic response: the role of the inhibition of CCL2/CCR2 axis

Preliminary results

Background and aims

There is a strong relationship between metabolism and immune system. Low-grade chronic inflammation is a consequence of a compromised management of excessive energy intake and represents a significant factor in the development of prevalent metabolic diseases such as obesity and NAFLD. Chemokines and its receptors are important factors for the interconnection between nutrient excess and derangements in immune system. These molecules play a crucial role in the inflammatory process through the recruitment of macrophages to metabolically compromised tissues and, consequently participate in the course of disease such as obesity, insulin resistance, in which inflammation is a key factor of its pathogenesis. For this reason, nowadays, chemokines and chemokines receptors have become attractive therapeutic targets. Accordingly, the aim of this study was to determine if the metabolic effects of a continuous and ubiquitous expression and secretion of CCL2 combined with energy surplus can be counteract by the absence of CCR2 (CCL2 receptor).

Material and Methods

Animal experimental models

We carried out this study using the CCL2 transgenic model previously described, and designed by our group using standard methods and proprietary technology from Ozgene (Bentley, WA, Australia). We also provide data from a novel animal model, a double genetically modified mouse, CCL2 overexpressor and CCR2 knockout mice. This strain was obtained by inbreeding CCL2 transgenic and CCR2 knockout mice. Finally, as controls, we used CCL2 knockout mouse conveniently backcrossed and purchased from Jackson Laboratory (Sacramento, CA, USA). They were housed under standard conditions and given a regular chow diet and water *ad libitum* until experiments began. To explore dietary effects, at 10 weeks of age, littermates for each model were equally and randomly assigned to two dietary groups (n=8); one fed with the regular chow diet (Teklad rodent diet; Harlan, Barcelona, Spain) and the other fed with a high-fat diet (FuttermittelfurMaüse; SSniff spezial diaten, Soest, Deutschland).

Sample collection

Blood was also obtained at the moment of sacrifice and collected into tubes containing EDTA. The relevant tissues were removed, flash-frozen and stored at 80°C until further analysis.

Histology

Microscopic examination was performed on the liver and epididymal adipose tissue. Tissues were removed, fixed for 24h in 4% phosphate-buffered formalin at room temperature, processed and embedded in paraffin for histological analyses. The area of adipocytes was quantified using AnaliSYS software (Soft Imaging System, Munster. Germany). The corresponding fractions of the liver were sectioned and stained with hematoxylin and eosin and steatosis was evaluated by estimating the percentage of area covered by fat droplets. Finally, sections of liver were used to detect F4/80 (rat anti-mouse macrophages/monocytes; Serotec, Oxford, UK). Images were acquired, and results were analysed with AnaliSYS software.

Western Blot analysis

Liver samples were weighted and homogenized in 5 ml/g of lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% CHAPS, 1 mM Pefabloc and 1% phosphatase inhibitor cocktail no. 2, all from Sigma–Aldrich Inc., Steinheim, Germany) using the Precellys 24 system (Bertin Technologies, France). Protein concentration was determined by 2D Quant kit (GE Healthcare, Piscataway, NJ, USA). After SDS-PAGE, gels were transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen, Barcelona, Spain). Detection antibodies were rabbit anti-AMPK (2532, Cell Signaling Tech., Danvers, MA, USA) and rabbit anti-pAMPK (2531, Cell Signaling Tech.) The secondary antibody was goat anti-rabbit-HRP (Dako, Glostrup, Denmark). Chemiluminescent detection was performed using the ECL Advance Western Blotting Detection kit (Amersham, GE Healthcare, Barcelona, Spain), and membranes were analysed in Chemidoc system (Bio-Rad, Madrid, Spain). Densitometric quantification of the immunoblotted membranes was performed with Image Lab software (Version 2.0 build11, Bio-Rad Laboratories).

Statistical analysis

Data were compared using Mann-Whitney tests. GraphPad Prism 5.03 software (GraphPad, San Diego, CA) was used to perform all statistical analyses. Unless otherwise indicated, values are expressed in mean ± SEM. A p-value <0.05 was considered statistically significant.

Results

Effects on body weight, food intake and on adipose tissue

When mice were fed a regular chow diet we did not observe significant differences in body weight increase neither in cumulative food intake among groups. In contrast, when fed a high fat diet, CCL2 overexpression induced weight gain and the absence of CCR2 in transgenic mice as well as the lack of CCL2 in control mice protects them from the development of obesity. There were not any significant differences in food intake; hence differences can be attributed to the presence of CCL2 (**Figure 1**).

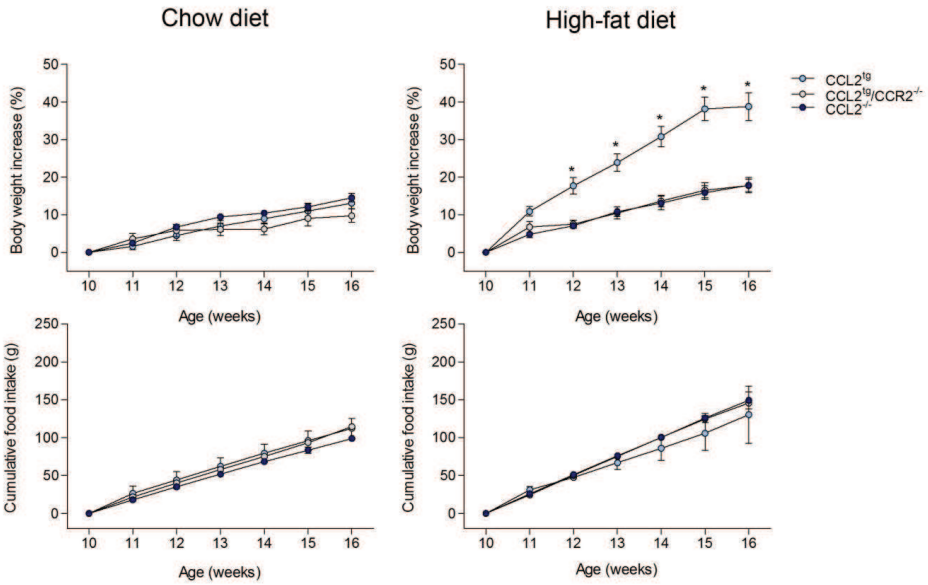

Figure 1. Body weight gain and cumulative food intake in mice fed either chow diet or high-fat diet.
*p<0.05 for differences between groups.

Overexpression of CCL2 increased the size of epididymal adipose tissue adipocytes, independently of the caloric content of the diet. The adipocyte size was higher in CCL2 transgenic mice compared with knockout mice. The differences are higher when mice were fed a high-fat diet (**Figure 2**).

Moreover, at the moment of sacrifice, selected types of adipose tissue depots were weighed. When mice were fed the regular diet we did not observe significant difference between the strains. However, high caloric intake combined with CCL2 overexpression resulted in a significant increase of all adipose tissue depots. Knockout mice fed a high-fat diet also showed higher adipose tissue weight compared to chow diet fed mice but this increase was significantly higher in transgenic animals (**Figure 3**).

The influence of CCR2 absence in dietary manipulations in the liver

We found no significant differences among strains in the appearance of liver tissue when mice were fed a chow diet. However, when mice where fed a high-fat diet, the hepatic lipid content correlated with the expression of CCL2, in other words, we found significantly high amount of lipid accumulation in the liver of transgenic mice, conversely, the absence of CCR2 or CCL2 prevented liver steatosis (**Figure 4**).

In addition, we also explored the activating phosphorylation of AMP-activated protein kinase (AMPK) (**Figure 5**). We did not find differences among strains when they were fed a regular diet. However, interestingly, the lack of CCR2 or CCL2 combined with a high-fat diet induced the activation of this protein. In transgenic mice, values did not change as a result of high-fat diet intake.

Finally, when F4/80 immunostaining was assessed (**Figure 6**), the number of F4/80 stained cells was higher in knockout mice than in the transgenic model in both dietary interventions. This could represent that the overexpression of CCL2 could affect macrophage function and could be related with the differences in the presence of liver steatosis.

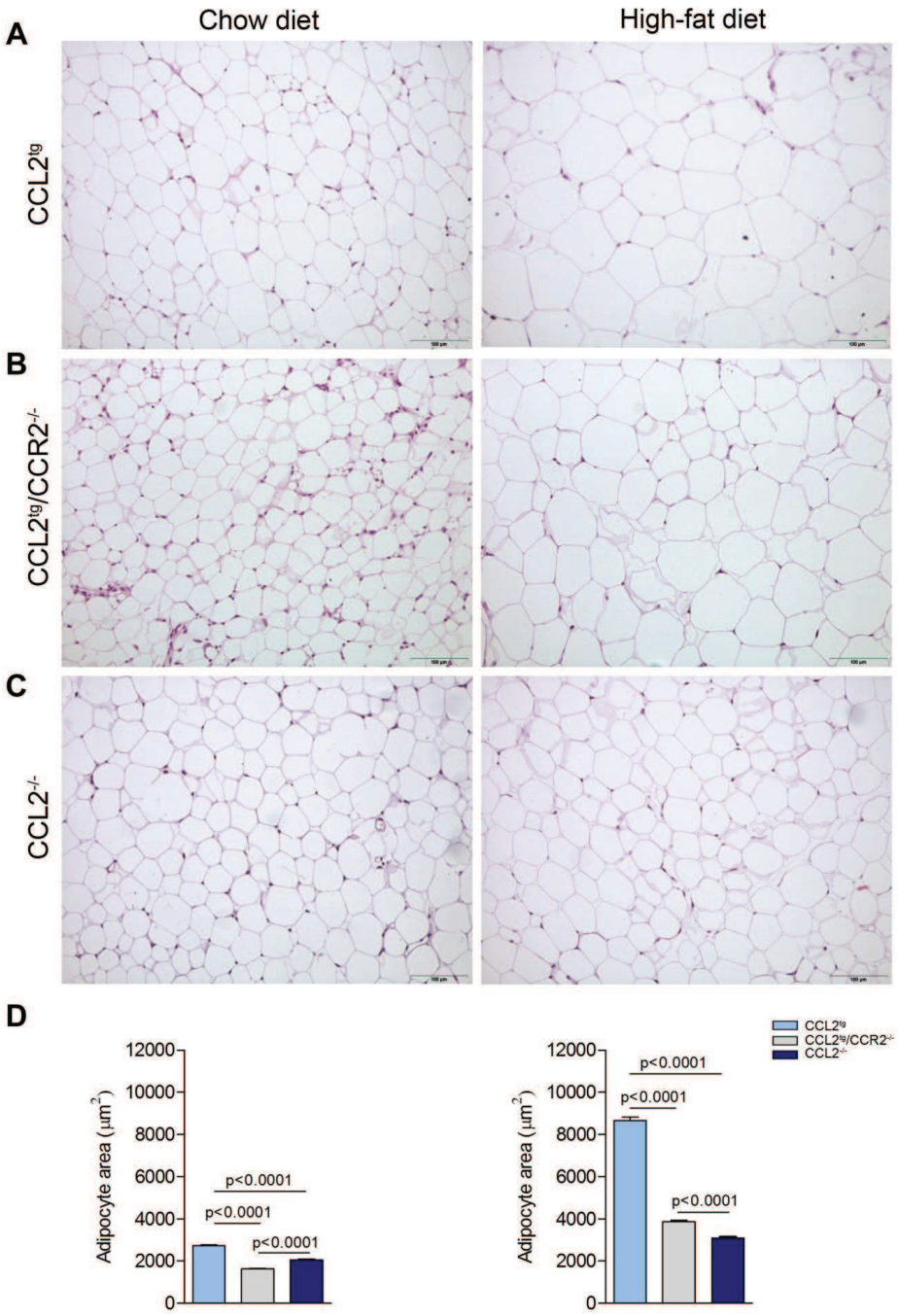

Figure 2. Epidydimal adipose tissue adipocyte size. Representative micrographs are shown for transgenic and knockouts animals (A, B and C, respectively) when fed a chow diet (left column) or high-fat diet (left column). Adipocyte area quantification (D) revealed that CCL2 overexpression increased adipocyte size independently of the administered diet.

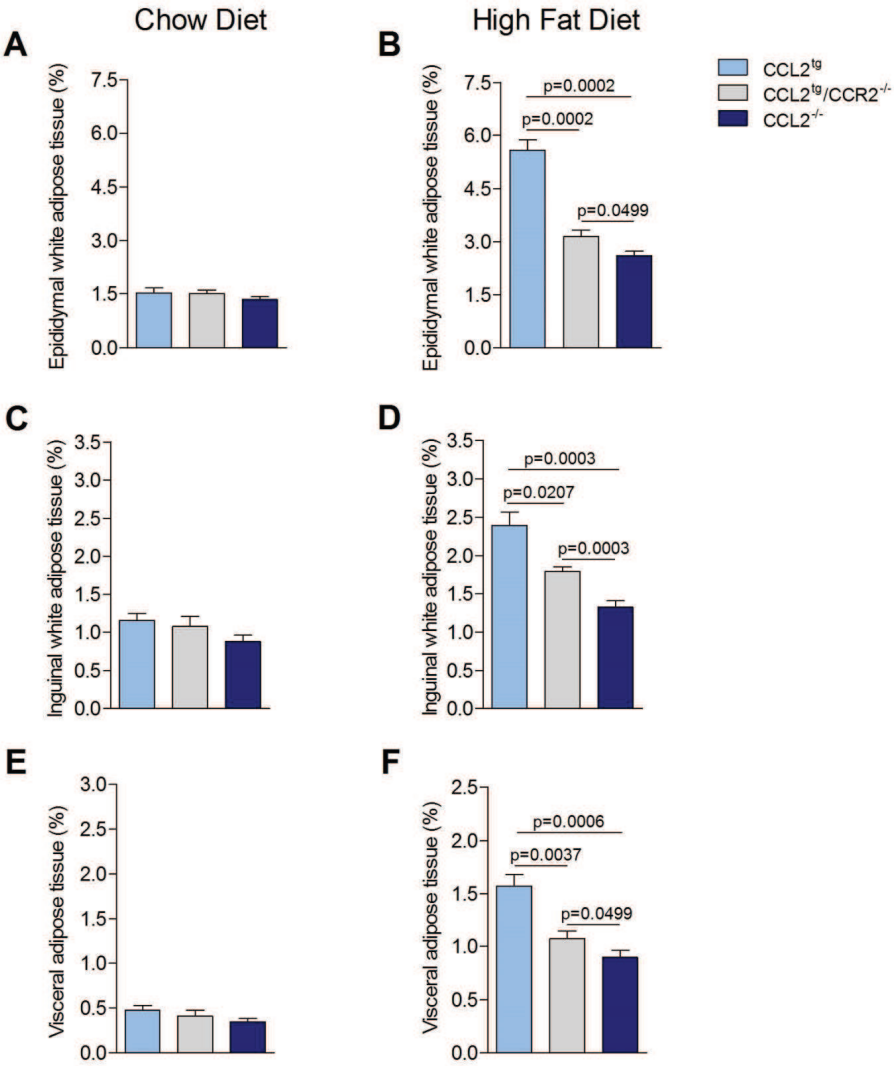

Figure 3. Adipose tissue depots weight in animals fed either chow diet (left column) or high-fat diet (right column). Of note, differences among strains were significantly evident with an energy surplus.

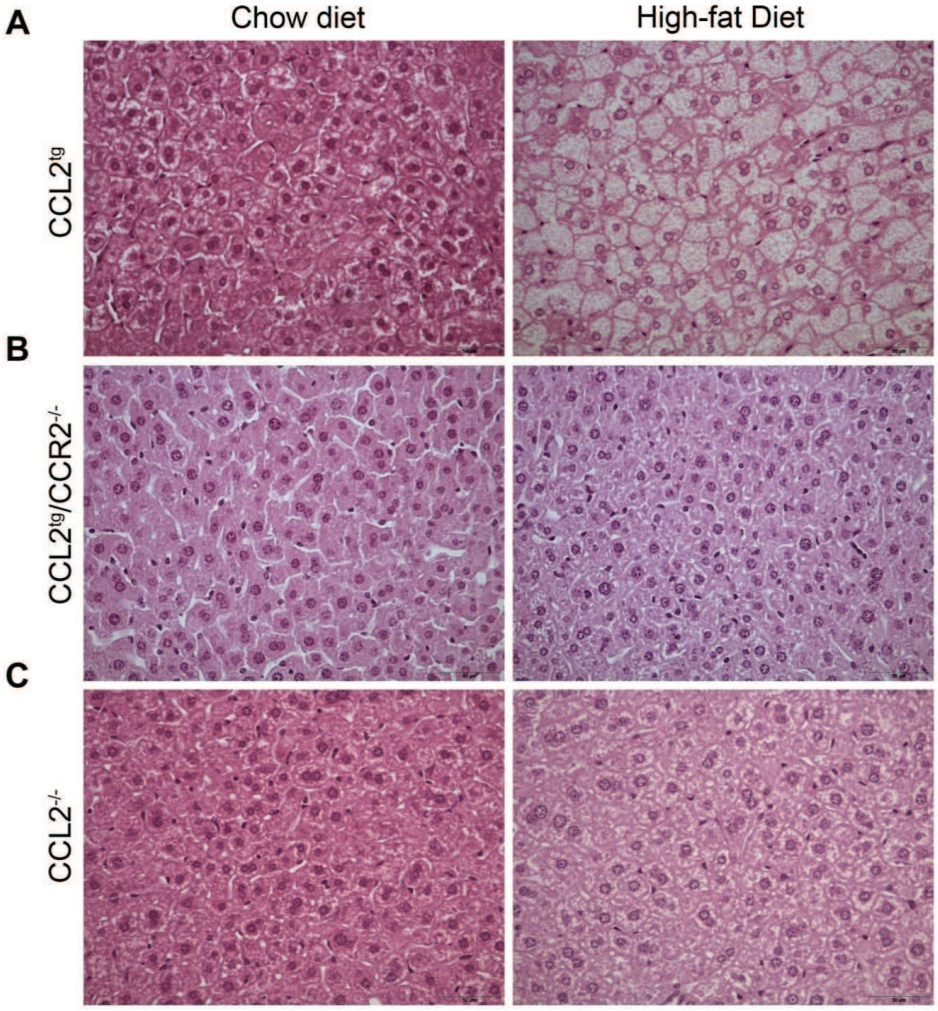

Figure 4. Appearance of liver tissue. Representative micrographs are shown for transgenic and knockouts animals (A, B and C, respectively) when fed a chow diet (left column) or high-fat diet (left column).

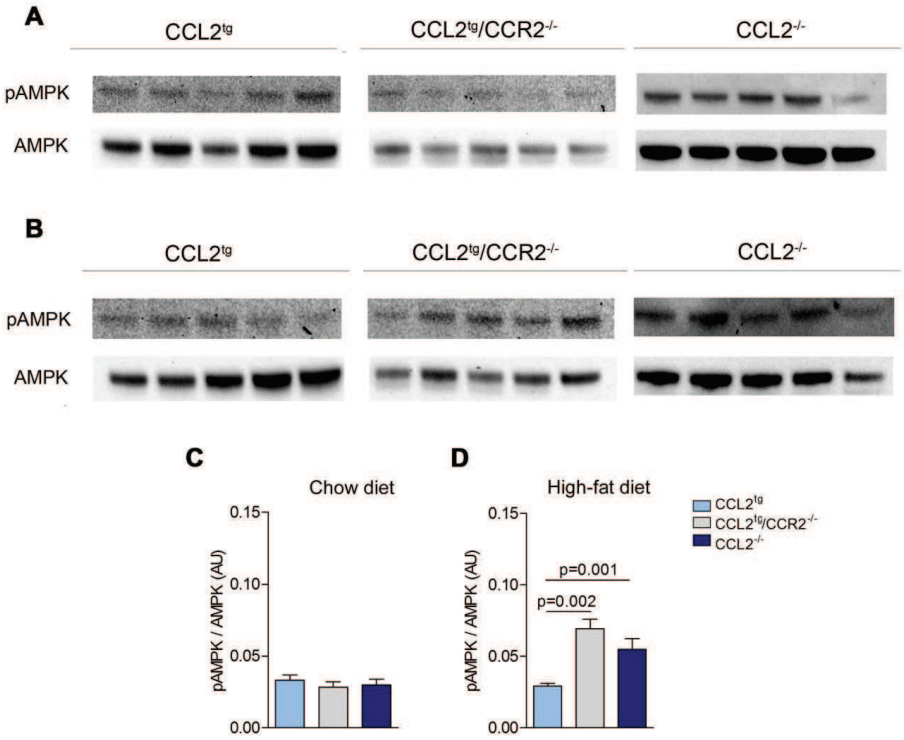

Figure 5. Activation of AMPK in the liver. We did not observe any difference when mice fed chow diet (A, C). However, the absence of CCR2 or CCL2 combined with energy surplus activated AMPK by increasing pAMPK expression in the liver (B, D).

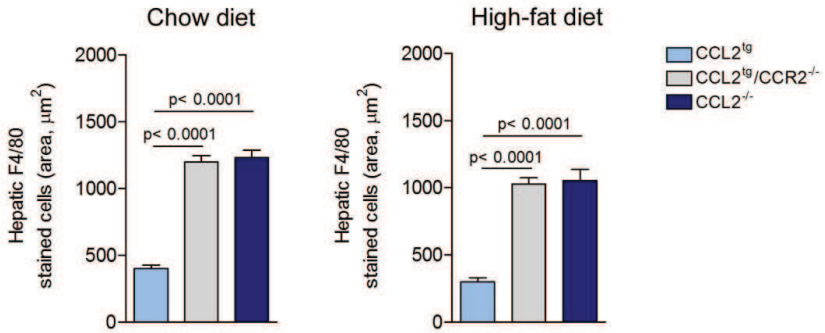

Figure 6. Quantification of macrophage infiltration in the liver. The lack of CCR2 or CCL2 increases the number of liver macrophages in both dietary groups.

DISCUSSION

Obesity, described by the WHO as the "epidemic of the XXI", has become the greatest worldwide public health concern [3]. The demographic, economic and epidemiological changes, resulting in unfavorable diets and in the decrease of physical activity, have driven to globally and alarming increase of its prevalence in both adults and children [19].

The consequences of obesity are many and diverge from higher risk of premature death to numerous health disorders such as T2DM, cardiovascular disease, NAFLD, some types of cancer, and a number of psychiatric disorders [25, 39].

NAFLD is an important comorbidity of obesity and is recognized worldwide as the most common cause of chronic liver disease in adults and children and its incidence and prevalence are constantly increasing [96, 101]. Furthermore, NAFLD is not a simple disease; it includes a spectrum of hepatic abnormalities which extends from simple steatosis without inflammation to NASH, a pathological entity associated with an increased risk for developing more serious diseases such as cirrhosis, liver failure and hepatocellular carcinoma [99].

Traditionally, obesity has been associated with NAFLD prevalence. Many studies claim that NAFLD prevalence may be greater than 90% in the presence of morbid obesity (BMI above 40) [87, 315]. However, this relationship between liver steatosis and obesity is not an absolute one. Some studies have shown that, although less prevalent than in obese patients, both NAFLD and NASH could be observed in individuals with a BMI in a normal range [316, 317]. Moreover, in our morbidly obese cohort the degree of steatosis was widely distributed and notably, steatosis was predominantly mild. More interestingly, a significant number of patients with extreme obesity never developed NAFLD and other liver alterations such as portal and/or lobular inflammation and fibrosis were primarily benign. For this reason, we aimed to better understand the pathogenesis of NAFLD searching for metabolic differences between these two groups, morbidly obese patients with and without steatosis.

The results showed that patients with steatosis presented alterations in the metabolism of carbohydrates and lipids and liver damage compared to the group of

individuals without steatosis. Accordingly, understanding the pathogenesis of NAFLD could be useful to identify biomarkers of disease progression and to clarify the effects and limitations of therapeutic regimes. Furthermore, new findings regarding the involvement of specific metabolic pathways could provide important potential targets for future therapeutic interventions. However, more studies are needed to achieve a reduction in morbidity and mortality from this disease.

Given the risk of progressive liver disease in obese patients, the significant number of individuals which are asymptomatic until their liver begins to fail and also the association between NAFLD with an increase of all-cause mortality, an early prognosis and an accurate diagnosis and staging of the disease has become an important health challenge [88].

Nowadays, although there are no clear recommendations whether liver biopsy is necessary to confirm the diagnosis of NAFLD, it still represents the gold standard to distinguish the different stages of fatty liver disease. However, it has several drawbacks because it is invasive, painful and a costly procedure associated with sampling error and variability. Moreover, the high prevalence of NAFLD in the general population make liver biopsy unsuitable as a diagnostic procedure, in other words, it is not practical to perform liver biopsy in all patients to screen for NAFLD or NASH. These shortcomings and limitations of liver biopsy highlight the great need to find a non-invasive method for the assessment of NAFLD [142].

Some data indicated that the use of some non-invasive imaging technologies such as proton magnetic resonance spectroscopy can be useful in the assessment of hepatic steatosis and could help to minimize the frequency of liver biopsy. However, the uses of these methods are limited by high costs, restricted availability, and morbidly obese patients usually did not fit into the apparatus [143, 145, 146].

On the other hand, liver enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT), are well recognized as liver injury markers and these liver biochemistries are performed widely. However, in regard to NAFLD these laboratory data remain controversial [318-320]. As an example, serum ALT is generally used as the population-wide

screening test to diagnose NAFLD. In contrast, Chalasani *et al.* elucidated that this measure is not accurate as patients with NAFLD or even advanced NASH and cirrhosis can exhibit ALT levels within the normal ranges [154]. In our study, we also corroborated these results; ALT could not differentiate between the different grades of steatosis and exhibited the worst performance when it was tested to discriminate between patients with or without steatosis. Moreover, despite several years of research, there is no clear evidence in the literature that any sophisticated algorithms of available biomarker are good enough to avoid liver biopsy [321, 322]. For these reasons, a great effort is now being made toward the identification and validation of novel biomarkers to assess NAFLD and NASH.

Metabolomics, the study of part or the entire set of small molecules in a biological sample, has emerged as a powerful tool for discovering novel biomarkers. One of the most important advantages of metabolomics technologies is that they can be used to identify a unique "metabolic signature" of disease through the detection of the changes in metabolite levels [160, 323].

Metabolomics has already identified new biomarkers for prostate cancer [324], Parkinson's disease [325] and type 2 diabetes mellitus among other important chronic diseases [326, 327]. Moreover, metabolomics technologies have recently provided some important insights into the pathogenesis of NAFLD [328-330]. However, these studies had some limitations such as a small sample size and not detailed models.

Accordingly, to address the urgent need for identification of novel diagnostic biomarkers which could facilitate the diagnosis and the treatment of NAFLD, we performed a metabolomics analysis of the plasma comparing obese patients without steatosis with their affected counterparts to obtain a comprehensive view of changes in several metabolic pathways and to identify disease-related patterns and biochemical perturbations.

Mechanisms responsible for fatty liver are still not fully elucidated but decreased capacity to oxidize fatty acids, increased delivery and transport of FFAs into the liver

and augmented hepatic fatty acid synthesis are likely to play a significant role in the pathogenesis of NAFLD and resulted in acquired mitochondrial dysfunction.

Mitochondrion plays an important role in hepatocyte metabolism, being the primary site for the oxidation of fatty acids and oxidative phosphorylation. NAFLD affects mitochondrial metabolism and metabolic pathways which can lead to perturbations in citric acid cycle [122]. Our exploratory study showed that lean controls exhibited significantly lower α-ketoglutarate plasma levels than obese patients and also the values were significantly higher in patients with steatosis compared with those without this lipid accumulation. Thus, we proposed plasma α-ketoglutarate as a potential diagnostic biomarker with higher predicted accuracy than commonly used laboratory tests which resulted not appropriate for clinical decisions. Thereby, monitoring mitochondrial function by the quantification of mitochondrial metabolite levels provided new insights into NAFLD pathophysiology.

The handling of fat and/or excessive energy intake not only leads to mitochondrial dysfunction but also encompasses the linkage of inflammation to the deleterious effects of the continuous excessive food consumption.

During the last decades, a high number of studies have come out demonstrating that immunity and metabolism are closely interconnected owing to inflammation is a key factor of the pathogenesis of several metabolic disorders such as obesity, insulin resistance and NAFLD [200].

Chemokine and their receptors, key mediators of inflammatory and immune cell trafficking, are involved in the maintenance of metabolic homeostasis and, consequently, have made a name for themselves as pleiotropic molecules involved in a range of physiological as well as pathological processes [230]. The importance of chemokines and their receptors has been appreciated in the research from multi-disciplinary backgrounds and the development of new therapeutic agents directed against chemokines or their receptors with proven effectiveness in the treatment of prevalent diseases [248, 331]. Thus, chemokines and their receptors are poised to make substantive impacts as both biomarkers and the basis for novel pharmaceutical strategies for human disease.

Accordingly, to better understand the pathogenesis of metabolic diseases and to propose novel therapeutic approaches, we created a novel animal model which overexpresses CCL2 in all tissues to assess the low-grade chronic inflammation in response to an excessive energy intake.

Many studies have demonstrated the role of CCL2 in obesity, diabetes and fatty liver due to its influence in the regulation of energy metabolism [252, 267]. Moreover, it has been reported the involvement of inflammation in mitochondrial function and autophagy [237, 261].

Using this model we could corroborate that the overexpression of CCL2 combined with a high fat diet predisposes to obesity and causes differences in adipocyte size, disturbance in glucose and lipid metabolism, premature death and liver alterations such as fatty liver and changes in mitochondria biogenesis and autophagy. Moreover, this overexpression affects macrophages function and plasticity. Thus, these preliminary data contribute to the knowledge about the relationship between inflammation and metabolism and suggested some mechanistic questions for future studies as well as possible therapeutic targets such as AMPK and MTOR. These two proteins play an important role in energy homeostasis through several pathways and could be crucial in the management of prevalent metabolic disorders.

As stated above, the modulation of the chemokine system arises as an attractive target for the treatment of inflammatory diseases. Moreover, studies with mice lacking specific chemokine ligand or receptor and/or pharmacological inhibitions of them have begun to provide new insights into the consequences of the management of diseases with an inflammatory background [231, 266].

CCL2 or CCR2 deficient mice are less predisposed to develop obesity and its related comorbidities; they display an improvement of insulin sensitivity, glucose tolerance, adiponectin expression and an attenuated expression of pro-inflammatory markers in different tissues. Moreover, the lack of these proteins ameliorated the presence of hepatic steatosis although fed a high-fat diet [248, 249]. All these results have driven to the development of CCR2 antagonists.

Hence, in order to determine if the deleterious metabolic effects caused by a continuous and ubiquitous overexpression of CCL2 combined with energy surplus could be counteract by the absence of CCR2, we created another animal model. In that case mice overexpressed CCL2 but, at the same time, they were CCR2 deficient.

This novel animal model showed less body weight gain, lower adipocyte size as well as less weight of adipose tissue depots. So, the absence of CCR2 prevented the development of obesity observed in the CCL2 overexpressor mice. Moreover, the absence of CCR2 also ameliorated hepatic steatosis probably by the activation of key proteins in energy metabolism such as AMPK and modulating macrophage function. So we could conclude that, as expected, all metabolic disturbances observed in the overexpressor model could be reverted by the inhibition of CCL2/CCR2 axis biologic function.

All this information could be really important to establish CCR2 modulators as a new class of therapeutic agents for the management of prevalent metabolic disease such as obesity, diabetes, insulin resistance and NAFLD. Actually, some CCR2 antagonists such as INCB-3344, RS-504393, TEI-KO3134 among others, have been patented by many companies and many of them are in the last stages of clinical trials with promising results [332, 333].

CONCLUSIONS

- ✓ The metabolic imbalance caused by obesity and NAFLD affects mitochondrial function and it can be detected in plasma metabolomics profile. Hence, monitoring mitochondrial function via the quantitative evaluation on mitochondrial metabolite abundances may be an important new avenue of research in obesity-related NAFLD.

- ✓ Plasma α-ketoglutarate as a surrogate biomarker of NAFLD is superior to common liver function tests in obese patients. Therefore, the measurement of this biomarker may potentiate the search for a therapeutic approach, may decrease the need for liver biopsy and may be useful in the assessment of disease progression.

- ✓ Overexpression of CCL2 in combination with excessive energy intake leads to a wide spectrum of metabolic disorders such as obesity, hepatic steatosis and mitochondrial dysfunction and also the manipulation of macrophage plasticity and autophagy.

- ✓ CCL2 or CCR2 inhibition could counteract these metabolic abnormalities becoming an interesting target for the design of new therapeutic strategies.

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UNIVERSITAT ROVIRA I VIRGILI
INFLAMMATION AND ENERGY METABOLISM IN OBESITY: THE SEARCH FOR BIOMARKERS AND NOVEL THERAPEUTIC STRATEGIES.
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SUPPLEMENTARY MATERIAL

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ORIGINAL ARTICLE

Mapping of the circulating metabolome reveals α-ketoglutarate as a predictor of morbid obesity-associated non-alcoholic fatty liver disease

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BACKGROUND: Obesity severely affects human health, and the accompanying non-alcoholic fatty liver disease (NAFLD) is associated with high morbidity and mortality. Rapid and non-invasive methods to detect this condition may substantially improve clinical care.
METHODS: We used liquid and gas chromatography–quadruple time-of-flight–mass spectrometry (LC/GC-QTOF-MS) analysis in a non-targeted metabolomics approach on the plasma from morbidly obese patients undergoing bariatric surgery to gain a comprehensive measure of metabolite levels. On the basis of these findings, we developed a method (GC-QTOF-MS) for the accurate quantification of plasma α-ketoglutarate to explore its potential as a novel biomarker for the detection of NAFLD.
RESULTS: Plasma biochemical differences were observed between patients with and without NAFLD indicating that the accumulation of lipids in hepatocytes decreased β-oxidation energy production, reduced liver function and altered glucose metabolism. The results obtained from the plasma analysis suggest pathophysiological insights that link lipid and glucose disturbances with α-ketoglutarate. Plasma α-ketoglutarate levels are significantly increased in obese patients compared with lean controls. Among obese patients, the measurement of this metabolite differentiates between those with or without NAFLD. Data from the liver were consistent with data from plasma. Clinical utility was assessed, and the results revealed that plasma α-ketoglutarate is a fair-to-good biomarker in patients ($n = 230$). Other common laboratory liver tests used in routine application did not favourably compare.
CONCLUSION: Plasma α-ketoglutarate is superior to common liver function tests in obese patients as a surrogate biomarker of NAFLD. The measurement of this biomarker may potentiate the search for a therapeutic approach, may decrease the need for liver biopsy and may be useful in the assessment of disease progression.

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Keywords: biomarker; citric acid cycle; energy; oxoglutaric acid; mitochondria; steatosis

INTRODUCTION

Obesity severely affects human health. The number of adults with morbid obesity (that is, a body mass index (BMI) $\geqslant 40\ kg\ m^{-2}$) is increasing, and the prevalence of obesity (>30% in some countries) is unacceptably high. Moreover, the incidence of obesity is increasing among children, and obesity-associated premature mortality rivals that of smoking.[1–4]

Morbidly obese patients undergoing bariatric surgery share a common metabolic background and similar environmental factors. We assume that possible genetic differences among these particular obese patients are likely negligible. Non-alcoholic fatty liver disease (NAFLD) is no longer a benign condition and is now considered an important co-morbidity in these patients.[5–7] The prognosis is pessimistic because of the risk of progressive liver disease (for example, non-alcoholic steatohepatitis, fibrosis, cirrhosis, liver failure and hepatocarcinoma).[8] There is a great need for NAFLD biomarker discovery because it is often difficult to determine when a liver biopsy is appropriate. A significant number of individuals are asymptomatic until their liver begins to fail, at which point conventional treatments are useless.

Current laboratory tests are insufficient and unreliable for the determination of NAFLD presence. Previous estimates using common liver function tests in the plasma, such as the determination of alanine aminotransferase levels, suggest that the prevalence of NAFLD should be nearly 100% in obese patients.[9] Evaluation by proton magnetic resonance spectroscopy is expensive and is currently being refined,[10] and patients of this size did not typically fit into the apparatus. However, the use of liver biopsy revealed in our patients that a significant portion did not display fatty infiltration of hepatocytes. In this study, we aimed to better understand NAFLD pathogenesis in obesity and to identify a non-invasive metabolite biomarker.

We hypothesised that the excess in energy intake alters anabolic and catabolic functions, especially in the liver, which may be detected in a plasma metabolomic profile. We used non-targeted metabolomics to compare obese patients without

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steatosis with their affected counterparts to identify differences between these groups. We expected numerous and low differences among the measured metabolites, which may explain the lack of previous metabolomics-based testing for these diseases.[11] However, *post hoc* analysis and further quantification resulted in the qualification and verification of the plasma α-ketoglutarate concentration as a good indicator of NAFLD in obese patients.

MATERIALS AND METHODS

Participants

Our local ethics committee approved the study protocol, and written informed consent was obtained from the participants (EPINOLS/12-03-29/3proj6). Patients fulfilling the criteria established for morbid obesity (BMI $\geqslant 40\ kg\ m^{-2}$), for which bariatric surgery was indicated after numerous failed attempts to lose weight using non-surgical means, were recruited from the outpatient clinic between 2006 and 2012. We first conducted a non-targeted metabolomic analysis in a limited group of patients with or without steatosis ($n=15$ in each group). Steatohepatitis, fibrosis and hepatocyte injury in these patients were histologically ruled out. These samples were sent to Metabolon Inc. (Durham, NC, USA) for analyses. No differences in the quality of life were observed between the groups, and the selected patients did not consume alcohol or any prescribed medication that could alter liver function, including vitamin supplements. The results of these non-targeted preliminary analyses prompted us to qualify and verify plasma α-ketoglutarate as a candidate biomarker to identify NAFLD in obese patients. For this purpose, we recruited 230 patients in which the only inclusion criterion was application for bariatric surgery, thus including a wide variety of conditions likely to be encountered in clinical practise. Blood was obtained immediately before surgery and after clinical nutrition evaluation. Portions of the liver were obtained during the surgical procedure after patient consultation to minimise risks and to limit the variability based on location of biopsy.[12] Bio-banked samples ($n=54$) from a group of age- and sex-matched lean, healthy controls (BMI $< 24\ kg\ m^{-2}$)[13] were used to assess differences in plasma α-ketoglutarate levels between lean and obese patients. Dietary advice and standardised overnight fasting were implemented to ensure uniformity and consistency. Plasma aliquots were anticoagulated with EDTA and were frozen at −80 °C within 2 h after collection.

Clinical data and analytic measurements

Relevant data were extracted from clinical records or were obtained using standardised guidelines and routine laboratory methods.[14] The BMI was calculated as the weight in kilograms divided by the height in metres squared. Histological alterations in the liver biopsies were evaluated in sections stained with haematoxylin and eosin. The degree of steatosis was evaluated using image analysis software and was expressed as percentages (AnalySIS image software system, Soft Imaging System, Munster, Germany). Patients were considered free of steatosis when values were $\leqslant 5\%$. Patients with steatosis were arbitrarily classified as mild: 6–30%; moderate: 31–60%; and severe: >61%.[8,15] Portions of the liver biopsies were also used to measure α-ketoglutarate in tissues from patients with or without steatosis ($n=6$ in each group). The shape and size of mitochondria were determined in portions of the liver using standard transmission electronic microscopy ($n=3$ in each group).

Non-targeted metabolomic platform

Specimens. Samples outsourced to Metabolon's laboratory were extracted upon arrival. The instrument and overall process variability was 4% and 9%, respectively.

Chromatography. Chromatographic conditions have been previously described.[16] In brief, the liquid chromatography–mass spectrometry (LC–MS, LC–MS^2) platform was based on a Waters ACQUITY UPLC (Waters Technologies, Milford, MA, USA) and a Thermo-Finnigan (San Jose, CA, USA) LTQ mass spectrometer, which consisted of an electrospray ionisation source and a linear ion-trap mass analyser. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion. The samples for gas chromatography/mass spectrometry (MS) analysis were derivatised and analysed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionisation. Metabolites were identified using a reference library of ~2800 standard chemical entries that included retention times, mass (m/z) and MS or MS^2 spectra.

Quantitative measurement of plasma α-ketoglutarate using gas chromatography–quadrupole time-of-flight mass spectrometry analysis

Sample pre-treatment. This method was developed in our laboratory. A surrogate standard was added to maximise technical precision during the injection and recovery during the extraction procedure. We used deuterated succinic acid (Isotec Stable Isotopes, Miamisburg, OH, USA) rather than deuterated α-ketoglutarate, which readily exchanges deuterium.[17] A solution of deuterated succinic acid (25 μl, $2\ mg\ l^{-1}$) was added to aliquots of plasma that were thawed on ice at 4 °C (50 μl), deproteinised with 400 μl of methanol, mixed using a vortex (2 min) and centrifuged (15 000 *g*, 15 min, 4 °C). The supernatant was dried and stored at −80 °C. Samples were derivatised using 30 μl of methoxyamine hydrochloride in pyridine ($30\ mg\ ml^{-1}$) and incubated 1.5 h at 37 °C with agitation. Then, 30 μl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich, Steinheim, Germany) was added with shaking and further incubated in darkness for 1 h before analysis. We found this derivatisation step to be a reproducible and robust method, independent of the matrix and without appreciable losses in yield that minimised the loss of α-ketoacids to decarboxylation.[18] A calibration curve of α-ketoglutaric acid (Fluka, St Gallen, Switzerland) was prepared (0–82.5 μM) immediately before each assay.

Chromatographic analysis. Samples (1 μl) were automatically injected onto a 7890A gas chromatograph coupled to a 7200 quadrupole time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, USA) equipped with a J&W Scientific (Folsom, CA, USA) HP5-MS column (19091S-433). Helium was used as a carrier gas at a flow rate of $1.5\ ml\ min^{-1}$ in constant-flow mode. The oven programme was set at an initial temperature of 70 °C that was increased to 190 °C at a rate of $12\ °C\ min^{-1}$ followed by an increase to 325 °C at a rate of $20\ °C\ min^{-1}$ and a final hold at 325 °C for 3.25 min. Ionisation was performed using electronic impact with an electron energy of 70 eV and an emission intensity of 35 μA. Raw data were processed using MassHunter B.05.00 (Agilent Technologies). Plasma α-ketoglutaric acid was quantified using a target ion and was identified using qualifier ions and the retention time. The molecular weight of the derivatised molecule, retention time, quantifier and qualifier ions, relative abundance, recovery, accuracy, precision and additional pertinent results are provided in Supplementary Figure S1. The limit of detection was 0.001 μM. Instrumental reproducibility was 2.2%. Standard curves for the analysis were reproducible and displayed R^2 values of $\geqslant 0.99$, which indicated linearity over the measured concentration range; we did not detect carryover.

Statistical analysis

Significantly altered metabolites, which were corrected for multiple testing, were defined using a P-value < 0.05 and a predesigned false discovery rate.[19] We performed Welch's t-tests and/or Wilcoxon's rank sum tests for pairwise comparisons. A repeated measurement analysis of variance was used in some instances. We used multivariate statistics to improve the refining and distilling of complex raw data and for pattern recognition. Random Forests is a supervised classification technique based on an ensemble of decision trees.[20] This method provides an unbiased estimate of how well one can predict sample classes in a new data set (prediction accuracy) and a selection of variables that make the largest contributions to the classification. We also used linear discriminant analysis as a classical method of classification and principal component analysis as an unsupervised data analysis, which measures the innate variation in data sets. Ingenuity pathway analysis, which is a web-based functional analysis, was used to explore biomolecular interaction networks to identify the signalling and metabolic pathways and to compare the affected pathways. Fisher's exact test was used to calculate a P-value to determine the probability of the association between the metabolites and the canonical pathway. The network score was based on the hypergeometric distribution and was calculated using the right-tailed Fisher's exact test. Logistic regression analysis and receiver operator characteristic curves described and assessed the binary classification.[11,21] The employed statistical software included the program 'R' (http://cran.r-project.org) and the SPSS 18.0 package (IBM, Madrid, Spain).

RESULTS

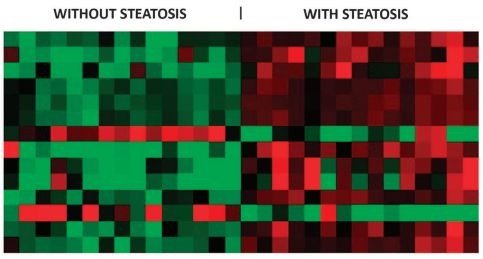
Plasma metabolites and obesity-related liver steatosis

Raw data. The baseline characteristics of the selected patients, raw data and an exhaustive list of the measured metabolites in untargeted metabolomic analyses are shown in Supplementary Tables S1 and S2. Most patients were female (73%) and BMI values ranged between 43.1 and 52.1 kg m^{-2}. Patients with steatosis showed significantly higher plasma cholesterol and triglyceride concentrations than those without steatosis. Common liver function tests revealed higher values in patients with steatosis, but significance was only reached for γ-glutamyl transpeptidase (Supplementary Table S1). We identified 316 metabolites of which 38 were significantly different between groups. Finally, 19 metabolites with the highest statistical difference were chosen using more stringent conditions for further consideration (Supplementary Figure S2). The relative abundance of perturbations in amino acids and lipid metabolism are shown in Figure 1a.

Interpretation. The citric acid cycle is the most important metabolic pathway for the energy supply and connects most metabolic pathways. The following alterations support the idea of defective liver function: (1) the levels of all three branched-chain amino acids (leucine, isoleucine and valine) were significantly increased in patients with steatosis; and (2) the levels of branched-chain keto acids (3-methyl-2-oxobutyrate, 3-methyl-2-oxovalerate and 4-methyl-2-oxopentanoate) were slightly elevated in the plasma from patients with steatosis. Steatosis may also sequester fatty acids from β-oxidation in liver cells using an alternative energy source, as shown by significant decreased levels of 3-hydroxybutyrate and a significant increase in the concentration of plasma α-ketoglutarate and succinylcarnitine in patients with steatosis. The mechanisms of lipolysis and gluconeogenesis appear to be affected (Supplementary Figure S3). Glucose metabolism is also altered in patients with steatosis. The significant decrease in the concentration of plasma 1,5-anhydroglucitol in these patients probably indicates long-term higher hyperglycaemia. In addition, hyperglycaemia-induced oxidative stress is likely in patients with steatosis, as indicated by the increased plasma levels of bradykinin and des-Arg9-bradykinin. The plasma level of the dicarboxylic acid 2-hydroxybutyrate (α–hydroxybutyrate), which is an early marker for impaired glucose regulation, was also significantly increased. Plasma glycocholate and taurocholate concentrations were higher in patients with steatosis, which indicates that the damaged livers were not functioning properly in the uptake of these compounds. The excretion of steroids is also limited in liver steatosis because these compounds are not effectively sulphated, which was indicated by significantly lower plasma levels of sulphated steroids (that is, 4-androsten-3β, 17-β-diol disulphate 1, 4-androsten-3β, 17-β-diol disulphate 2, 5α-androstan-3β, 17β-diol disulphate, 5α-pregnan-3β, 20α-diol disulphate, pregnen-diol disulphate, pregnen steroid monosulphate, andro steroid monosulphate 2 and 21-hydroxypregnenolone disulphate).

a

PATHWAY	SUBPATHWAY	COMPOUND
Amino acid	Creatine metabolism	creatine
	Cysteine, methionine, SAM, taurine metabolism	2-hydroxybutyrate (AHB)
	Glycine, serine and threonine metabolism	beta-hydroxypyruvate
	Valine, leucine and isoleucine metabolism	isoleucine
		valine
		leucine
Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism	1,5-anhydroglucitol (1,5-AG)
Energy	Krebs cycle	alpha-ketoglutarate
Lipid	Medium chain fatty acid	caprate (10:0)
		caprylate (8:0)
	Sphingolipid	stearoyl sphingomyelin
	Sterol/Steroid	andro steroid monosulfate 2*
		cholesterol
Nucleotide	Pyrimidine metabolism, uracil containing	uridine



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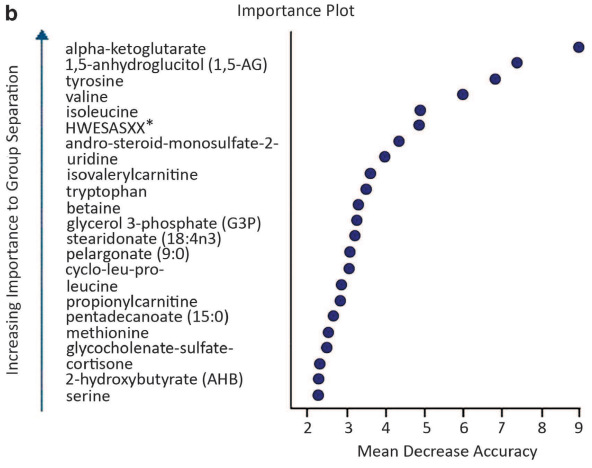


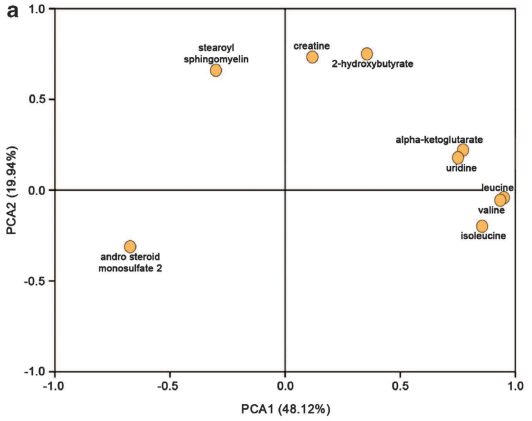
Figure 1. (**a**) Heat map of the relative plasma concentration of selected metabolites that may distinguish obese patients with or without steatosis (green, lower; red, greater). (**b**) Random (decision) Forest analysis was used as implemented in the R package software as a framework to create a large number of particular models built around the presence or absence of steatosis.

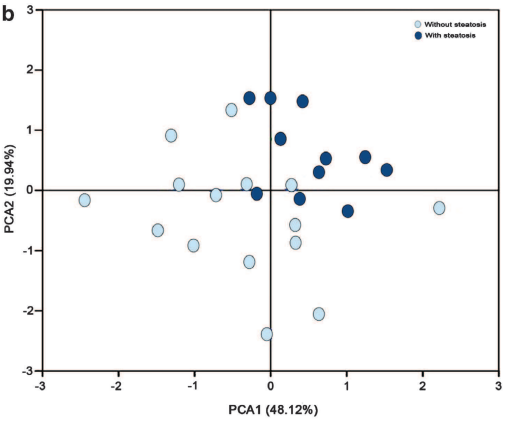
Pattern recognition

Random Forest analysis resulted in a predictive accuracy of >80% to distinguish patients with or without steatosis and revealed plasma α-ketoglutarate as the primary differentiator in a ranked list of metabolites in order of their importance in the classification scheme (Figure 1b). The application of linear discriminant analysis and principal component analysis yielded similar results for group clustering and pattern recognition (Figure 2), and logistic regression and receiver operator characteristic analyses produced a list of possible biomarkers.

Metabolite interaction networks

We uploaded the metabolite lists (with kyoto encyclopedia of genes and genomes IDs) onto an ingenuity pathway analysis server to identify the biological pathways and functions of the biomolecules of interest. The top-associated network functions were limited to scores of >24. We highlighted networks that were similar and that shared identical functions, that is, lipid metabolism, amino-acid metabolism, molecular transport and small molecule biochemistry. Only tRNA charging ($P = 0.0007$) and isoleucine degradation ($P = 0.0009$) were significantly different among the top canonical pathways. Interactions and regulatory networks are depicted in Figure 3, which integrates most of the altered metabolites based on their biochemical relationships in obese patients with steatosis. These plots indicate that L-glutamic acid upregulates α-ketoglutarate (2-oxoglutaric acid) and suggest a role of branched-chain amino-acid transaminase. Predicted disturbances, which probably result in α-ketoglutarate accumulation in the plasma, are depicted in Supplementary Figure S4.



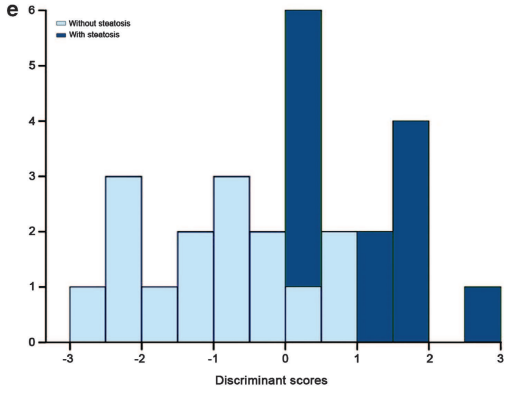


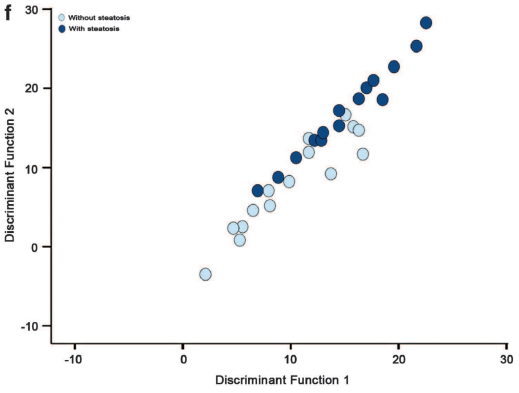
c Logistic regresion

	p-value	OR	95% CI Lower	95% CI Upper
PCA 1	0.044	4.675	1.044	20.946
PCA 2	0.023	7.098	1.310	38.47

d Receiver operating characteristic curve

	Area	p-value	%Sensitivity	% Specificity
PCs	0.842	0.003	80	86.7
α-ketoglutarate	0.903	0.001	80	93.5





g Logistic regresion

	p-value	OR	95% CI Lower	95% CI Upper	R^2 Nagelkerke
DF1	0.036	0.306	0.101	0.927	0.684
DF2	0.014	3.251	1.275	8.288	

h Receiver operating characteristic curve

	Area	p-value	%Sensitivity	% Specificity
PCs	0.900	<0.001	80	84.0
α-ketoglutarate	0.903	<0.001	80	93.5

Figure 2. Model plots constructed using principal component analysis (**a** and **b**) and linear discrimination analysis (**e** and **f**). Logistic regression analysis and receiver operator characteristic curves indicated the presence of a pattern for the selection of candidate biomarkers and group clustering. As shown in the calculations (**c**, **d**, **g** and **h**), plasma α-ketoglutarate was the most qualified for this purpose.

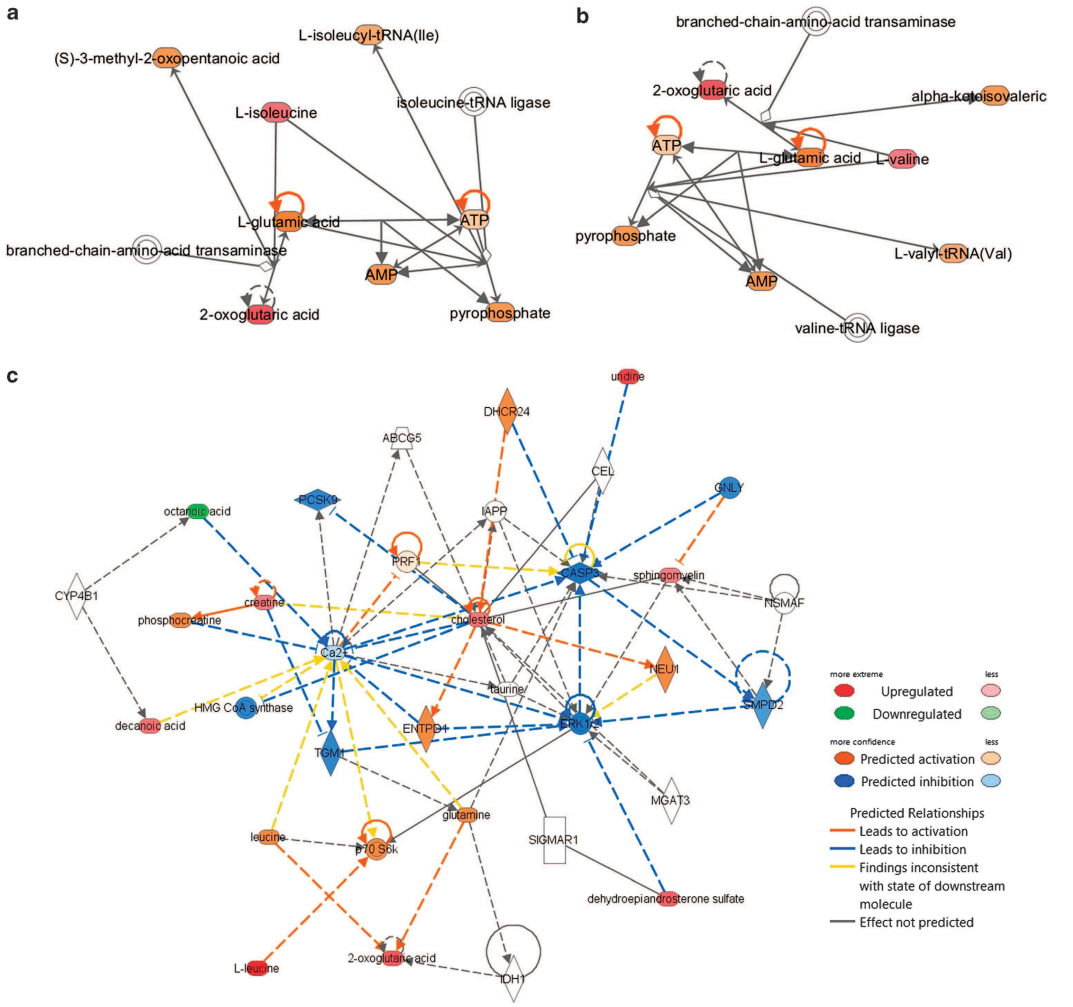


Figure 3. Interaction and regulatory networks associated with steatosis in morbidly obese patients as identified using the ingenuity pathway analysis. Plots are depicted for the top-associated network functions (score >24, **a** and **b**) and top canonical pathway (**c**).

These results indicate a role for mitochondrial dysfunction. Our preliminary findings also indicate that in patients without steatosis the mean diameter of the mitochondria was slightly lower, the matrix was more electron dense and the crests were more visible compared with patients with steatosis. More importantly, toroidal mitochondria were abundant and only observed in non-steatotic patients and there was a significantly higher accumulation of autophagosomes in patients with steatosis. It is therefore plausible that the detection of perturbations in the citric acid cycle (CAC) may facilitate the evaluation of mitochondrial functions in physiology and disease.[22]

Plasma α-ketoglutarate as a biomarker

The obtained results identified and qualified plasma α-ketoglutarate as a diagnostic biomarker.[23] We calculated, with 95% confidence, that the true area under the curve of the reported receiver operator characteristic curve for plasma α-ketoglutarate ranged from 0.90 to 0.96 with a specificity of 0.93 at a fixed sensitivity of 0.8 (Figure 2). However, these results were calculated using a low number of carefully selected patients. To decrease uncertainty or margin of error in the relevance of these measurements, we extended the analysis to a broad range of morbidly obese patients (Table 1) in which the degree of steatosis was widely distributed. Notably, steatosis was predominantly mild, and the other liver alterations were primarily benign (Supplementary Figure S5). Approximately 25% of our patients were insulin sensitive, but this condition was unrelated to the presence of NAFLD. Plasma α-ketoglutarate was slightly but significantly associated with homoeostasis model assessment values ($\rho = 0.25$, $P = 0.01$). We calculated that at least 115 cases would be required to determine whether plasma α-ketoglutarate outperforms other commonly used biomarkers using an inferential approach.[24] These minimum requirements were increased to 230 cases to ensure validity in a target group in which

Table 1. Selected clinical characteristics and laboratory variables in obese patients segregated by the degree of steatosis

	Without steatosis (n = *76*)	*Mild steatosis* (n = *86*)	*Moderate steatosis* (n = *52*)	*Severe steatosis* (n = *16*)
Clinical characteristics				
Male, *n* (%)	8 (10.5)	12 (13.9)	10 (19.2)	4(25.0)
BMI, kg m^{-2}	47.0 (44.1–51.0)	45.9 (43.2–49.5)	48.1 (44.1–51.1)	44.5 (42.9–47.8)
Laboratory variables[a]				
Total cholesterol, mmol l^{-1}	4.0 (3.4–4.9)	4.5 (3.9–5.4)	4.9 (4.2–5.5)[b]	4.2 (3.5–5.6)
HDL cholesterol, mmol l^{-1}	0.9 (0.7–1.1)	0.9 (0.7–1.0)	0.9 (0.8–3.4)	0.8 (0.5–1.1)
LDL cholesterol, mmol l^{-1}	2.4 (1.9–3.0)	2.7 (2.6–3.3)	3.0 (2.3–3.4)	2.6 (1.9–3.0)
Triglycerides, mmol l^{-1}	1.6 (1.2–1.9)	2.2 (1.6–2.7)[c]	2.0 (1.7–3.0)[b]	2.5 (1.6–3.0)
NEFAs, mEq l^{-1}	1.1 (1.0–1.4)	1.2 (1.0–1.5)	1.1 (0.8–1.6)	1.0 (0.9–1.7)
Glucose, mmol l^{-1}	7.1 (5.9–8.4)	7.4 (6.2–10.0)	7.9 (7.0–9.8)[b]	7.6 (6.7–13.4)
Insulin, pmol l^{-1}	74.6 (41.9–117.6)	79.3 (42.5–122.3)	82.7 (56.8–137.3)	91.5 (40.8–172.2)
HOMA-IR	3.3 (1.9–6.1)	3.9 (2.6–6.1)	3.9 (2.7–8.0)	3.61 (2.02–11.6)
AST, μKat l^{-1}	0.5 (0.3–0.8)	0.5 (0.4–0.7)	0.9 (0.6–1.1)[b,d]	1.1 (0.6–2.0)[e,f]
ALT, μKat l^{-1}	0.5 (0.4–0.6)	0.4 (0.4–0.7)	0.9 (0.5–1.2)[b,d]	0.6 (0.4–1.9)
GGT, μKat l^{-1}	0.2 (0.2–0.3)	0.3 (0.2–0.8)	0.4 (0.3–0.6)[b]	0.4 (0.3–0.8)[e]
LDH, μKat l^{-1}	2.5 (2.2–2.9)	2.7 (2.3–3.3)	3.2 (2.6–4.0)[b,d]	4.0 (2.8–6.7)[e,f]
Total bilirubin, mmol l^{-1}	7.0 (5.0–10.0)	8.0 (5.3–11.8)	8.5 (4.5–11.0)	9.5 (5.8–20.8)
Leptin, ng ml^{-1}	68.5 (55.4–97.5)	73.7 (51.9–97.5)	83.2 (59.5–114.1)	87.8 (75.2–185.4)
Adiponectin, μg ml^{-1}	3.6 (2.4–4.3)	2.6 (1.8–4.2)	2.4 (1.8–3.9)	2.2 (1.6–3.4)
CRP, mg l^{-1}	6.6 (5.0–10.9)	9.2 (6.7–13.2)	12.7 (8.1–18.5)[b]	11.4 (5.9–14.4)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, γ-glutamyl transpeptidase; HOMA-IR, homoeostasis model assessment-estimated insulin resistance; LDH, lactate dehydrogenase; NEFAs, non-esterified fatty acids. Significant differences (at least $P < 0.05$) in comparisons. [a]Values are given as median (interquartile range). [b]Without steatosis vs moderate steatosis. [c]Without steatosis vs mild steatosis. [d]Mild steatosis vs moderate steatosis. [e]Without steatosis vs severe steatosis. [f]Mild steatosis vs severe steatosis.

positive and negative outcomes are not equally distributed and in which the addition of fibrosis and/or inflammation may complicate the interpretation. A significant association between circulating triglycerides and steatosis was observed in these patients ($\rho = 0.42$, $P < 0.001$; see also Table 1), which is similar to the association observed with plasma α-ketoglutarate levels ($\rho = 0.49$, $P < 0.001$). Lean controls exhibited significantly ($P < 0.001$) lower (1.1 μM (0.82–1.37)) plasma α-ketoglutarate levels than obese patients (7.5 μM (5.5–10.8)) without overlap (median (interquartile range)). The values were also significantly higher in obese patients with steatosis than in those without steatosis, indicating the potential to discriminate between the different stages in the progression of these conditions (Figure 4). These differences were also observed in liver tissue; in samples without steatosis, α-ketoglutarate concentration was significantly ($P = 0.017$) lower (22.1 μM per 100 mg dry weight (11.3–31.3)) than in those with steatosis (57.8 μM per 100 mg dry weight (32.8–63.1)). Among the measured variables, only the plasma α-ketoglutarate concentration exhibited significant agreement with the degree of steatosis, which may differentiate between mild, moderate and severe steatosis. Although comparisons were significant, data are not shown for severe steatosis because the number of patients was considered too low to yield relevance. The area under the curve of the receiver operator characteristic curves exhibited a fair-to-good clinical utility (Figure 4). Other common laboratory liver function tests failed to discriminate between patients with or without NAFLD. Interestingly, the clinically considered 'gold standard', alanine aminotransferase, exhibited the worst performance. In contrast, γ-glutamyl transpeptidase performed relatively well (Supplementary Figures S6 and S7). We subsequently constructed multivariate models that combined the values of the available biomarkers, but the performance was lower than plasma α-ketoglutarate alone. Conversely, the addition of plasma α-ketoglutarate to any of these models improved the performance (Figure 5). The optimal clinically useful threshold (that is, the plasma concentration) rather than the mathematically optimal threshold is difficult to assign. The choice is highly dependent on the intended use. Ideally, all patients with steatosis should be correctly classified, but this may come at the cost that some patients may be incorrectly classified as free of steatosis. For example, 8 or 4 μM (which represent two mathematically possible candidates) yield a similar positive predictive value (>80%), but 8 μM provides high specificity (>85%) and 4 μM provides high sensitivity (>97%).

DISCUSSION

There is currently no clinically useful plasma surrogate for the assessment of hepatic steatosis in obesity. Multivariate metabolomics analyses provide meaningful information owing to the simultaneous global assessment of hundreds of endogenous metabolites in a biological sample. To perform studies in this context, it is important to ensure the maintenance of preanalytical aspects. Similar nutritional status and similar food intake are necessary because dietary factors have an important role as a causative factor of NAFLD.[25–27] As we predicted, inflammation does not appear to be a prominent factor,[28,29] but neither obesity nor NAFLD are simple, monogenic disorders. Therefore, we chose clinically controlled bariatric patients who exhibited a similarly high BMI.

The accumulation of lipids in hepatocytes induced a relative energy deficit, reduced liver function and disturbed insulin resistance, which is apparently accompanied by an acquired and reversible mitochondrial dysfunction.[30] Our data also partially support the importance of a newly described mechanism (lipophagy) that links lipolysis and regulation of intracellular lipid stores.[31] Notably, the deficit in sulphonation that we have observed in NAFLD patients may be clinically relevant. This is because phase II drug-metabolising enzymes maintain cellular homoeostasis via the metabolism of several endogenous molecules that may facilitate metabolic disorders and may result in the improper management of xenobiotics and endobiotics.[32,33] Therefore, the search for NAFLD diagnostics in obesity should focus on mechanisms of energy homoeostasis, mitochondrial biogenesis, fatty acid oxidation and glucose metabolism, which may require new strategies.[34] The results obtained from the plasma analysis are consistent with those obtained in the livers of different animal models and human studies, which demonstrate that steatosis is the cause or consequence of disturbances in hepatic lipid and glucose metabolism that decreases the ability to obtain energy.[8,25,35,36] The metabolic imbalance caused by obesity and

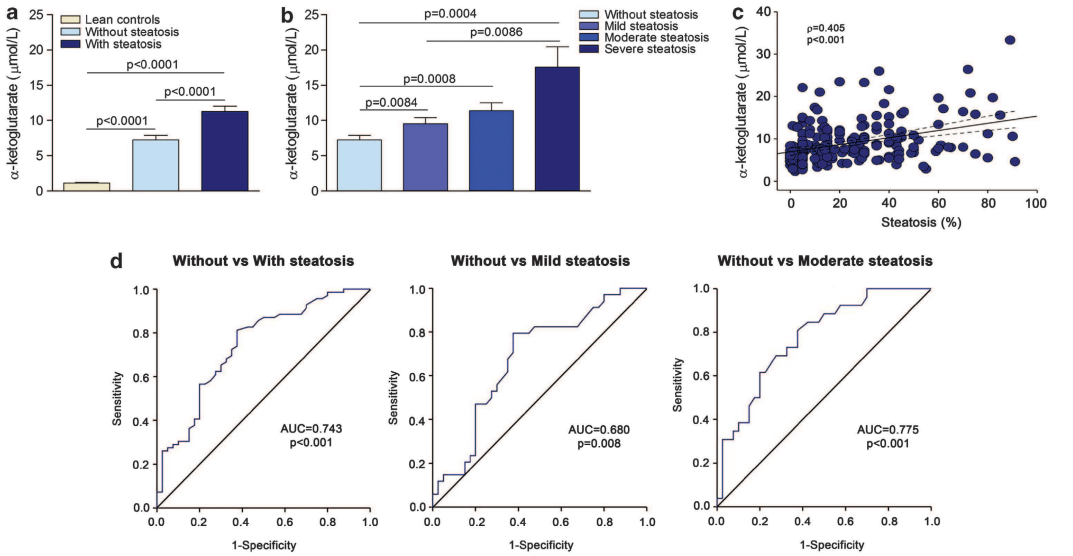
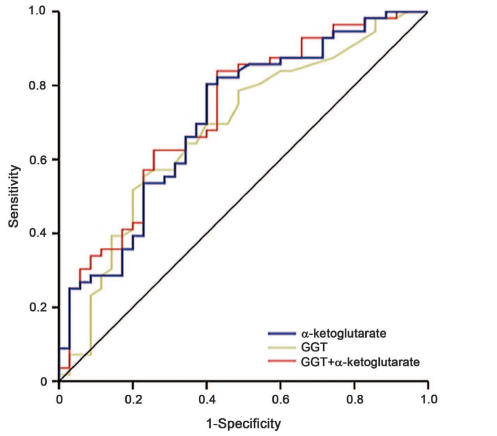


Figure 4. The mean plasma α-ketoglutarate concentration was significantly lower in lean controls and patients without steatosis (**a**) and displayed significant agreement with the degree of steatosis (**b** and **c**). The area under the curve of the receiver operator characteristic curves were significant, and clinical utility was approximately fair to good; for this purpose, the number of patients with severe steatosis was considered too low to yield relevance (**d**).



Variable	Area	Error	P-value	95% CI Lower	95% CI Upper	Sensitivity (%)	Specificity (%)
α-Ketoglutarate	0.743	0.050	<0.001	0.645	0.840	80.0	62.5
GGT	0.685	0.058	0.003	0.571	0.799	80.0	47.5
GGT+α-Ketoglutarate	0.727	0.055	<0.001	0.619	0.834	80.0	57.0

Figure 5. Multimetabolite biomarker models should provide more information than a single biochemical measurement, as assessed using receiver operator characteristic curves. However, predictive scores from multivariate models that combined several variables were not superior to plasma α–ketoglutarate alone, as exemplified by the combination with plasma γ-glutamyl transpeptidase (GGT), which displayed the best performance of commonly used laboratory biomarkers.

NAFLD affects mitochondrial metabolism and metabolic pathways, which are important for recycling α-ketoglutarate into and out of the mitochondrion and allow for the continuous production of intracellular messengers.[37] Finally, it has been recently described in a model of mitochondrial dysfunction (*PINK1* deficiency) that increased expression of α-ketoglutarate is the most prominent and early effect, probably representing a compensatory mechanism.[38]

Monitoring mitochondrial function via the quantitative evaluation of mitochondrial metabolite abundances may be an important new avenue of research in obesity-associated NAFLD. This is mainly because CAC metabolites have a central role in catabolic and anabolic functions (that is, it is amphibolic in nature). As in other non-communicable diseases, it is most likely adequate to adopt the view that steatosis is governed by a pivotal regulatory role of metabolic reprogramming in cell fate decisions.[39,40]

Plasma α-ketoglutarate levels may distinguish lean controls from obese patients with a 'predictive accuracy' of 100% and predict obese patients with or without NAFLD better than commonly used biomarkers. This result supports the potential clinical utility of plasma α-ketoglutarate levels. However, additional validation in other patients from an identical target population is required. Preliminary results in ongoing validation studies suggest that this novel biomarker deserves further evaluation and development and that this biomarker should be added to clinical practise. In addition, pilot studies indicate the usefulness of performing serial measurements in the same patient, which do not require high specificity or sensitivity, to monitor disease progression and/or the response to treatment. The major limitation of our results is the confinement to morbid obesity. We believe this is a blood test that provides a solution for a major unmet clinical need but implementation of a new biomarker is difficult and requires validation. Regardless of the attractiveness of plasma α-ketoglutarate as a biomarker, the high costs of validation hamper the introduction of new biomarkers and therefore commercial considerations may ultimately determine the clinical use.[41] However, commonly used laboratory tests do not appropriately guide clinical decisions. Imaging studies are also not possible for patients of this size, and patients do not readily accept more invasive studies.

We also confirmed the potential of metabolomics to influence patient care. Metabolomics is designed to delineate biological processes, and the selection of metabolites for development as clinical biomarkers should be performed *a priori* rather than *post hoc*. Our results indicate that this approach is not necessarily true if compound quantification follows metabolomics, which is a strategy that is currently feasible and is not time consuming. This study successfully utilised a metabolomics approach to provide new insights into the consequences of NAFLD in obese patients and supports the possible translational relevance of plasma α-ketoglutarate as a biomarker of a condition that carries an enormous burden of disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies this paper on International Journal of Obesity website (http://www.nature.com/ijo)

Supplementary Information corresponding to the manuscript:

Mapping of the circulating metabolome reveals α-ketoglutarate as a predictor of morbid obesity-associated nonalcoholic fatty liver disease

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Table S1. Baseline clinical characteristics and laboratory variables of obese patients with or without liver steatosis used for the first nontargeted metabolomics approach.

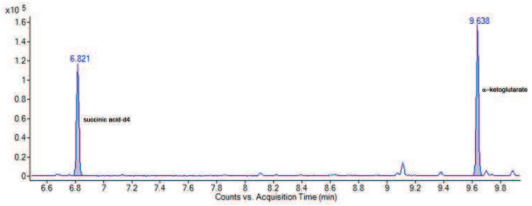
	Without steatosis (n=15)	With Steatosis (n=15)	p-value
Clinical characteristics			
Male, n (%)	4 (26.7)	4 (26.7)	1.000
BMI, kg/m^2	48.2 (44.6-52.1)	44.5 (43.1-46.2)	0.058
Laboratory variables			
Total cholesterol, mmol/L	3.9 (3.4-4.9)	4.8 (4.3-5.8)	**0.013**
HDL-cholesterol, mmol/L	0.9 (0.8-1.0)	0.9 (0.7-1.0)	0.624
LDL-cholesterol, mmol/L	2.4 (1.7-2.9)	2.8 (2.6-3.4)	**0.031**
Triglycerides, mmol/L	1.2 (1.0-1.6)	2.1 (1.6-2.7)	**0.019**
NEFAs, mEq/L	1.2 (1.0-1.7)	1.1 (0.8-1.5)	0.296
Glucose, mmol/L	7.0 (5.6-8.4)	9.2 (7.0-10.7)	0.077
Insulin, pmol/L	80.2 (67.2-119.2)	58.3 (35.0-152.8)	0.694
HOMA-IR	4.3 (2.8-7.1)	5.5 (1.8-9.6)	0.963
Albumin, g/L	35.0 (32.1-38.3)	36.4 (32.4-40.6)	0.457
AST, μKat/L	0.5 (0.3-0.8)	1.1 (0.6-1.1)	0.064
ALT, μKat/L	0.5 (0.3-0.6)	0.8 (0.5-1.3)	0.057
GGT, μKat/L	0.3 (0.2-0.5)	0.8 (0.3-1.1)	**0.009**
LDH, μKat/L	2.5 (1.8-2.8)	3.3 (2.6-4.5)	0.064
Total bilirubin, mmol/L	7.0 (6.0-7.8)	8.0 (6.0-10.8)	0.321
Leptin, ng/mL	80.2 (53.2-111.3)	78.7 (52.6-90.4)	0.485
Adiponectin, μg/mL	2.3 (1.6-3.3)	1.8 (1.5-4.1)	0.983
CRP, mg/L	6.0 (5.0-10.3)	9.3 (5.0-18.3)	0.297

Values are given as median (interquartile range). ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, γ-glutamyl transpeptidase; LDH, lactate dehydrogenase; NEFAs, nonesterified fatty acids.

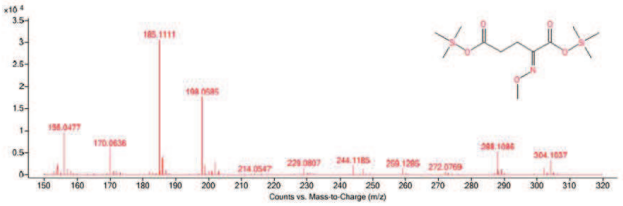
A

Retention time (min)	Quantifier ion				Qualifier ions				
	Ion	Theoretical m/z	Observed m/z	Mass error (ppm)	Ion	Theoretical m/z	Observed m/z	Mass error (ppm)	Relative abund. (%)
Succinic acid-d4									
6.818	M-CH_3	251.1073	251.1082	-3.6	M-(Me_3Si-OH)	176.0806	176.0800	2.8	29.3
α-Ketoglutarate									
9.644	M-(Me_3Si-OCH_3)	198.0586	198.0585	0.5	M-(OCH_3)	288.1087	288.1086	0.3	27.5
					M-CH_3	304.1036	304.1037	-0.3	18.0

B



C



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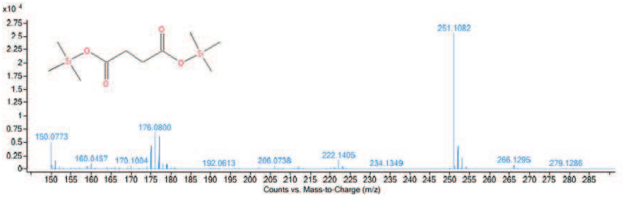

E

Concentration level[a], mg/l (*μM*)	Mean recovery (%)	Mean accuracy (%)	Intraday precision (%)	Interday precision (%)
0.52 (*3.6*)	-	117.1	5.1	9.3
0.72 (*4.9*)	99.7	101.7	3.3	10.9
5.52 (*37.8*)	99.3	91.1	3.7	10.4
40.52 (*277.3*)	94.0	92.3	1.2	10.4
100.52 (*688.0*)	96.5	101.3	1.5	8.5

[a] Final concentration of the spiked plasma pool, with addition of 0, 0.2, 5, 40 and 100mg/l of α-ketoglutaric acid standard.

Figure S1. Relevant analytical data obtained during the chromatographic analysis of plasma α-ketoglutarate, which was quantified using a target ion and identified using qualifier ions and the retention time (A). Extracted ion chromatogram of both, deuterated succinic acid (as internal standard) and α-ketoglutaric acid (B). Subtracted accurate mass spectra of derivatized succinic and α-ketoglutaric acid are also provided (C, D). The values for the recovery, accuracy, and imprecision (E) were considered acceptable.

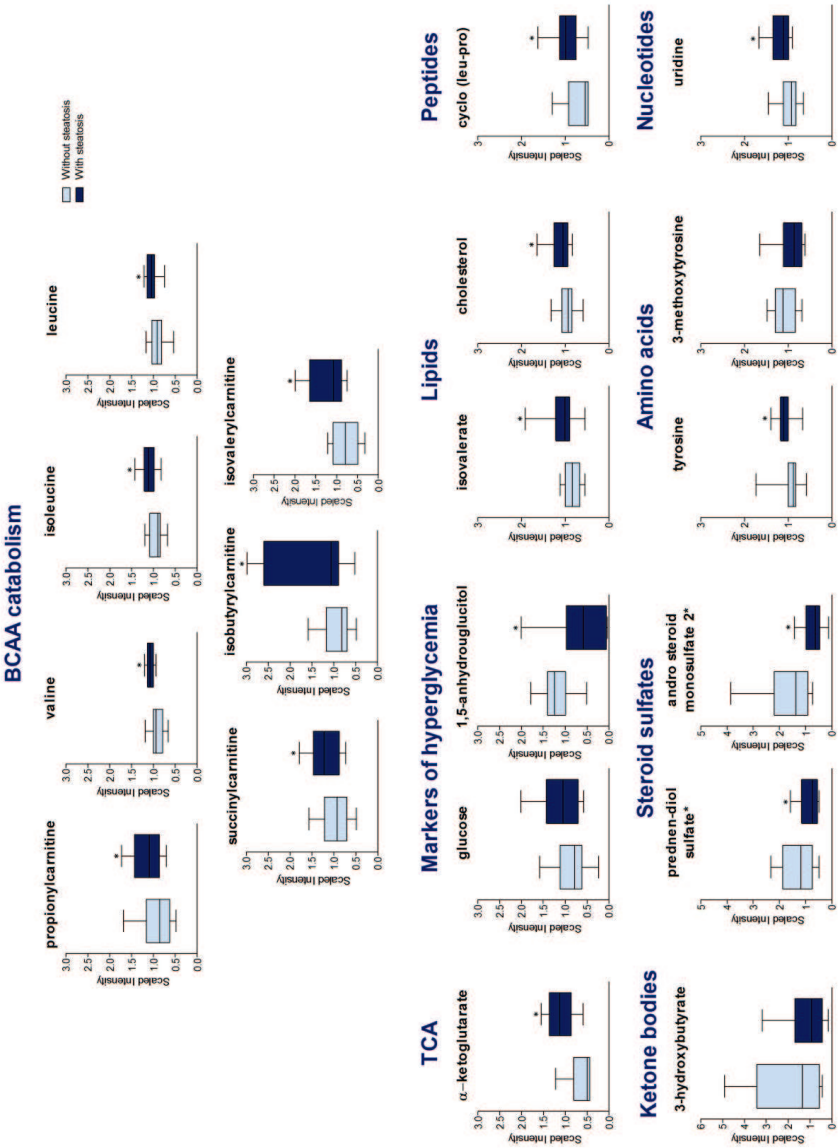

Figure S2. Among the selected metabolites, 14 were overexpressed in the plasma of obese patients with steatosis and five were overexpressed in patients without steatosis.

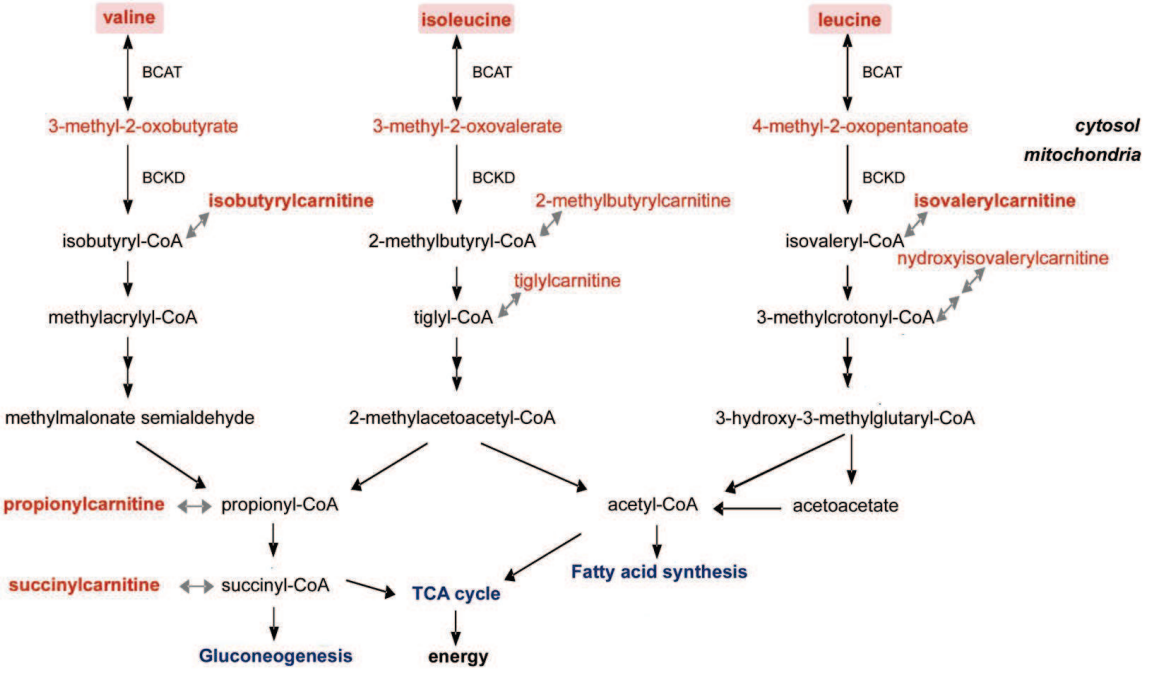

Figure S3. Morbidly obese patients with significant nonalcoholic fatty liver disease exhibited increased (in red) plasma levels of branched-chain amino acids and downstream metabolites. Valine, leucine, and isoleucine were significantly increased, which may represent a possible energy supplement during reduced β-oxidation.

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INFLAMMATION AND ENERGY METABOLISM IN OBESITY: THE SEARCH FOR BIOMARKERS AND NOVEL THERAPEUTIC STRATEGIES.
Esther Rodríguez Gallego
Dipòsit Legal: T 1337-2015

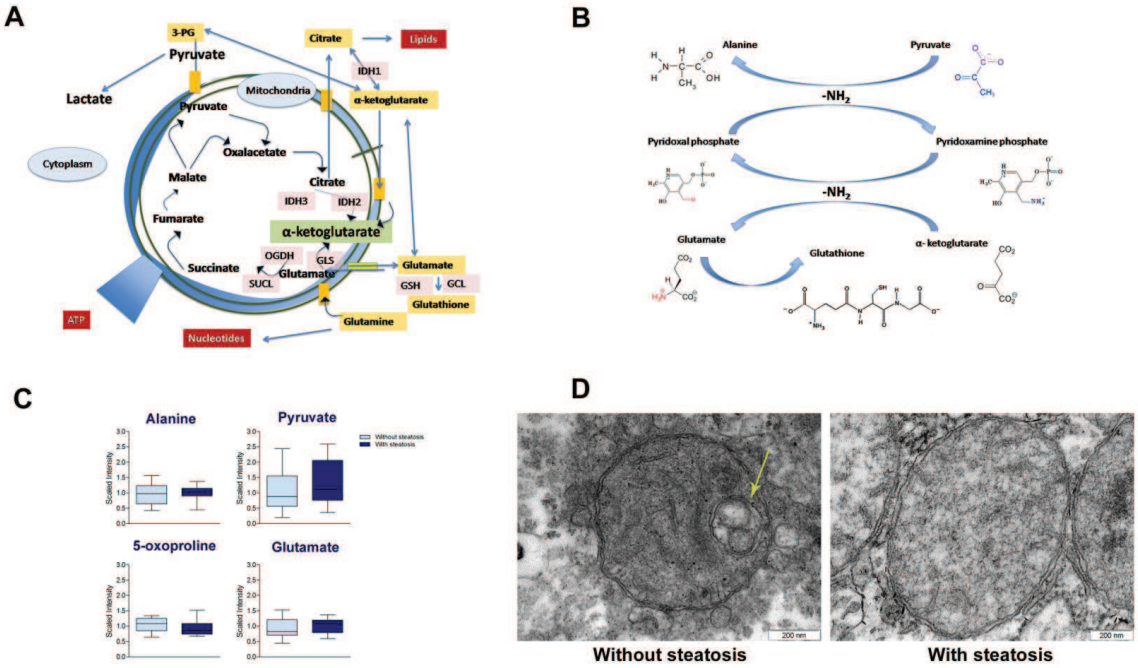

Figure S4. Predicted disturbances that likely result in the accumulation of α-ketoglutarate in the plasma. (A) The isocitrate dehydrogenase (IDH) reaction is carefully regulated to avoid the depletion of isocitrate, and, therefore, an accumulation of α-ketoglutarate. The 2-oxoglutarate or α-ketoglutaratedehydrogenase (ODGH) complex catalyzes the overall conversion of α-ketoglutarate to succinyl-CoA and CO_2 during the citric acid cycle. Succinate-CoA ligase (SUCL) facilitates the flux of molecules into other metabolic pathways by controlling the interconversion between succinyl-CoA and succinate. This control is important because succinyl-CoA is a necessary intermediate for many biosynthetic reactions. The substrates of glutamate synthase (GLS) that are required to produce L-glutamate are L-glutamine, α-ketoglutarate, NADPH, and H^+. Glutathione synthetase (GSS) and glutamate-cysteine ligase are responsible for glutathione biosynthesis. Among the glutamate sources for glutathione synthesis (B), the transamination of α-ketoglutarate to glutamate *via* alanine aminotransferase is relevant in the presence of liver disease. In this reaction, the amino group from alanine is transferred to pyridoxal phosphate to form pyridoxamine phosphate and pyruvate. In turn, the pyridoxamine phosphate provides the amino group that results in the formation of glutamate from α-ketoglutarate. However, glutathione malfunction is unlikely because the circulating amount of associated metabolites was similar in both groups of patients (C). Accordingly, we found changes in mitochondrial shape and size (n=3), which indicates a higher degree of autophagy in livers with steatosis, and the exclusive and frequent appearance of toroidal mitochondria, *i.e.*, the so-called "holes", in patients without steatosis (D).

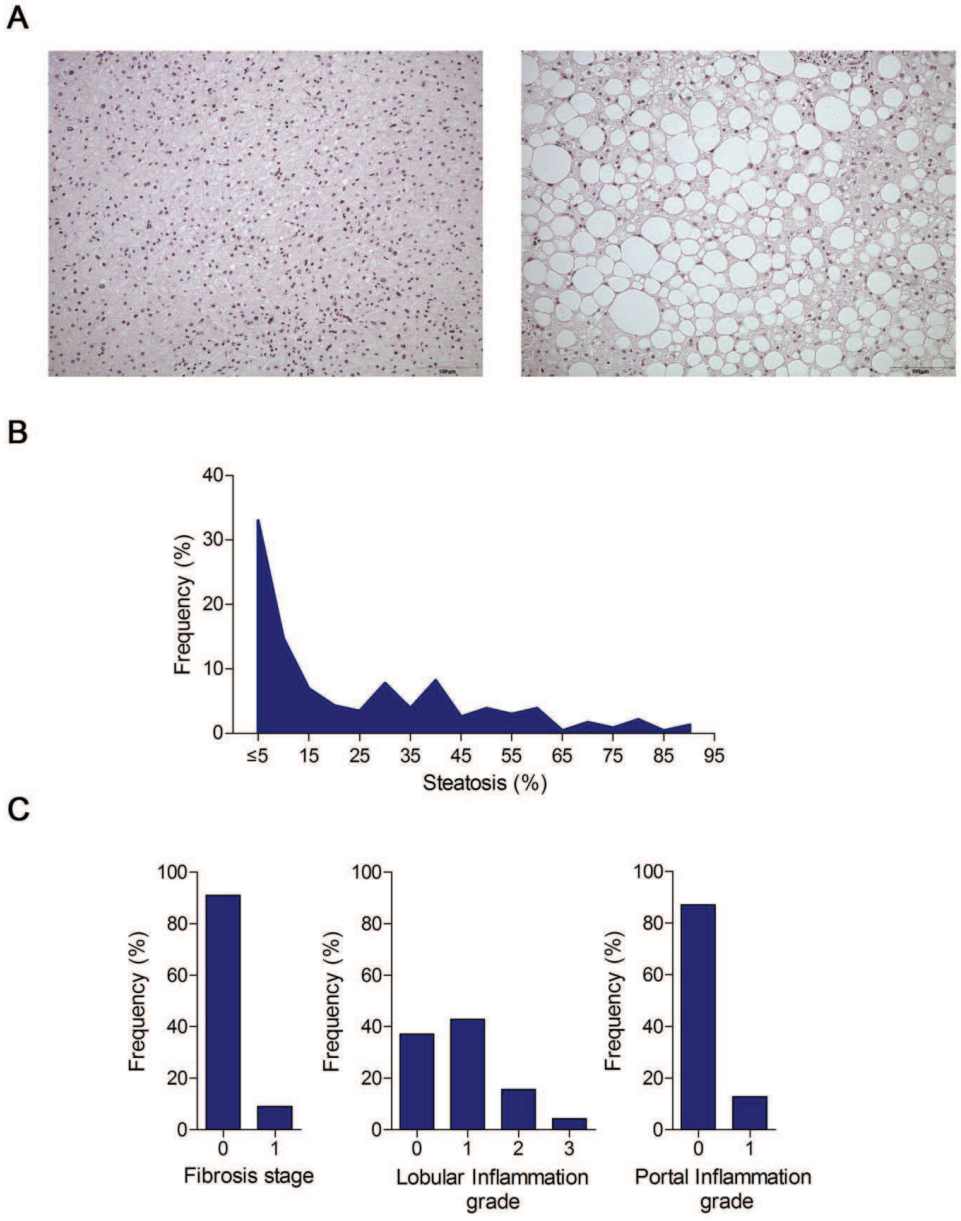

Figure S5. Histological examination of patient livers revealed significant differences among obese patients (A). The distribution of steatosis was represented as a percentage of patients with a given value of fatty infiltration. The amount of patients without steatosis was grouped in one point (≤5; B). The distribution of fibrosis and inflammation is shown in C.

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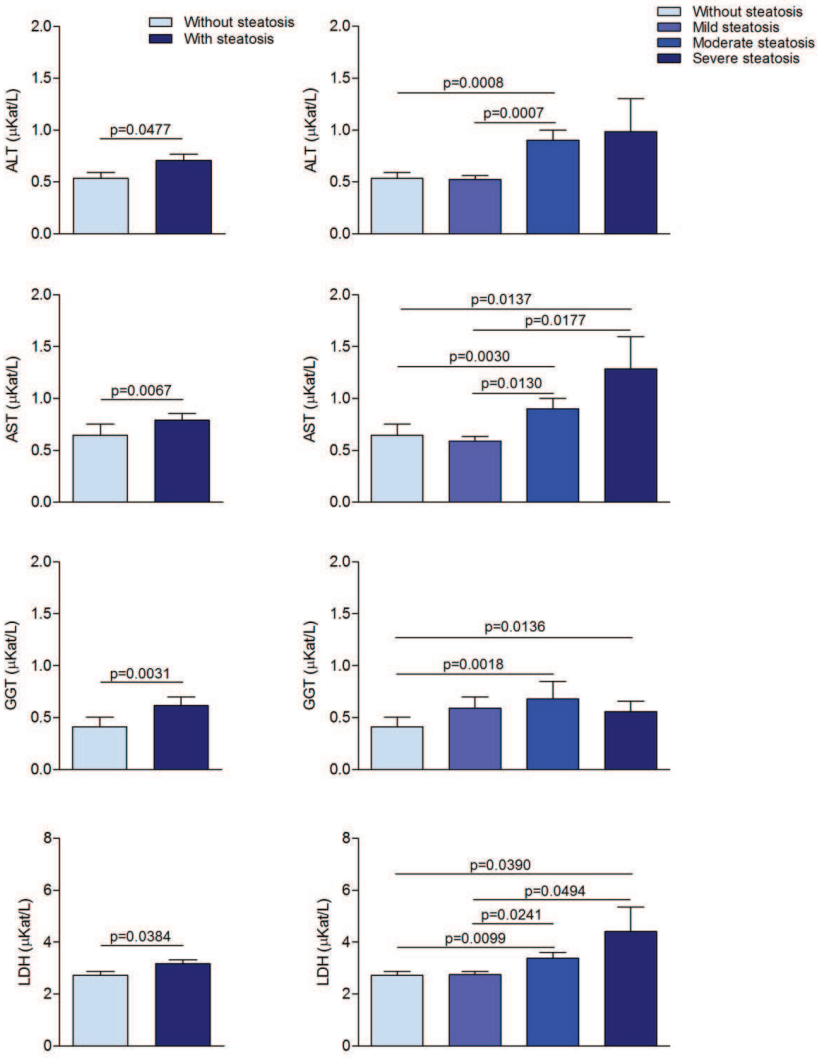
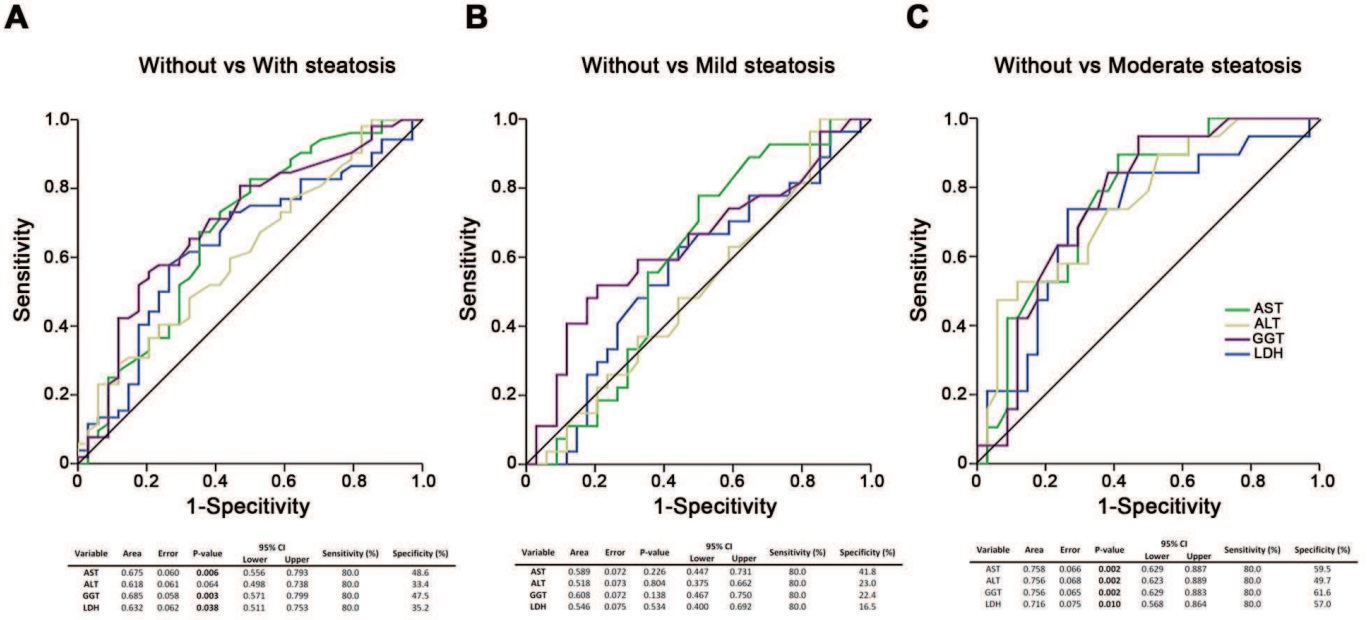


Figure S6. The plasma concentrations of selected metabolites from routine laboratory measurements in the study population depict differences between patients with and without steatosis and differences in arbitrarily considered degrees of steatosis.



Variable	Area	Error	P-value	95% CI Lower	95% CI Upper	Sensitivity (%)	Specificity (%)
AST	0.675	0.060	**0.006**	0.556	0.793	80.0	48.6
ALT	0.618	0.061	0.064	0.498	0.738	80.0	33.4
GGT	0.685	0.058	**0.003**	0.571	0.799	80.0	47.5
LDH	0.632	0.062	**0.038**	0.511	0.753	80.0	35.2

Variable	Area	Error	P-value	95% CI Lower	95% CI Upper	Sensitivity (%)	Specificity (%)
AST	0.589	0.072	0.226	0.447	0.731	80.0	41.8
ALT	0.518	0.073	0.804	0.375	0.662	80.0	23.0
GGT	0.608	0.072	0.138	0.467	0.750	80.0	22.4
LDH	0.546	0.075	0.534	0.400	0.692	80.0	16.5

Variable	Area	Error	P-value	95% CI Lower	95% CI Upper	Sensitivity (%)	Specificity (%)
AST	0.758	0.066	**0.002**	0.629	0.887	80.0	59.5
ALT	0.756	0.068	**0.002**	0.623	0.889	80.0	49.7
GGT	0.756	0.065	**0.002**	0.629	0.883	80.0	61.6
LDH	0.716	0.075	**0.010**	0.568	0.864	80.0	57.0

Figure S7. The performance of clinically used biomarkers to distinguish patients with or without steatosis (A) and the indicated degree of steatosis (B, C), which was considered approximately fail to poor as assessed by the area under the ROC curves. The number of patients with severe steatosis was considered too low to yield relevant comparisons.

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Research Article

Ubiquitous Transgenic Overexpression of C-C Chemokine Ligand 2: A Model to Assess the Combined Effect of High Energy Intake and Continuous Low-Grade Inflammation

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Excessive energy management leads to low-grade, chronic inflammation, which is a significant factor predicting noncommunicable diseases. In turn, inflammation, oxidation, and metabolism are associated with the course of these diseases; mitochondrial dysfunction seems to be at the crossroads of mutual relationships. The migration of immune cells during inflammation is governed by the interaction between chemokines and chemokine receptors. Chemokines, especially C-C-chemokine ligand 2 (CCL2), have a variety of additional functions that are involved in the maintenance of normal metabolism. It is our hypothesis that a ubiquitous and continuous secretion of CCL2 may represent an animal model of low-grade chronic inflammation that, in the presence of an energy surplus, could help to ascertain the afore-mentioned relationships and/or to search for specific therapeutic approaches. Here, we present preliminary data on a mouse model created by using targeted gene knock-in technology to integrate an additional copy of the CCl2 gene in the Gt(ROSA)26Sor locus of the mouse genome via homologous recombination in embryonic stem cells. Short-term dietary manipulations were assessed and the findings include metabolic disturbances, premature death, and the manipulation of macrophage plasticity and autophagy. These results raise a number of mechanistic questions for future study.

1. Introduction

Excessive energy intake is a part of the current human lifestyle that leads to a state of chronic systemic low-grade inflammation, which is thought to play a role in the development of atherosclerosis, cancer, and other noncommunicable diseases. At the same time, it is also plausible that the long-term consequences of prolonged inflammation exacerbate the deleterious effects of continuous nutrient surplus [1–3].

The immune system and metabolism are closely interconnected [4, 5]. During inflammation, the whole body is under metabolic stress, and energy excess management could

compromise the relationships among metabolism, oxidation, and inflammation. We reasoned that searching for an adequate animal model [6] might allow us to better understand disease pathogenesis.

Chemokines are promising candidates for the design of such a model. Some of the functions of chemokines are associated with the migration of immune cells, and chemokines are important for the correct functioning of metabolism. In humans, C-C chemokine ligand 2 (CCL2; formerly referred as MCP-1 or monocyte chemoattractant protein-1) could be a marker of inflammation; it is overexpressed in noncommunicable diseases and is involved in a variety of metabolic functions [7]. Actually, CCL2 modifies lipid and glucose metabolism and contributes to insulin resistance and hepatic steatosis [8–11]. Of note, circulating chemokines cause and maintain metabolic disturbances that may be reversed by anti-inflammatory drugs, and the role of chemokines is likely a causal and predisposing factor [12, 13]. Rather than local overexpression [14–17], it is now recognised that CCL2 protein and mRNA are expressed in the vast majority of tissues, suggesting both a systemic production and the ability to respond *in situ* to inflammatory stimuli [18, 19].

Therefore, we hypothesised that challenging an animal model that systemically overexpresses CCL2 with diets rich in fat and cholesterol could help to assess the role of chronic inflammation in response to excessive energy intake. We then proceeded to integrate a copy of the *Ccl2* gene in the Gt(ROSA)26Sor (commonly referred to as ROSA26) locus of the mouse genome via homologous recombination in embryonic stem cells (ES) to generate targeted transgenic mice [20–22] that overexpress CCL2 in all tissues. Preliminary data are promising and suggest a number of mechanistic questions for future study.

2. Material and Methods

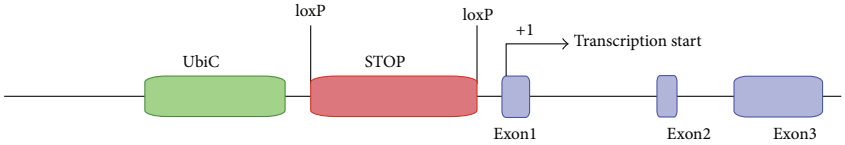
2.1. Animal Handling. All procedures and experimental protocols were examined and approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili. Basic protocols for tissue collection, diets, allocation concealment and metabolic assessment of the mice have been already described in detail [6, 18, 23]. Strains were backcrossed >10 generations to C57BL/6J mice and maintained homozygously. Littermates without mutations were used as controls (WT). We also provide data from knockouts (KO) of CCL2 (conveniently backcrossed), which were purchased from the Jackson Laboratory (Sacramento, CA). Dietary experiments began at 10 weeks of age, when all strains display similar phenotypes. To avoid possible effects of immature adipocyte modelling, most results were obtained in different groups after 6 or 14 weeks of treatment (16 and 24 weeksold, resp.). To explore dietary effects, mice from each group were fed either chow (Teklad rodent diet; Harlan, Barcelona, Spain) or a high-fat diet (FuttermittelfürMaüse; SSniff spezial diäten, Soest, Deutschland) and caged indefinitely under supervision. The breeding of all experimental populations was performed in our own facilities, and the progenies were maintained under close surveillance. The animals were not kept under germ-free conditions.

2.2. Targeted Transgenic (TG) Mice. The transgenic model was generated via a gene targeted inducible knock-in (KI), that is, a line with a duplicated gene, approach using standard methods and proprietary technology from Ozgene (Bentley, WA, Australia). The mRNA sequence corresponding to the mouse *Ccl2* gene (NM_011333 and ENSMUSG00000035385) is located on chromosome 11. The gene has 3 exons spread over approximately 3 Kb. The gene fragment was obtained from C57BL/6 genomic DNA (PCR primers AGCAAGATGATCCCAATGAGTAGGC and GAGGTGGTTGTGGAAAAGGTAGTGG) to be inserted by gene targeting into the ROSA26 locus. Upstream regulatory elements are important in the transcriptional regulation of *Ccl2* gene. Human ubiquitin promoter (Ubic) was chosen for the transgene to produce a high-level of expression. A loxP-flanked STOP cassette prevents the transcription of the gene following the UbiC promoter (See Figure 1 and Supplementary Materials S1 and S2 available online at http://dx.doi.org/10.1155/2013/953841). The STOP cassette can be removed using Crerecombinase. PGK-Neo-SD-IS, a selection cassette, is inserted downstream of the *Ccl2* gene to enrich homologous recombination events. The ROSA26 locus is conserved between mice and humans. The location is autosomal (chromosome 6) and is actively transcribed in most tissues (Figure 1). Moreover, epigenetic inactivation is unlikely [21, 24–26].

The combination of gene targeting and ES cell technology exploiting homologous recombination provides advantages over other techniques [27–31] (Supplementary Material S3). Mice are available upon request.

2.3. Immunopathology Studies and Assessment of Liver Steatosis. Portions of organs and tissues were either frozen in nitrogen or fixed in 4% phosphate-buffered formalin for 24 h at room temperature, washed twice with water, stored in 70% ethanol at 4°C, and embedded in paraffin for histological analyses. Primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and Serotec (Oxford, UK) [18, 32]. Detection was performed with the ABC peroxidase system (Vector, Burlingame, CA) using DAB (Dako, Glostrup, Denmark) as the substrate. To assess specificity, primary antibodies were omitted in the controls. Liver steatosis was assessed as previously described [6].

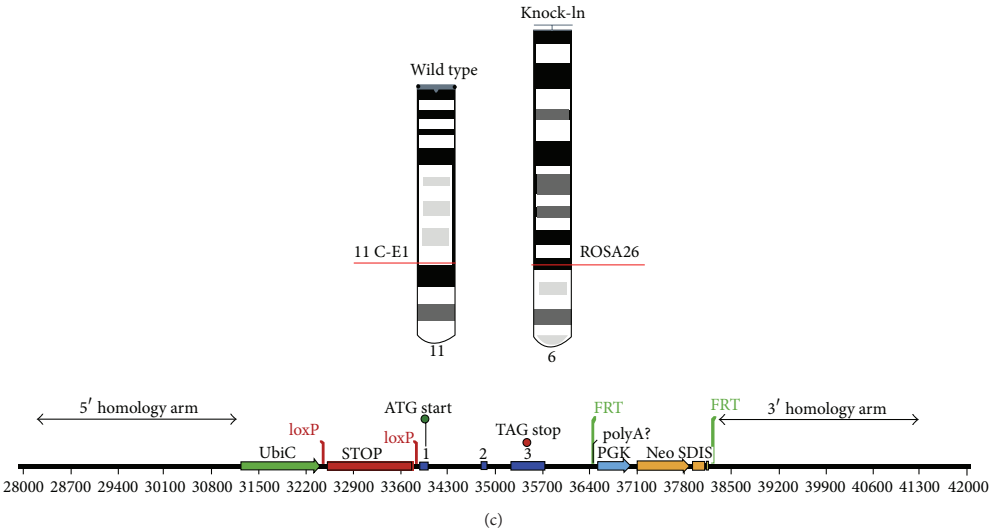
2.4. Laboratory Measurements. We measured murine CCL2 in plasma, serum, and tissues by ELISA (Peprotech, London, UK), according to the instructions of the manufacturer. Recombinant human CCL2 antigen was used as the calibrator for assay standardisation, and we found weak cross-reactivity with other chemokines, especially CCL7. The intraassay coefficients of variation were <3.2%, and the interassay of variation was <9.1%. Other biochemical measurements were performed in automated analysers using commercially available reagents as described [6, 33]. Selected tissues were homogenised using the Precellys 24 system (Izasa, Barcelona,



(a)

4681 ATTCTGGCCG TTTTTGGCTT TTTTGTTAGA CGCCACCATG CATATAACTT CGTATAGCAT
4741 ACATTATACG AAGTTATGGC GCGCCGATCC TCGGGGACAC CAAATATGGC GATCTCGGCC
4801 TTTTCGTTTC TTGGAGCTGG GACATGTTTG CCATCGATCC ATCTACCACC AGAACGGCCG
4861 TTAGATCTGC TGCCACCGTT GTTTCCACCG AAGAAACCAC CGTTGCCGTA ACCACCACGA
4921 CGGTTGTTGC TAAAGAAGCT GCCACCGCCA CGGCCACCGT TGTAGCCGCC GTTGTTGTTA
4981 TTGTAGCTGC TACTGTTATT TCTGGCACTT CTTGGTTTTC CTCTTAAGTG AGGAGGAACA
5041 TAACCATTCT CGTTGTTGTC GTTGATGCTT AAATTTTGCA CTTGTTCGCT CAGTTCAGCC
5101 ATAATATGAA ATGCTTTTCT TGTTGTTCTT ACGGAATACC ACTTGCCACC TATCACCACA
5161 ACTAACTTTT TCCCGTTCCT CCATCTCTTT TATATTTTTT TTCTCGAGGG ATCTTTGTGA
5221 AGGAACCTTA CTTCTGTGGT GTGACATAAT TGGACAAACT ACCTACAGAG ATTTAAAGCT
5281 CTAAGGTAAA TATAAAATTT TTAAGTGTAT AATGTGTTAA ACTACTGATT CTAATTGTTT
5341 GTGTATTTTA GATTCCAACC TATGGAACTG ATGAATGGGA GCAGTGGTGG AATGCCTTTA
5401 ATGAGGAAAA CCTGTTTTGC TCAGAAGAAA TGCCATCTAG TGATGATGAG GCTACTGCTG
5461 ACTCTCAACA TTCTACTCCT CCAAAAAAGA AGAGAAAGGT AGAAGACCCC AAGGACTTTC
5521 CTTCAGAATT GCTAAGTTTT TTGAGTCATG CTGTGTTTAG TAATAGAACT CTTGCTTGCT
5581 TTGCTATTTA CACCACAAAG GAAAAAGCTG CACTGCTATA CAAGAAAATT ATGGAAAAAT
5641 ATTCTGTAAC GTTTATAAGT AGGCATAACA GTTATAATCA TAACATACTG TTTTTTCTTA
5701 CTCCACACAG GCATAGAGTG TCTGCTATTA ATAACTATGC TCAAAAATTG TGTACCTTTA
5761 GCTTTTTAAT TTGTAAAGGG GTTAATAAGG AATATTTGAT GTATAGTGCC TTGACTAGAG
5821 ATCATAATCA GCCATACCAC ATTTGTAGAG GTTTTACTTG CTTTAAAAAA CCTCCCACAC
5881 CTCCCCCTGA ACCTGAAACA TAAAATGAAT GCAATTGTTG TTGTTAACTT GTTTATTGCA
5941 GCTTATAATG GTTACAAATA AAGCAATAGC ATCACAAATT TCACAAATAA AGCATTTTTT
6001 TCACTGCATT CTAGTTGTGG TTTGTCCAAA CTCATCAATG TATCTTATCA TGTCTGGATC
6061 TGACATGGTA AGTAAGCTTG GGCTGCAGGT CGAGGGACCT AATAACTTCG TATAGCATAC
6121 ATTATACGAA GTTATGCGGC CGCGAATTCA AAGCAGAGCC ACTCCATTCA CACTTGTGGT

(b)



(c)

Figure 1: A STOP sequence flanked by loxP sites was inserted between the Ubiquitin promoter and the mouse *Ccl2* gene (a). The sequences of both the STOP cassette (bold) and the loxP sites (underlined) are shown later (b). The wild-type allele for *Ccl2* gene is located in the region 11 C-E1 of chromosome 11 and the transgenic vector (bottom) is inserted in the ROSA26 locus of chromosome 6 (c). The procedure is designed to avoid chromosomal instabilities.

Spain) with prefilled bead tubes in the buffer of choice. Fractions of the homogenised liver were immunoblotted as described [34], using antibodies and reagents from Santa Cruz Biotechnology (Heidelberg, Germany).

2.5. Transmission Electron Microscopy. Small pieces of the liver were immediately fixed in a 2% glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.4. Samples were then post-fixed in 1% osmium tetroxide (OsO_4) for 2 h and dehydrated in sequential steps of acetone prior to impregnation in increasing concentrations of the resin in acetone over a 24 h period. Semithin sections (500 nm) were stained with 1% toluidine blue. Ultrathin sections (70 nm) were subsequently cut using a diamond knife, double-stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (Hitachi, Tokyo, Japan).

2.6. Characterisation of Mouse Bone Marrow-Derived Macrophages. The methods were performed as previously described [35]. Bone marrow cells were isolated by removing leg bones from WT and TG mice (aged 10 weeks) and were cultured for 24 hours. Floating cells were removed, and the remaining attached cells were analysed. Cells were further cultured in DMEM supplemented with 10% inactivated foetal calf serum, 50 μM beta-mercaptoethanol, and 1000 U/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF) or 25 ng/mL human macrophage colony-stimulating factor (M-CSF) (ImmunoTools, Friesoythe, Germany) to provide polarised activation of cells into M1 and M2 as a simplified descriptor of their functional plasticity. To assess the effect of activation, macrophages were treated with 100 ng/mL *E. coli* 055:B5 lipopolysaccharide (LPS) for 24 hours and were compared with the respective untreated controls. After this treatment, supernatants from M1 (GM-CSF) and M2 (M-CSF) macrophages were tested for the presence of CCL2, tumour necrosis factor-α (TNFα), and interleukin 10 (IL-10) using ELISA (BioLegend, Inc., Madrid, Spain). Total RNA was extracted using the RNeasy kit (Qiagen, Barcelona, Spain) and was retrotranscribed using the Reverse Transcription System kit (Applied Biosystems; Invitrogen, Barcelona, Spain). Oligonucleotides for selected genes were designed according to the Roche software for quantitative real-time PCR (Universal Probe Roche library), which was performed using a LightCycler 480 (Roche Diagnostics, Barcelona, Spain). The assays were performed in triplicate, and the results normalised according to the expression level of TATA-binding protein mRNA. C-C chemokine receptor type 2 (CCR2 or CD192), TNFα, inhibin beta A (INHBA), inducible nitric oxide synthase (iNOS), C-C chemokine receptor type 7 (CCR7), and Egl nine homolog 3 (EGLN3) were chosen as M1 markers. Arginase (ARG), EMR1/F4/80, insulin growth factor-1 (IGF1), IL-10, the mannose receptor CD206, and growth arrest-specific 6 (GAS6) were chosen as M2 markers.

2.7. Statistical Analyses. The normality of the distributions was assessed using the Kolmogorov-Smirnov method. Variables were compared using Mann-Whitney tests or Kruskal-Wallis one-way analysis adjusted for multiple testing. Unless otherwise indicated, the values in the figures represent the mean and SEM obtained in groups of 8 mice. The χ^2 test was used to compare categorical variables. For all measurements, we used either SPSS (SPSS Inc., Chicago, IL) or GraphPad Prism software (http://www.graphpad.com/scientific-software/prism/).

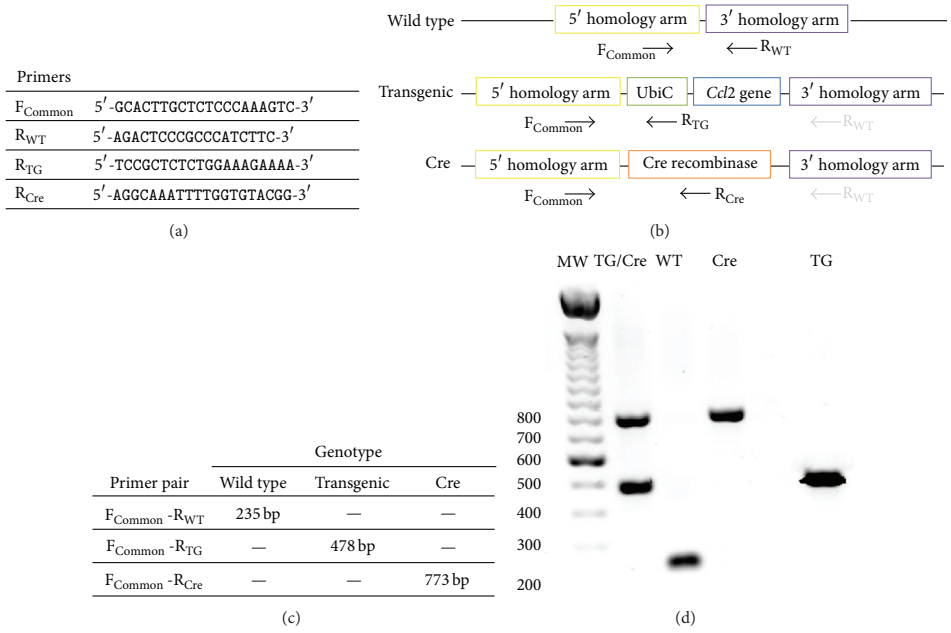
3. Results

3.1. Targeted Transgenic Mice Do Not Display Physical Abnormalities. The resulting mice for the targeted mutation are viable, fertile, and normal in size and weight. The animals do not display apparent behavioural or reproductive defects. The transgene insertion of a single copy occurs at a defined site, which allows for easy genotyping (Figure 2) and eliminates possible instabilities, independent segregation during breeding, and unpredictable positions in the chromosomes.

An additional advantage of this strategy is the Cre/lox recombination system that facilitates tissue-specific overexpression. The Ubic is conditioned by an Lox-Stop-Lox (LSL) element that is activated by Cre-mediated excision using the appropriate, tissue-specific Cre strain.

3.2. Transgenic Mice Overexpress CCL2 in Selected Tissues, and Circulating Protein Is Increased with respect to Controls. Consistently, transgenic mice displayed more CCL2 protein in all tissues examined with respect to WT animals. The differences increased with age, and there were minor relative differences among tissues (Figure 3). We confirm that CCL2 was immunologically detected in all selected tissues of the transgenic mice. The CCL2 mRNA expression in the transgenic mice was also higher in different types of cells with respect to WT mice. The amount of CCL2 expression was higher after the designed period of exposure to a diet with a high fat content. Of note, the serum and plasma CCL2 were also higher in transgenic mice than in WT mice, which is most likely caused by CCL2 secretion by multiple tissues. In accordance with previous observations, the plasma concentrations differed from the serum concentration. The differences are likely caused by coagulation and handling, but the differences were not statistically significant in transgenic mice. Notably, CCL2 was also detected in KO mice, but with less intensity. This is most likely due to quantitatively minor cross reactivity, as described in the methods.

3.3. Dietary Factors Influence Body Weight and Adipocyte Size. When mice were fed a regular chow diet, we did not observe significant differences in body weight increase among groups. The cumulative food intake was identical for the three strains examined. In contrast, when fed a high fat diet, both transgenic animals and WT animals developed obesity. Of note, the C57BL/6J male mouse is a commonly used model of diet-induced obesity [36]. The effect of CCL2 overexpression was apparent immediately after the ingestion of the high calorie diet, and the weight increased more rapidly than in WT mice. The absence of CCL2, however, protected the KO mice from excessive weight gain. The lack of



Primer pair	Genotype		
	Wild type	Transgenic	Cre
F_{Common} -R_{WT}	235 bp	—	—
F_{Common} -R_{TG}	—	478 bp	—
F_{Common} -R_{Cre}	—	—	773 bp

(c)

Figure 2: Simplified strategy for genotyping that includes the sequence of each primer (a), the reaction proposed for each primer (b), and the expected PCR products for each strain (c). The method is designed for the concomitant use of all primers and a representative gel is shown in (d).

significant differences in the food intake excluded any effect of CCL2 on appetite (Figure 4).

Overexpression of CCL2 also increased the size of the adipocytes. Data are presented for epididymal adipose tissue (Figure 5), but the effect was similar in other adipose tissues. The adipocyte size was significantly higher in CCL2 transgenic animals compared with WT and KO animals fed with both diets, but the difference was higher when mice were fed a diet with a high caloric content. When different types of adipose tissue were weighed, we found that the mice fed a chow diet showed no significant differences between the strains, with the possible exception of inguinal tissue. Conversely, the addition of fat to the diet resulted in a significant increase in the weight of white adipose tissue from other depots in mice with CCL2 overexpression. Notably, there was no effect on the weight of brown adipose tissue (Figure 6 and Supplementary Material S4). However, these differences among groups in adipose tissues weight disappeared when mice were fed with a high fat diet for 14 weeks. These results are probably indicating an already reported effect of adipose tissue remodelling on the consequences of high-fat dietary intake [37] (Supplementary Material S5).

3.4. Diet-Induced Disturbances in Glucose and Lipid Metabolism. Glucose tolerance tests (a proxy for insulin resistance) were unaffected in strains fed the chow diet during the experimental period of 6 weeks. However, WT littermates, KO, and transgenic mice displayed abnormal values when fed a high-fat diet, confirming the effect of diet in the pathogenesis of insulin resistance and suggesting that this short-term intervention is not adequate to investigate a possible role, if any, of CCL2 in the generation of glucose and lipid disturbances. Moreover, there were no differences among the strains in the plasma glucose levels after 6 hours of fasting, and after 3 hours in the fasting state, we found that the plasma glucose baseline concentrations were significantly higher in CCL2 overexpressing mice with respect to CCL2 deficient animals. This effect was more evident in the transgenic mice (Supplementary Material S6) but differences in plasma glucose disappeared after 14 weeks of dietary treatment suggesting immature adipose tissue remodelling [38].

When these tests were performed in animals fed a high-fat diet for a longer experimental period of 14 weeks in which adipose tissue is already well modelled, the lack of differences in insulin tolerance was maintained, probably indicating that the effect of CCL2 overexpression in the pathogenesis of insulin resistance is negligible.

However, results in the absence of CCL2 indicate that this chemokine may modify glucose metabolism and therefore we cannot discard the effects under a more intense metabolic stress [9]. Variations in plasma cholesterol and triglycerides concentrations were minimal among the strains at 16 weeks old. A high-fat diet significantly increased the amount of circulating cholesterol, an effect that was higher in CCL2 overexpressing mice. Conversely, there were unexpected, and most likely not representative, changes in the plasma

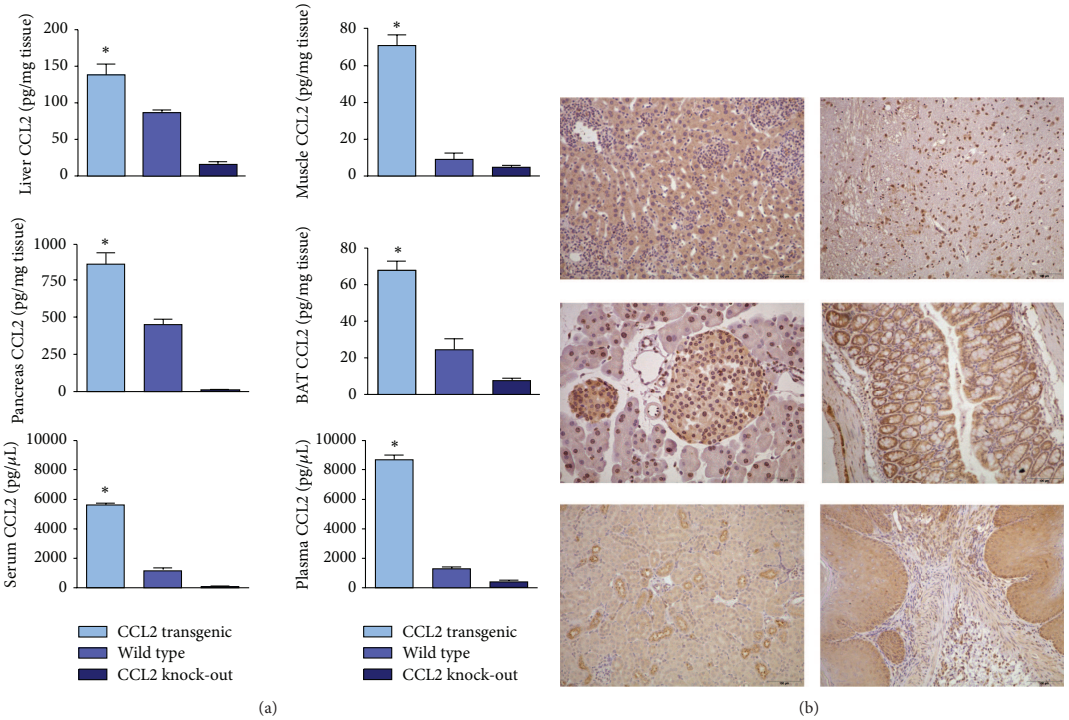


Figure 3: Overexpression of CCL2 with respect to wild type and knockout was observed in all selected tissues (extracts) in the transgenic mice as measured by ELISA. Differences were also observed in plasma and serum and there was cross-reactivity with similar chemokines that could explain the detection of CCL2 in KO mice (a). CCL2 was also detected by immunochemistry in different types of cells (b). $^*P < 0.005$; Micrographs in the left column are representative for liver, pancreas, and kidney. Those in the right column were for brain, intestine, and stomach.

triglycerides concentration of these mice as a consequence of dietary manipulations (data not shown).

3.5. The Influence of CCL2 and Dietary Manipulations in the Liver. When fed the chow diet, mice did not display significant differences among strains in the appearance of their liver tissue. The steatosis scores did not detect significant differences among strains, although some minor variations were detected (Figure 7) that did not correlate with the hepatic lipid content (data not shown). When mice were fed a high-fat diet, we found a certain amount of lipid accumulation in WT mice, but this lipid accumulation was significantly more evident in transgenic mice. Conversely, there was no accumulation of lipids in KO mice (Figure 7). Therefore, the effect of CCL2 under these conditions is directly related to the amount of tissue CCL2 disposal; the absence of CCL2 prevents liver steatosis, and overexpression of CCL2 predisposes the liver to steatosis. We also found that the expression of fatty acid synthase in the liver increased significantly in all strains when fed a high-fat diet, but there were no significant differences in the comparisons between transgenic and KO mice. We also explored the activating phosphorylation of AMP-activated protein kinase (AMPK), and values did not change as a result of high-fat diet in transgenic mice and were significantly higher in KO mice compared with transgenic mice (Supplementary Material S7).

When the livers were examined for the presence of F4/80 antigen, a widely accepted marker of macrophages, we found that both dietary fat and overexpression of CCL2 modify the size, number and morphology of liver macrophages (Figure 8 and Supplementary Material S8). Of note, F4/80 stained cells were more frequent in KO mice, a finding that merits further study because these results could represent a change in function and could be responsible for the differential effects of CCL2 in liver steatosis. We then explored the influence of both CCL2 and diet in mitochondrial biogenesis. Based on the appearance of the matrix, the mitochondria are healthier in mice fed a chow diet than in those fed a high-fat diet. The matrix was also consistently less electron-dense in transgenic mice. We also found altered fusion dynamics. In transgenic mice fed a chow diet, the process was unbalanced towards mitochondrial fusion, but the dietary manipulation significantly elicited a shift towards fission. The changes were similar in WT mice, but the effect of diet was

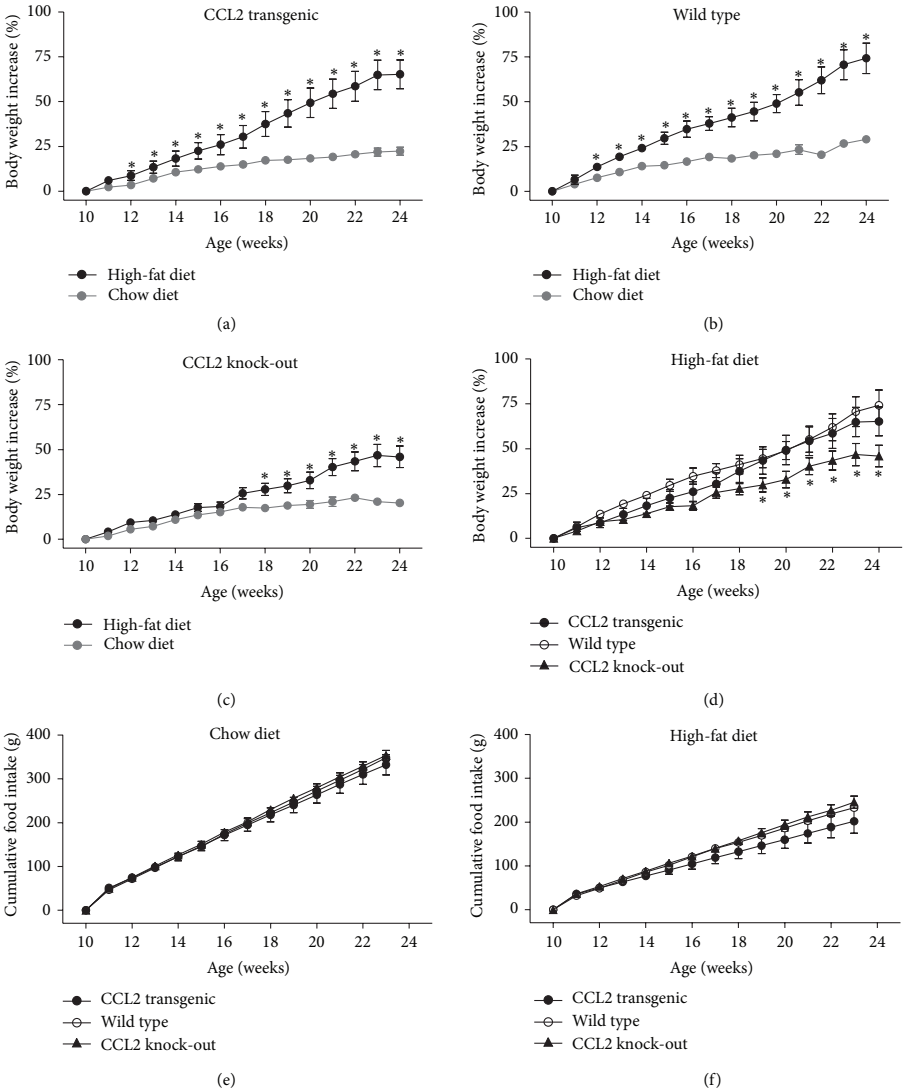


FIGURE 4: The effect of high-fat diet in body weight increase was evident in transgenic and wild-type mice ((a), (b)), but the different increase was immediate after dietary manipulation in transgenic. This effect was negligible in knockout mice (c). The combination of these effects with high-fat diet (d) shows similar results to facilitate comparison. These findings are not due to differences in the cumulative food intake ((e), (f)) indicating that CCL2 probably has no effect on appetite. $^{*}P < 0.05$.

quantitatively less evident than in transgenic mice. In KO mice, however, there were more mitochondria per cell, and fusion and fission were correctly balanced and apparently not altered by differences in diet. These findings strongly support further mechanistic studies, which may link the expression of CCL2 with mitochondrial biogenesis, inflammation, and energy management. According to our results, these putative mechanisms are related to the autophagic response, which was clearly enhanced in transgenic mice. Conversely, most liver cells in WT and KO displayed no evidence of autophagy (Figure 9).

3.6. Transgenic Mice That Overexpress CCL2 Die Prematurely When Fed High-Fat Diet. The transgenic mice fed a high-fat diet died prematurely between 10 and 14 months. The mice progressively decreased activity, reduced food intake

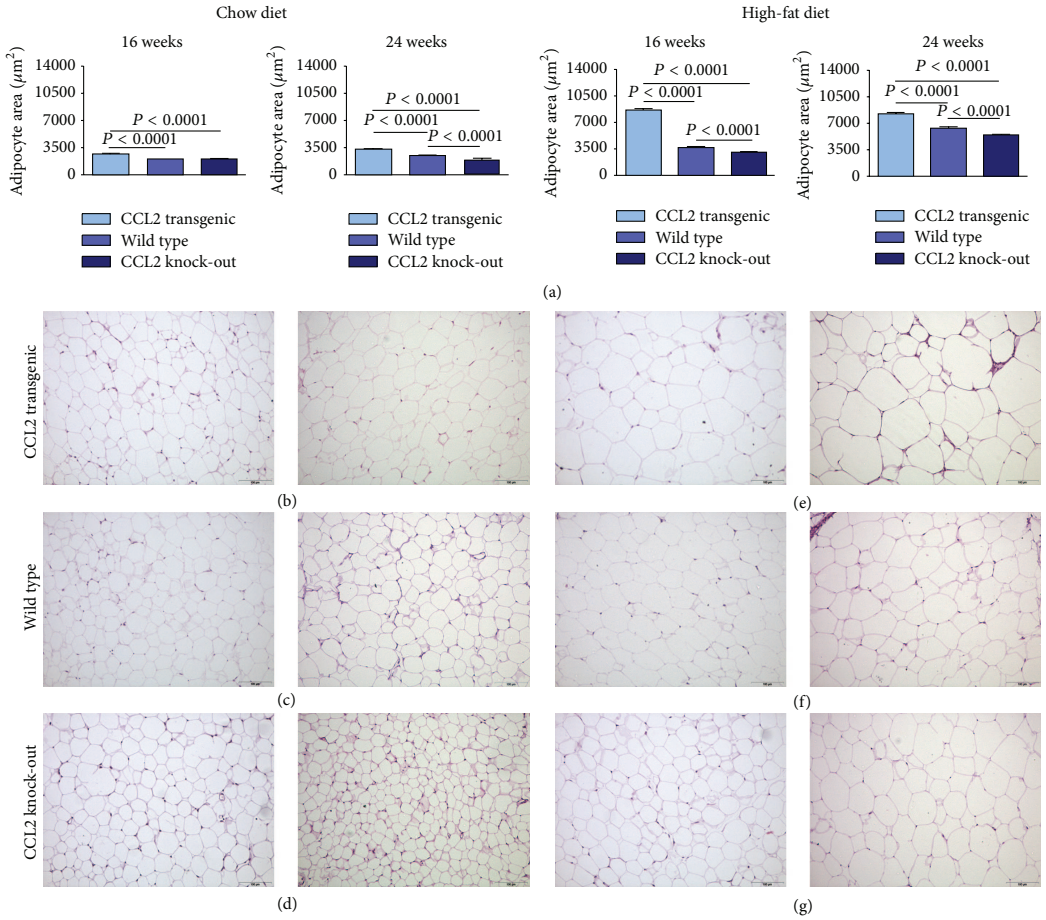


Figure 5: The size of the adipocytes was significantly higher in transgenic mice than in wild-type and knockout mice and the effect was observed with both dietary interventions regardless of the duration of the dietary treatment (6 or 14 weeks) (a) but it was more intense when mice were fed a high-fat diet. For clarity, values are indicated only for adipocytes in epididymal white adipose tissue. Representative micrographs are shown for transgenic, wild-type, and knockout animals ((b), (c) and (d), resp.) when fed a chow diet and for the corresponding animals fed a high-fat diet ((e), (f), (g)) at 16 and 24 weeks' old.

and the appearance of frailty became evident. There was also a casualty in the transgenic mice fed chow diet, but it was sudden, unexpected, and without a prior decrease in weight or activity. Among the casualties, one was also observed in the WT group fed a high-fat diet (Supplementary Material S9). A full autopsy was performed, and the cause of death was uncertain. There was neither cancer nor arteriosclerosis in these animals, but there were some cutaneous, superficial, and localised lesions in the skin accompanied with local loss of hair. There was also no evidence of sepsis. The only remarkable findings were limited to the spleen and the liver. The size and weight of the spleen was consistently higher in the transgenic mice fed high-fat diet. The presence of splenomegaly in these transgenic mice was consistent with the presence of giant cells that were identified as megakaryocytes (Factor VIII positive staining) and other proliferative signs. The weight of the liver was also higher in the transgenic mice, which is most likely due to the higher presence of steatosis. In the liver, there were signs of regenerative cells and increased apoptosis. Ongoing studies with higher sample sizes and the inclusion of females have been designed to further ascertain this point.

3.7. Bone Marrow Macrophages of Transgenic Mice: Expression of Selected Cytokines and mRNA. The CCL2 mRNA expression in the bone marrow macrophages was higher in transgenic than in WT mice, irrespective of stimulation with either GM-CSF (M1, pro-inflammatory) or M-CSF

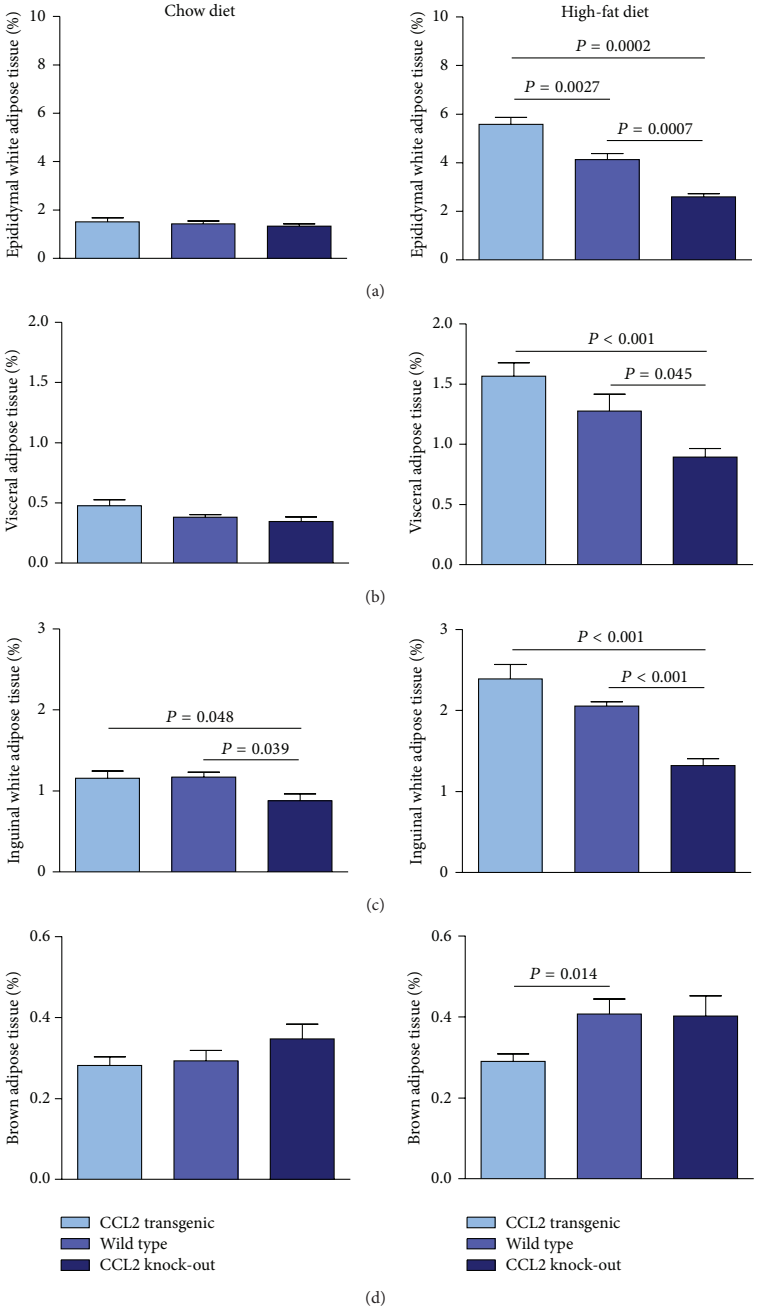


Figure 6: The effect of CCL2 expression in the weight of adipose tissue ((a)–(d)) of animals fed either chow (left column) or high-fat diet (right column). Of note, differences among strains were more evident during energy surplus and no change was observed in brown adipose tissue.

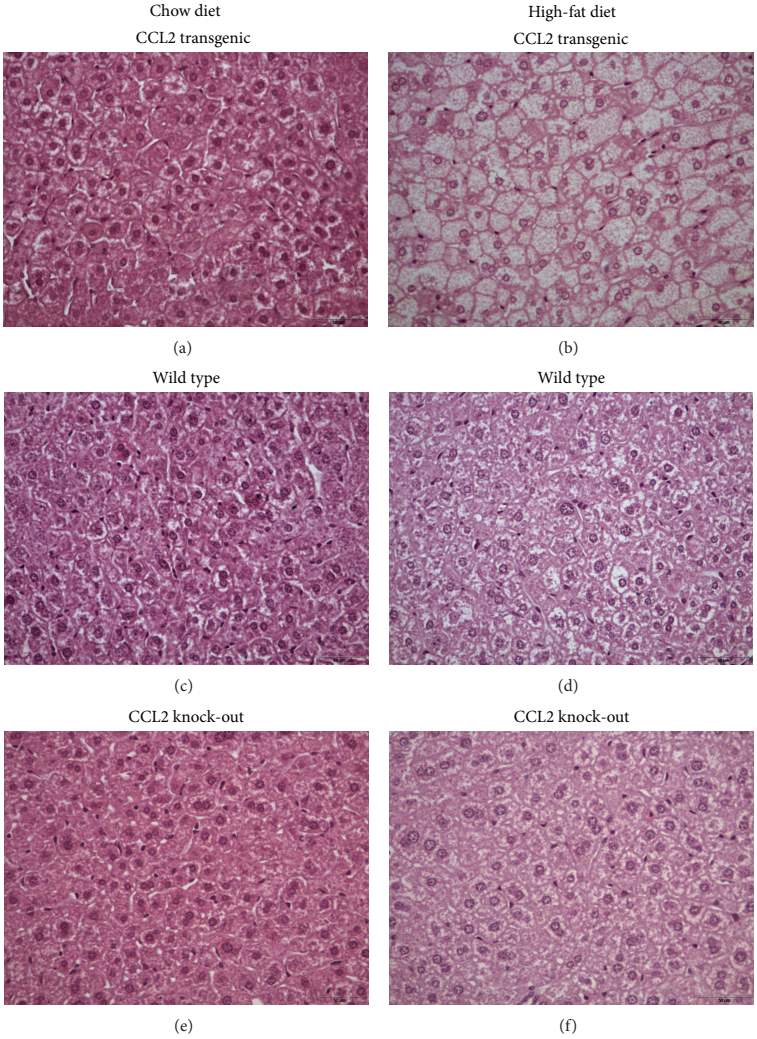


Figure 7: We found no significant differences among strains in the appearance of liver tissue when mice were fed a chow diet (left column (a), (c), (e); transgenic, wild-type, and knockout mice resp.). Representative micrographs show in the right column that a high fat diet produces steatosis in transgenic mice (b), dispersed lipid droplets in the liver of wild type mice (d), and no change in knockout mice (f).

(M2, phagocytic). The mRNA expression of the selected M2 markers was similar, with either low or undetectable expression in the GM-CSF macrophages without differences between transgenic and WT mice. The expression of the selected M1 markers was practically identical in the GM-CSF macrophages from TG and WT mice, with the notable exception of CCR7. Surprisingly, the expression of this chemokine receptor was significantly lower in TG mice, indicating lower pro-inflammatory activity. The expression of the M1 markers in M-CSF macrophages showed a unique and significant decrease in CCR2 mRNA expression; however, some M2 markers, including CD 206, GAS6, and IGF1, were also underexpressed. IL-10 expression also decreased, but the differences were not statistically significant. The results suggest that CCL2 overexpression may alter macrophage polarisation. Consequently, the secretion of selected cytokines was examined in macrophages that were treated with LPS and were compared with the relevant controls. The CCL2 secretion was higher in TG mice with both treatments compared with the WT mice and was 2–4 fold higher (2–4-fold change)

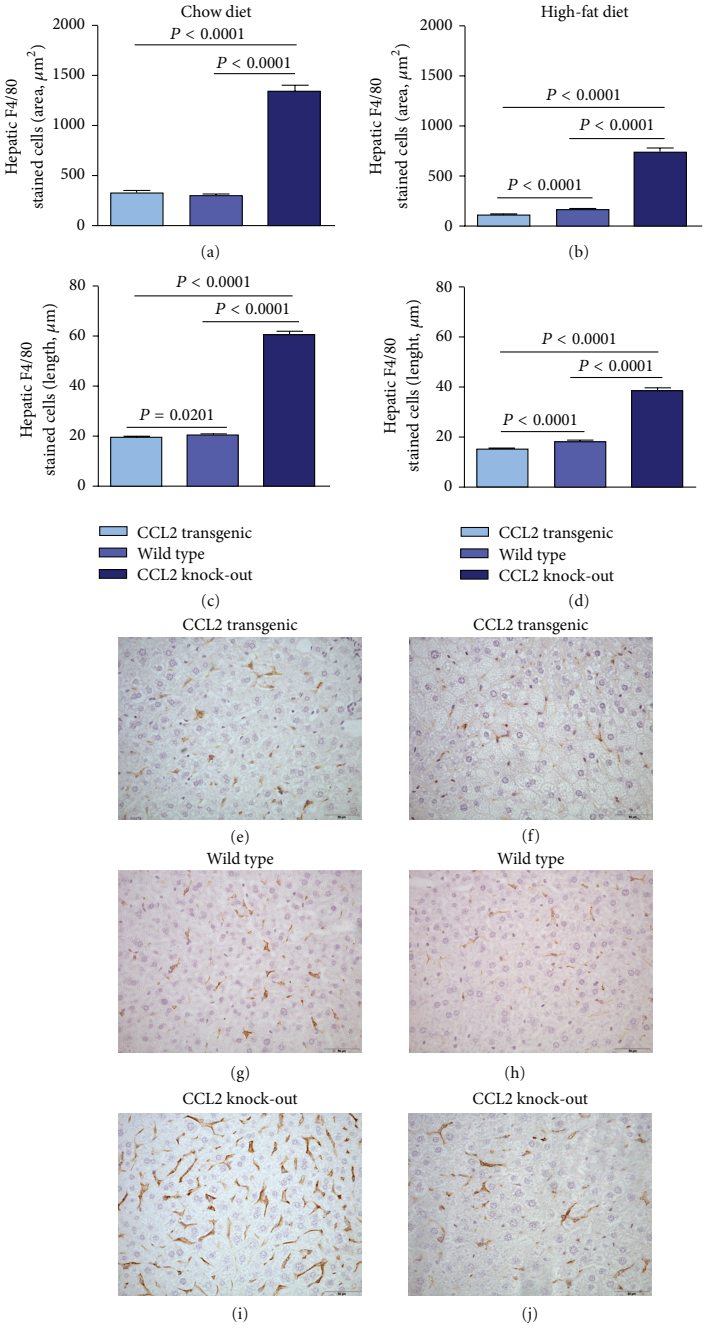


Figure 8: Dietary fat (right column) and CCL2 expression modify the size, number, and morphology of liver macrophages with respect to those fed a chow diet (left column) as assessed with F4/80 staining. Values for stained area and length of macrophages ((a)–(d)) are illustrated with representative microphotographs from transgenic ((e), (f)), WT ((g), (h)) and KO mice ((i), (j)).

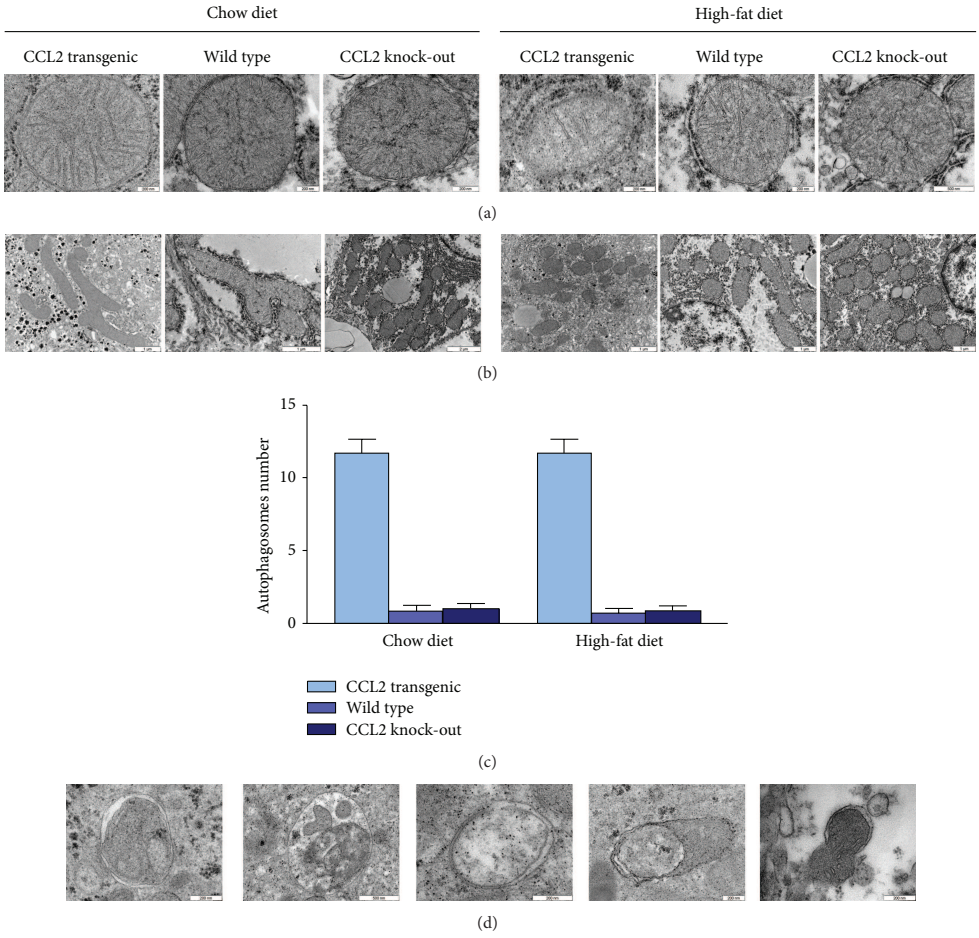


Figure 9: The appearance of mitochondria was affected by the dietary manipulation and the expression of CCL2 as shown in representative microphotographs (a) and these changes were accompanied by a significant effect in fusion-fission balance (b). The number of autophagosomes per cell was counted and was significantly higher in transgenic mice. Further, these were rare in both WT and KO and independent of diet (c). The heterogeneous nature of autophagic elements is illustrated in (d) (photographs obtained in transgenic mice).

in M-CSF macrophages. The IL-10 secretion was clearly detectable only in LPS-treated animals. The concentration in the supernatant was higher in TG than in WT mice, and the differences were statistically significant in GM-CSF macrophages. Finally, TNFα secretion was ostensibly higher in LPS-treated animals and significantly higher in TG mice with respect to the relevant controls (Figures 10 and 11).

4. Discussion

The transgenic mice developed in this study systemically overexpress CCL2. These animals were created to assess the combined effect of the recruitment of circulating monocytes in all tissues and the response to the stimuli of high dietary fat and energy ingestion. The hypothesis was that the continuous overexpression of this chemokine could promote or worsen common pathological conditions, and as animal model could be useful for assessing the pathogenic mechanisms and therapeutic approaches [39].

Fertility, growth, and physical appearance were identical to the controls. CCL2 overexpression did not result in abnormalities in the mice that were fed a regular chow diet. However, adding fat to the diet during a short period of time caused differences in body weight, adipocyte size, disturbances in glucose and lipid metabolism, premature death, and liver alterations that included a higher predisposition to fatty liver disease and significant changes in mitochondrial biogenesis and autophagy. Additionally, we explored bone

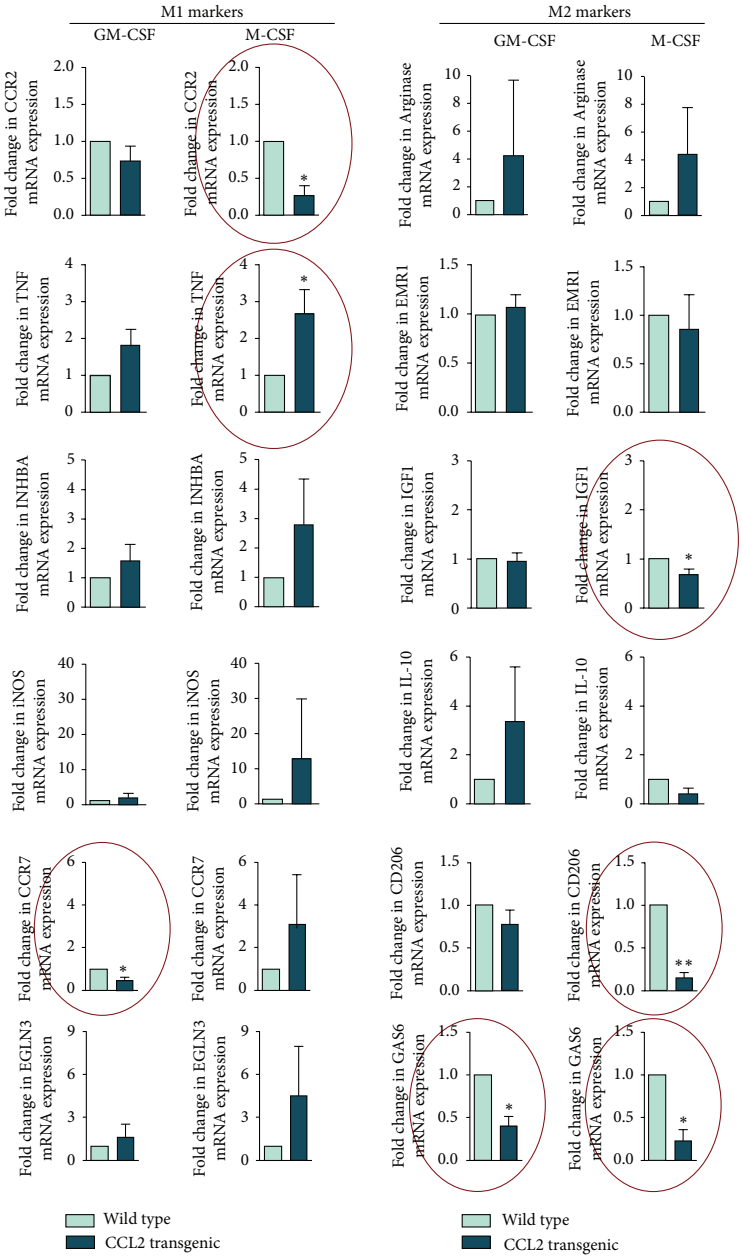


Figure 10: Relative mRNA expression in transgenic mice with respect to WT mice of selected markers for M1 and M2 macrophages in cells treated *in vitro* with either GC-MSF or M-CSF. Acronyms used were C-C chemokine receptor type 2 (CCR2), TNFα, Inhibin, beta A (INHBA), inducible nitric oxide synthase (iNOS), C-C chemokine receptor type 7 (CCR7), Egl nine homolog 3 (EGLN3), Arginase (ARG), EGF module-containing mucin-like hormone receptor EMR1 (F4/80), insulin growth factor-1 (IGF1), IL-10, the mannose receptor CD206, and Growth arrest-specific 6 (GAS6).

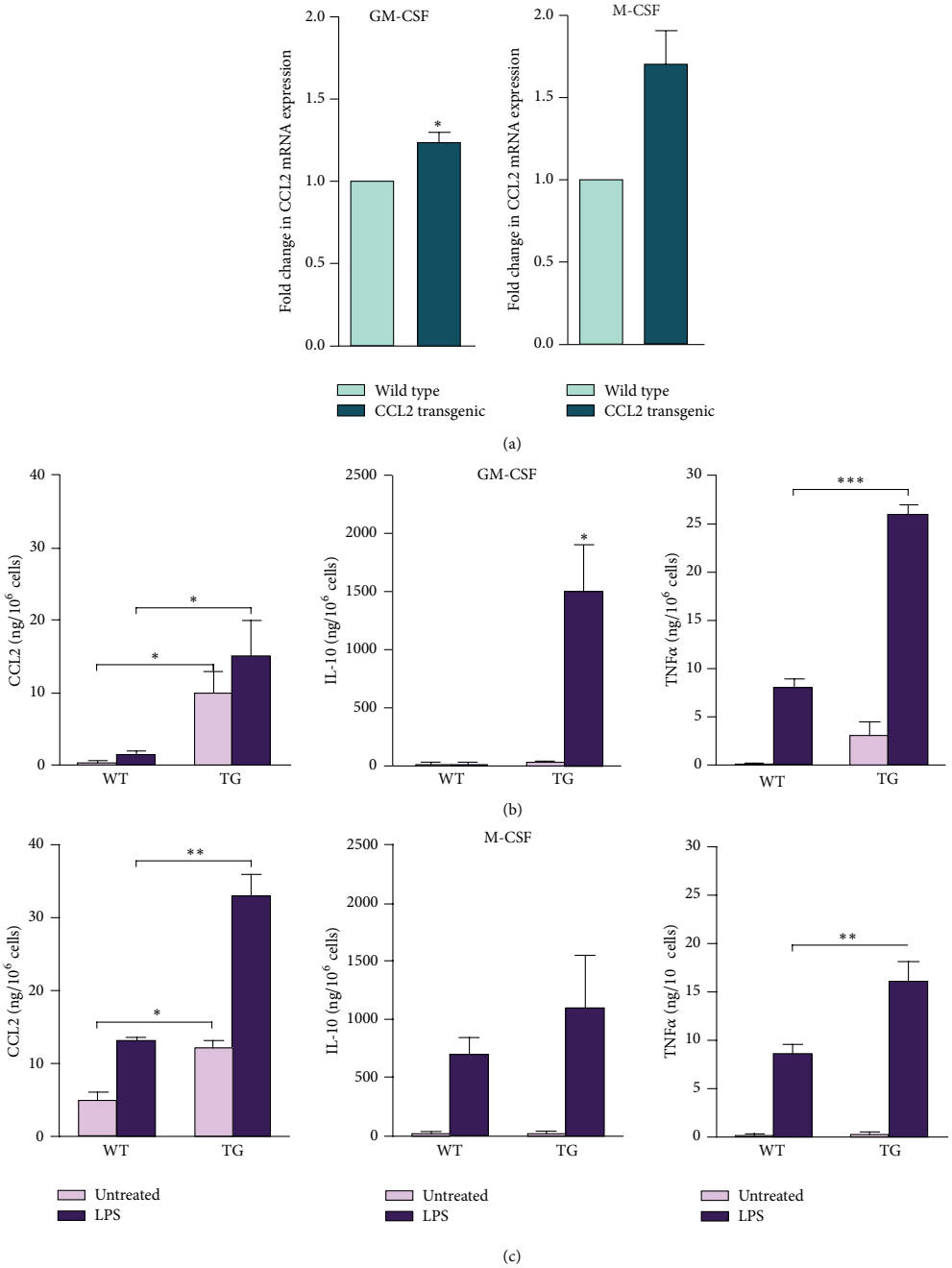


FIGURE 11: The relative CCL2 mRNA expression (a) and the secretion in supernatants of selected cytokines in bone marrow-derived macrophages of transgenic and WT cells treated *in vitro* with either GC-MSF (b) or M-CSF (c).

marrow macrophages under different *in vitro* conditions, and we found that CCL2 overexpression affects functional plasticity.

In previous studies, CCL2 has been considered a chemokine secreted by adipose tissue (adipokine), but systemic CCL2 overexpression regulates white adipose tissue (WAT) mass and size without apparent effects in brown adipose tissue (BAT). WAT serves primarily as lipid storage, and BAT is used for heat generation. The balance between the two adipose tissues affects the whole-body energy homeostasis, and the development and severity of obesity [40]. A higher production of CCL2 is not only a consequence of obesity but is most likely an exacerbating factor of diet-induced alterations. The roles of CCL2 in the aetiology of obesity and diabetes, the regulatory mechanisms, and the effect of therapies that inhibit CCL2 production have been recently reviewed [4, 41]. We have also found differences between fat cells in different adipose tissue depots and the heterogeneity of adipocytes within the same depots. Further examination of this issue is necessary because a different pattern of gene expression could explain the differential development of various types of adipose tissue [42, 43]. Moreover, this is closely associated with the pattern of fat distribution, the extent of obesity, and consequently the impact of different fat depots on the severity of metabolic complications [44, 45].

The size and number of hepatic macrophages significantly differs between transgenic and KO mice when detected with antibodies directed against F4/80. Curiously, this is an extracellular antigen of unknown function that belongs to a subgroup of the G-protein-coupled receptors [46]. The changes in macrophages morphology could represent concomitant changes in function and whether the macrophages are resident or recruited. This is further substantiated by the fact that these transgenic mice were prone to develop fatty liver disease and the KO mice were protected. The role of increased CCL2 is not yet understood, but the recruitment of macrophages seems to be important in different animal models. In KO mice there is an increased expression of peroxisome proliferator-activated receptors accompanied by the induction of fatty acid metabolism-related genes and the inhibition of pro-inflammatory cytokine production [47–49]. We confirmed that the effect of fat in the pathogenesis of fatty liver disease [49, 50] is influenced by the amount of available CCL2 and that the linkage between chemokines and hepatic lipid metabolism is plausible.

The characterisation of bone marrow-derived cells in the transgenic mice indicates that CCL2 overexpression affects the transition in the secretory function of macrophages (or the M1-M2 paradigm as a simplified descriptor of the functional plasticity). This is illustrated by differences in GM-CSF and M-CSF, which are cytokines that differentiate macrophages *in vitro* with distinct morphology and inflammatory function [51, 52]. The modulation of the phenotypic and functional differences in macrophage polarisation by CCL2 overexpression denotes a shift towards lower pro-inflammatory activity [53]. CCL2 decreased the expression of CCR7 in M1 and decreased the expression of CCR2, IGF1, CD206, IL-10, and GAS6 in M2. In cells under LPS treatment, however, CCL2 overexpression increased the secretion of IL-10 and TNFα with respect to WT controls. These changes could represent a quantitatively determinant factor in the development of macrophage-induced metabolic alterations. It should be highlighted that a high percentage of total body resident macrophages are present in the liver and that adipose tissue is a major site for the accumulation of recruited macrophages [54, 55].

Notably, CCL2 is involved, directly and/or through the induced metabolic alterations, in mitochondrial biogenesis and autophagy. We add CCL2 to the growing list of nonessential regulators of mechanisms that divide and fuse mitochondria [56]. The balance between rejuvenation and elimination of damaged mitochondria via autophagy is affected by both the presence of CCL2 overexpression and the increased availability of energy. The antagonistic and balanced activities of the fusion and fission machineries are constantly providing responses to inflammation to tightly regulate homeostasis of the organism [57, 58]. This is expected because mitochondrial diseases are associated with metabolic alterations. Apparently, there is a shift towards fusion in CCL2 overexpression to maximise ATP synthesis. Contrarily, morphological findings in CCL2 deficient mice, which are independent of high-fat diet, suggest a perfect balance [59, 60]. A certain unbalance is expected in inflammatory conditions and other energy-dependent disturbances via mitochondrial dysfunction [61, 62]. This is important because mitochondria and the access to energy (calorie restriction or increased dietary fat) play a pivotal role mediated by the mechanistic target of Rapamycin (MTOR) in deciding whether liver cells live or die [63].

In transgenic mice, autophagy was increased with respect to WT and KO mice, which is particularly important because autophagy affects immune responses as a result of degradative, biogenetic, and secretory activities that respond to various inputs via MTOR [64, 65]. Autophagy might control the infection of certain pathogens but also prevents excessive inflammatory reactions in the host [66]. As shown in autophagy-deficient macrophages, autophagy removes a number of proinflammatory stimuli [67–69]. Therefore, increased liver autophagy during CCL2 overexpression could be interpreted as an effort from the host to avoid the deleterious action of continuous inflammation.

Links between autophagy and inflammation have also been found in immune functions affecting several diseases, opening a new dimension in the understanding of the multifactorial basis of noncommunicable diseases. For example, increasing macrophage autophagy protects patients with advanced atherosclerosis [70]. It has also been reported that CCL2 controls the extent of autophagy in human prostate cancer [71], and autophagy is pivotal for the survival and differentiation of monocytes [72].

Finally, CCL2 overexpression resulted in premature death when combined with a high-energy intake. These findings require more extensive examination, and the cause of death remains obscure. Mice progressively lost interest in the environment, reduced activity, and their intake of food decreased. No chronic disease was evident, and there were no signs of sepsis or major infection. It is tempting to consider the possibility of premature aging, and future investigations will

include the characterisation of a senescence-associated secretory phenotype, particularly in pro-inflammatory cytokine enrichment [73] and the pro-inflammatory phenotype that accompanies aging [74, 75].

5. Conclusions, Perspectives, and Limitations

This animal model raises a number of questions about the prevalent diseases responsible for limiting the quality of modern life. Additionally, this model provides a link between inflammation and metabolism and suggests targets for the management of diseases in which there is a clear CCL2 overexpression. Specifically, this model can help to uncover the role of CCL2 in mitochondrial dysfunction, autophagy, and functionality of macrophages and aging in combination with excessive energy intake. Information gained could be useful for designing new mechanism-based therapeutic strategies.

None of the described effects appear in mice that are fed a regular diet, and this fact highlights the importance of calorie restriction for health. Therefore, the nutrient-sensing MTOR pathway seems to be crucial for the management of noncommunicable diseases. Consequently, drugs modulating MTOR are obvious candidates for assessment. For example, experiments on cancer, aging, and viral infections strongly suggest that this is the case for metformin [76–78]. This antidiabetic drug activates AMPK and inhibits MTOR with potent antiinflammatory actions. The usefulness of rapamycin, an MTOR inhibitor, and similar drugs in cancer prevention has been assayed [79]. Aspirin decreases inflammation, inhibits the MTOR pathway, decreases cancer incidence, and may reduce the burden of atherosclerosis [13, 80]. Lastly, although studies are scarce, angiotensin-II-blockers and beta-blockers, widely used in hypertensive patients, can also prevent the activation of the MTOR pathway and the incidence of chronic diseases [81].

The potential indications for these drugs are mostly related to chronic diseases in which inflammation plays a crucial role. This animal model could be used to further select candidates and suggests a number of mechanistic questions for future study. Particularly, we consider this model as a valuable contribution to our evolving comprehension of the interphase between autophagy and inflammation. However, we acknowledge that care must be taken in analysing the results of studies performed in animal models and that further research effort is necessary to fully characterize our observations. To name a few, possible effects of sex should be studied and metabolic alterations should be confirmed with the use of metabolic cages and more specific methods to detect significant differences. Particularly, CCL2 may have a higher influence if there is a relative contribution from different type of cells, particularly from immune cells [72].

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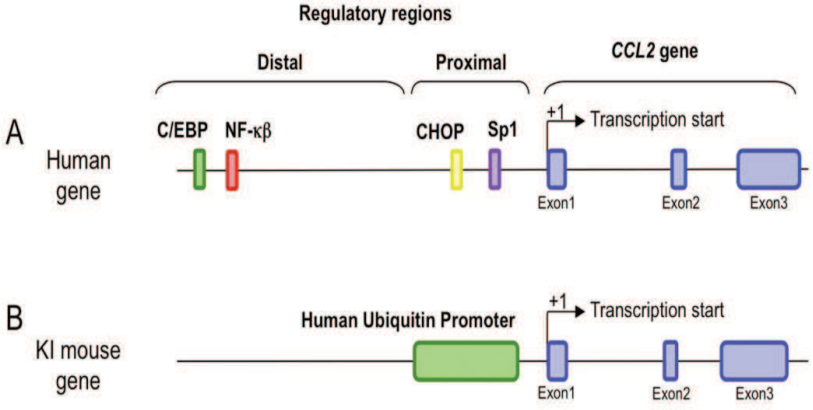
Supplementary material S1, comparison between human and murine CCL2 regions, corresponding to the manuscript:

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation?

Esther Rodríguez-Gallego, Marta Riera-Borrull, Anna Hernández-Aguilera, Roger Mariné-Casadó, Anna Rull, Raúl Beltrán-Debón, Fedra Luciano-Mateo, Javier A. Menéndez[1], Alejandro Vazquez-Martin[1], Juan J. Sirvent[2], Vicente Martín-Paredero[3], Angel L. Corbí[4], Elena Sierra-Filardi[4], Gerard Aragonès, Anabel García-Heredia, Jordi Camps, Carlos Alonso-Villaverde[5], Jorge Joven*

A, displays a schematic view of human *CCL2* gene region that includes its regulatory region and the most prominent elements identified. The *Ccl2* gene transgenic vector includes the murine CCL2 gene and the comparative position of human ubiquitin promoter (B); sequence is provided in C.



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3841 TCGCAGCCGG GATTTGGGTC GCGGTTCTTG TTTGTGGATC GCTGTGATCG TCACTTGGTG
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4681 ATTCTGGCCG TTTTTGGCTT TTTTGTTAGA CGCCACCATG CATATAACTT CGTATAGCAT
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Supplementary material S2, details of the sequence of other main elements used in the construction of the vector, corresponding to the manuscript:

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation?

Esther Rodríguez-Gallego, Marta Riera-Borrull, Anna Hernández-Aguilera, Roger Mariné-Casadó, Anna Rull, Raúl Beltrán-Debón, Fedra Luciano-Mateo, Javier A. Menéndez[1], Alejandro Vazquez-Martin[1], Juan J. Sirvent[2], Vicente Martín-Paredero[3], Angel L. Corbí[4], Elena Sierra-Filardi[4], Gerard Aragonès, Anabel García-Heredia, Jordi Camps, Carlos Alonso-Villaverde[5], Jorge Joven*

The sequence of main elements used in the construction of the vector is described according to the following, color-based legend. For clarity, flanking regions were also included.

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MCS 3475-3495
UbiC promoter 3500-4709
loxP site 4724-4757
STOP cassette 4768-6101
loxP site 6102-6135
genomic fragment of *CCL2* gene (frag) 6150-9056
exon1 6468-6628
exon2 7374-7491
exon3 7817-8343
SV40pA 9090-9318
FRT site 9331-9378
PGK promoter 9388-9906
Neo CDS 9974-10777
Splice donor (SD) 10799-11005
Instability signal (IS) 11006-11059
FRT site 11090-11137
MCS 11209-11230
3'homology arm 11231-14260

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 121 GACGGGGAAA GCCGGCGAAC GTGGCGAGAA AGGAAGGGAA GAAAGCGAAA GGAGCGGGCG
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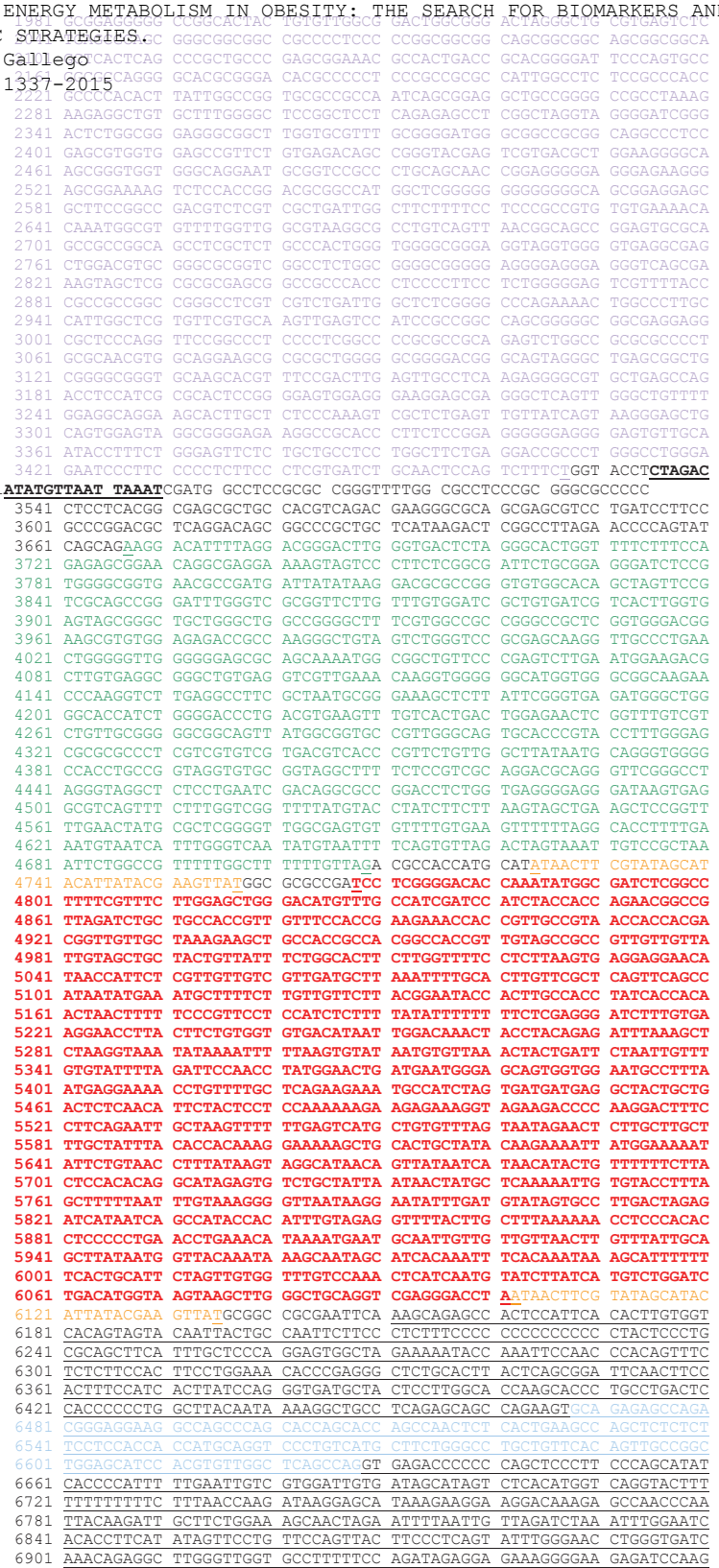

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Supplementary material S3, details on the construct design, corresponding to the manuscript:

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation?

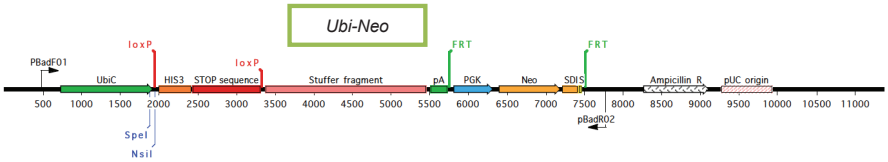
Esther Rodríguez-Gallego, Marta Riera-Borrull, Anna Hernández-Aguilera, Roger Mariné-Casadó, Anna Rull, Raúl Beltrán-Debón, Fedra Luciano-Mateo, Javier A. Menéndez[1], Alejandro Vazquez-Martin[1], Juan J. Sirvent[2], Vicente Martín-Paredero[3], Angel L. Corbi[4], Elena Sierra-Filardi[4], Gerard Aragonès, Anabel García-Heredia, Jordi Camps, Carlos Alonso-Villaverde[5], Jorge Joven*

The Knock-in Construct Design, from plasmid to electroporation

1. Overview

1.1 Vector backbone

The following plasmid (named *Ubi-Neo*) was used for construction of the *targeting vector*.



1.2 Construction fragments

Named *frag arm*, contained the genomic fragment of the gene to be introduced (*Ccl2*).

2. *frag arm* (Generation, Diagnostics and Sequencing)

2.1 Production

Using the following primers, the genomic fragment of *Ccl2* gene was amplified by PCR.

Template: C57BL/6 genomic DNA

P_01 5' *AGAATTC*AAAGCAGAGCCACTCCATTCACAC

EcoRI Homology

P_02 5' *TGAATTC*TCCCTCCCTCTTTATTGGACCGAAG

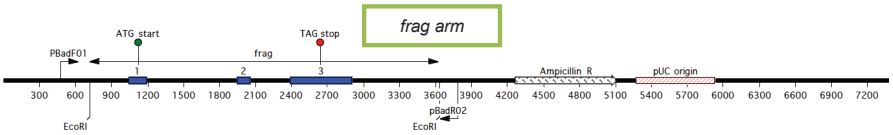
EcoRI Homology

Product: 2921 bp

2.2 Cloning into pPCR

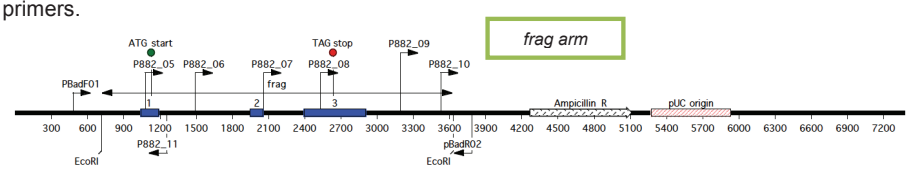
a) Sequencing with PBadF01 or PBadR02

The PCR product was cloned into pPCR and screened by sequencing to confirm clones with the correct insert.



b) Full Sequencing

Once end sequencing identified clones, a full sequence was completed using the following primers.

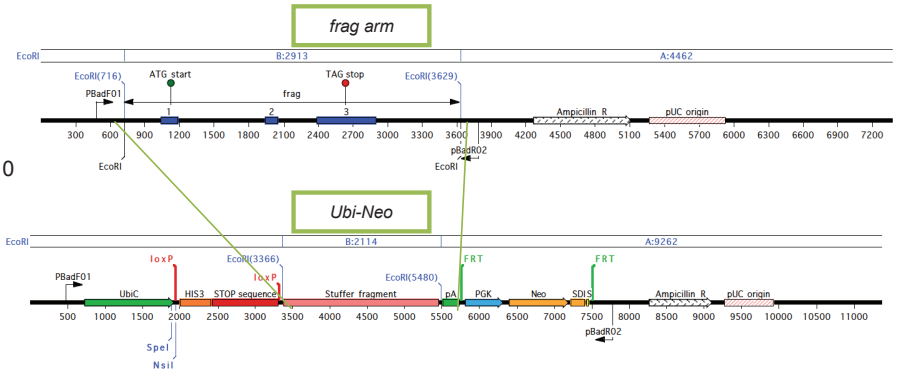


2.3 Cloning into the targeting vector

a) Restriction enzyme digest for cloning

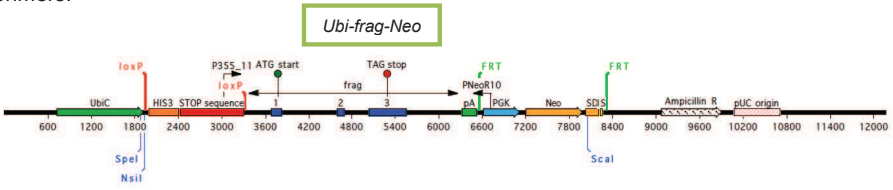
Both *frag arm* vector (containing Ccl2 gene) and the *Ubi-Neo* vector, were digested with EcoRI for cloning *Ccl2* gene into *Ubi-Neo* vector (just where the *Stuffer fragment* was placed).

The resulting vector was named *Ubi-frag-Neo*



b) Screen by Sequencing

Clones containing the insert could be screened by end sequencing with P335_11 and PNeoR10 primers:

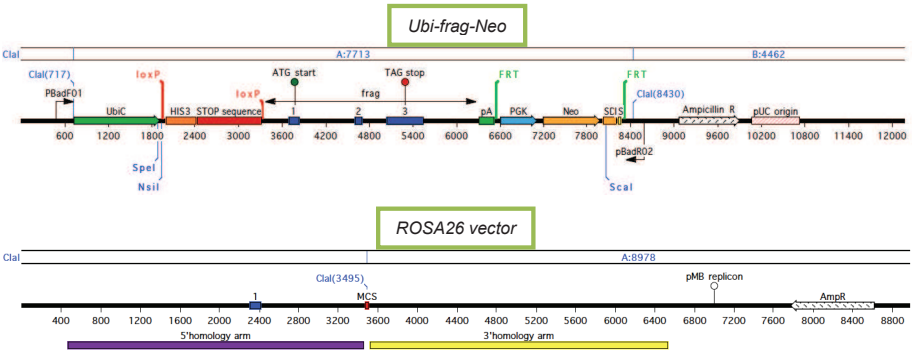


3. Cloning into the ROSA 26 vector

3.1 Cloning into the targeting vector

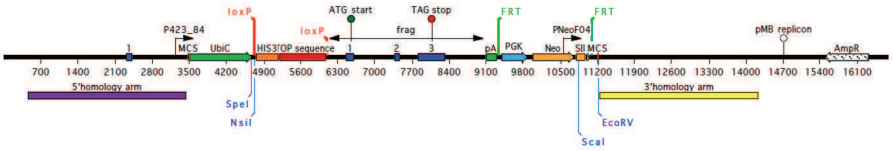
a) Restriction enzyme digest for cloning

ClaI restriction enzyme was used for cloning the constructed fragment (*Ubi-frag-Neo*) into the *ROSA26 vector* (which contained homologous sequences to ROSA26).



b) Screen by Sequencing

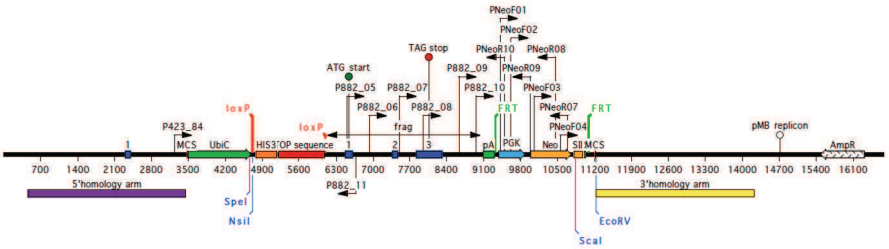
Clones that contained the insert (the *targeting vector*), were screened by end sequencing with the following primers



4. Complete *Targeting Vector*

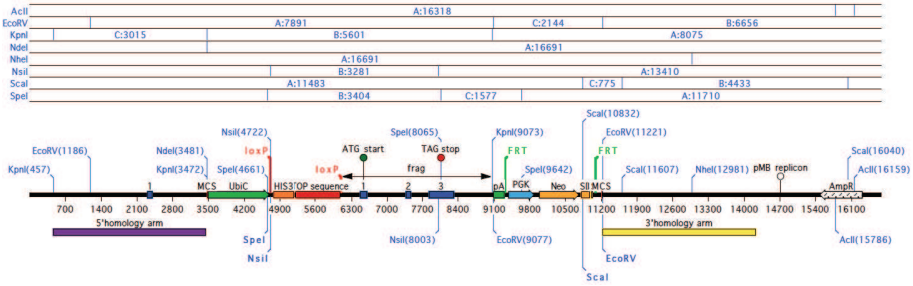
4.1 Final sequencing

To assess the *targeting vector* integrity, final sequencing was performed using the primers identified in the following graphic:



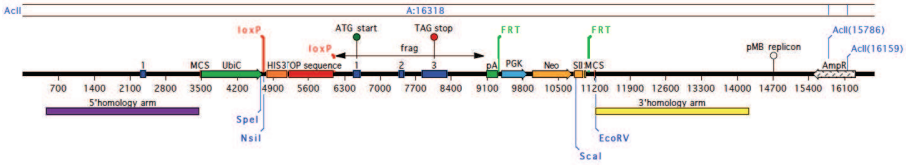
4.2 Restriction enzyme digest for Quality Control

Targeting vector was digested with several restriction enzymes as quality control strategy:



4.3 Restriction enzyme digest for electroporation

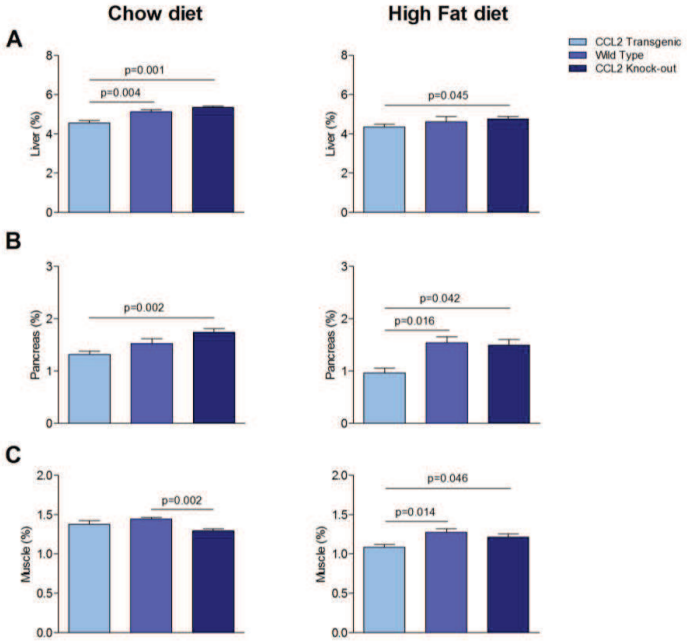
Finally, *targeting vector* was digested with AclI to obtain a suitable vector for electroporation into stem cells



Supplementary material S4, Weight of selected tissues and organs, corresponding to the manuscript:

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation?

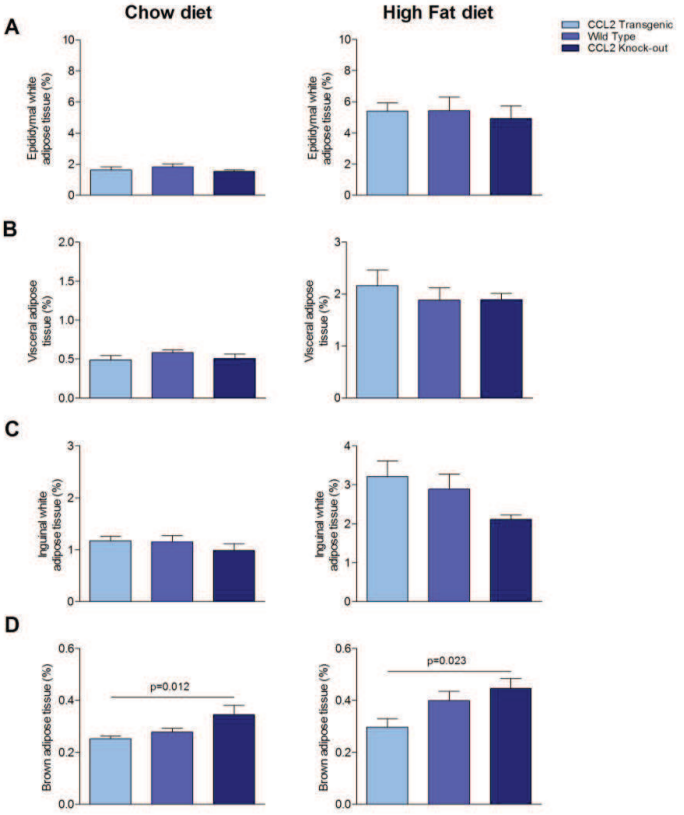
Esther Rodríguez-Gallego, Marta Riera-Borrull, Anna Hernández-Aguilera, Roger Mariné-Casadó, Anna Rull, Raúl Beltrán-Debón, Fedra Luciano-Mateo, Javier A. Menéndez[1], Alejandro Vazquez-Martin[1], Juan J. Sirvent[2], Vicente Martín-Paredero[3], Angel L. Corbí[4], Elena Sierra-Filardi[4], Gerard Aragonès, Anabel García-Heredia, Jordi Camps, Carlos Alonso-Villaverde[5], Jorge Joven*



Supplementary material S5, Weight of adipose tissues in the three strains after 14 weeks of dietary tratment with either chow diet or high fat diet treatment, corresponding to the manuscript:

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation?

Esther Rodríguez-Gallego, Marta Riera-Borrull, Anna Hernández-Aguilera, Roger Mariné-Casadó, Anna Rull, Raúl Beltrán-Debón, Fedra Luciano-Mateo, Javier A. Menéndez[1], Alejandro Vazquez-Martin[1], Juan J. Sirvent[2], Vicente Martín-Paredero[3], Angel L. Corbi[4], Elena Sierra-Filardi[4], Gerard Aragonès, Anabel García-Heredia, Jordi Camps, Carlos Alonso-Villaverde[5], Jorge Joven*

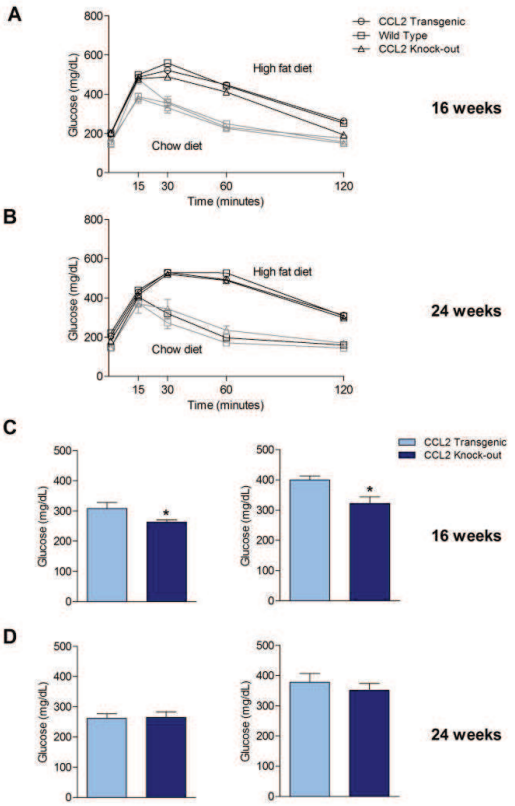


Supplementary material S6, disturbances in glucose metabolism, corresponding to the manuscript:

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation?

Esther Rodríguez-Gallego, Marta Riera-Borrull, Anna Hernández-Aguilera, Roger Mariné-Casadó, Anna Rull, Raúl Beltrán-Debón, Fedra Luciano-Mateo, Javier A. Menéndez[1], Alejandro Vazquez-Martin[1], Juan J. Sirvent[2], Vicente Martín-Paredero[3], Angel L. Corbí[4], Elena Sierra-Filardi[4], Gerard Aragonès, Anabel García-Heredia, Jordi Camps, Carlos Alonso-Villaverde[5], Jorge Joven*

Oral glucose tolerance tests displayed a significant effect of high fat, high-cholesterol diet on insulin resistance even with a short-term manipulation. However, differences among strains fed the same diet were negligible either in 16 an 24 weeks old (A, B). Plasma glucose was higher in CCL2 overexpressors than in other strains and the effect of diet was also more evident (C). These differences disappeared when the dietary tratement was for 14 weeks (D). * P<0.05 with respect to relevant pair.

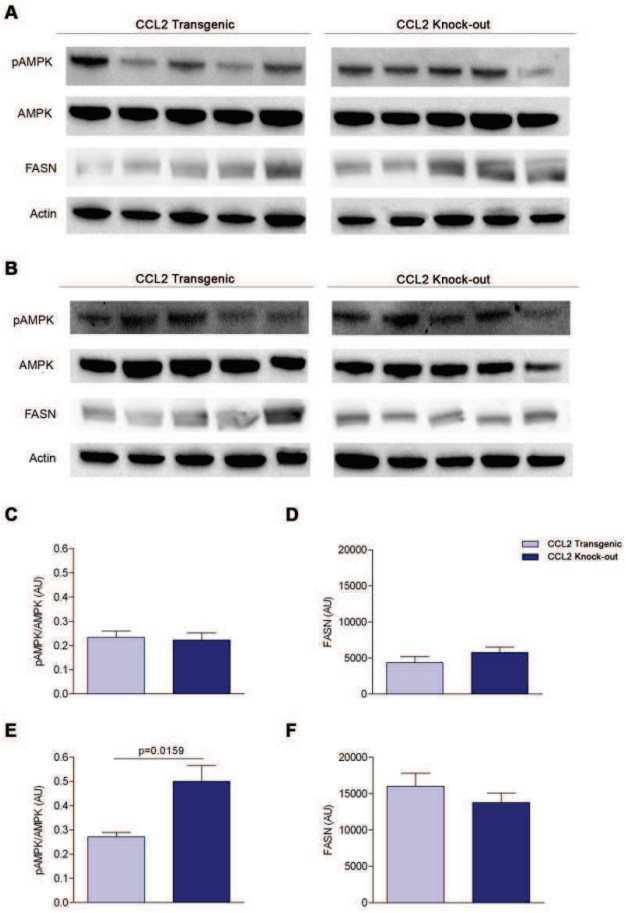


Supplementary material S7, expression of fatty acid synthase and AMPK in the liver, corresponding to the manuscript:

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation?

Esther Rodríguez-Gallego, Marta Riera-Borrull, Anna Hernández-Aguilera, Roger Mariné-Casadó, Anna Rull, Raúl Beltrán-Debón, Fedra Luciano-Mateo, Javier A. Menéndez[1], Alejandro Vazquez-Martin[1], Juan J. Sirvent[2], Vicente Martín-Paredero[3], Angel L. Corbi[4], Elena Sierra-Filardi[4], Gerard Aragonès, Anabel García-Heredia, Jordi Camps, Carlos Alonso-Villaverde[5], Jorge Joven*

Representative immunoblottings in the liver of transgenic and KO mice fed chow diet (A,C,D) and high-fat diet (B,E, F) of FASN and activated AMPK and the respective calculations (C-F) .

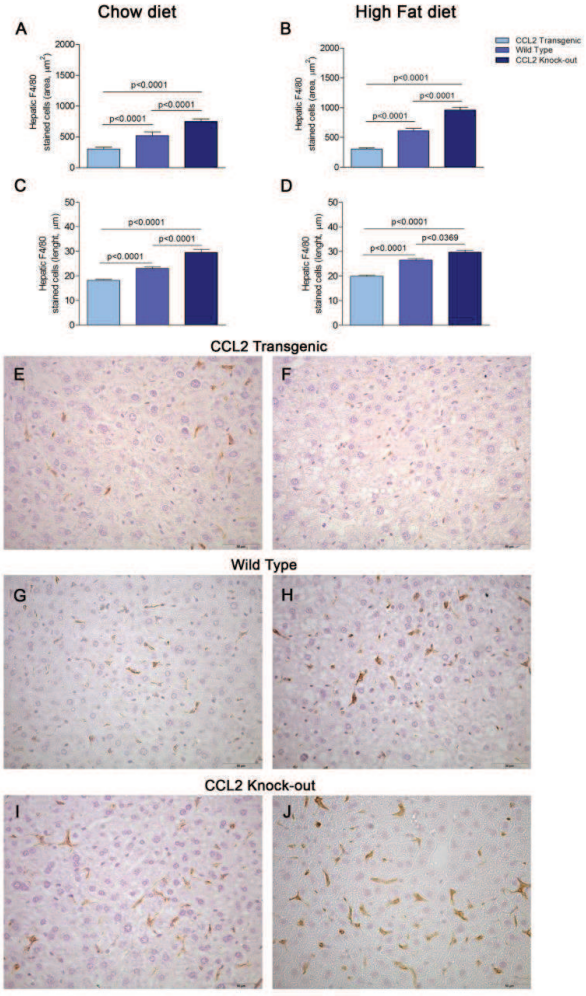


Supplementary material S8 The effect of high fat diet and overexpression of CCL2 in the size, number and morphology of liver macrophages after 14 weeks of dietary treatment, corresponding to the manuscript:

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation?

Esther Rodríguez-Gallego, Marta Riera-Borrull, Anna Hernández-Aguilera, Roger Mariné-Casadó, Anna Rull, Raúl Beltrán-Debón, Fedra Luciano-Mateo, Javier A. Menéndez[1], Alejandro Vazquez-Martin[1], Juan J. Sirvent[2], Vicente Martín-Paredero[3], Angel L. Corbí[4], Elena Sierra-Filardi[4], Gerard Aragonès, Anabel García-Heredia, Jordi Camps, Carlos Alonso-Villaverde[5], Jorge Joven*

Dietary fat (right column) and CCL2 expression modify the size, number and morphology of liver macrophages with respect to those fed a chow diet (left column) as assessed with F4/80 staining. Values for stained area and length of macrophages (A-D) are illustrated with representative microphotographs from transgenic (E,F), WT (G,H) and KO mice (I,J).

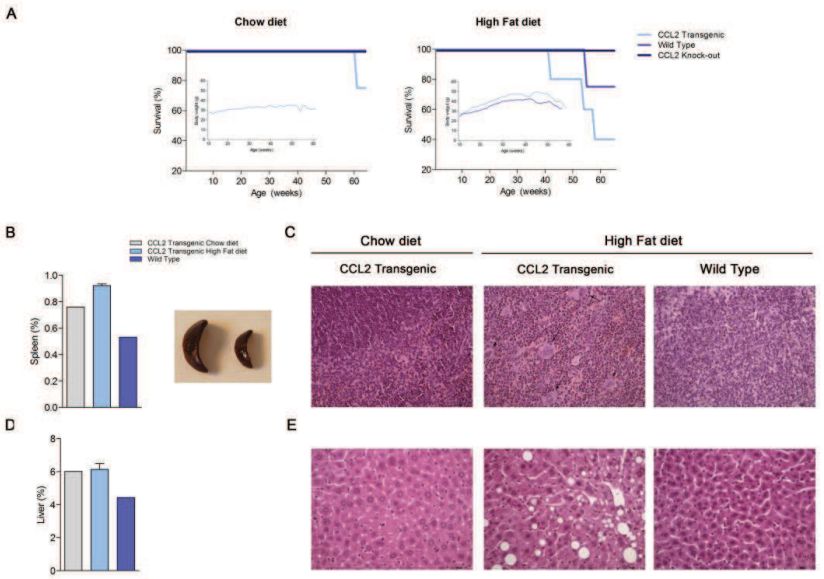


Supplementary material S9, Transgenic mice that overexpress CCL2 die prematurely when fed high-fat diet, corresponding to the manuscript:

Ubiquitous transgenic overexpression of C-C chemokine ligand 2: a model to assess the combined effect of high energy intake and continuous low-grade inflammation?

Esther Rodríguez-Gallego, Marta Riera-Borrull, Anna Hernández-Aguilera, Roger Mariné-Casadó, Anna Rull, Raúl Beltrán-Debón, Fedra Luciano-Mateo, Javier A. Menéndez[1], Alejandro Vazquez-Martin[1], Juan J. Sirvent[2], Vicente Martín-Paredero[3], Angel L. Corbí[4], Elena Sierra-Filardi[4], Gerard Aragonès, Anabel García-Heredia, Jordi Camps, Carlos Alonso-Villaverde[5], Jorge Joven*

Transgenic mice fed high-fat diet died prematurely after a progressive decrease in weight and activity (A,B). The size of the spleen in transgenic animals was higher than in controls and there were abundant megakariocytes (C, D). The livers were also higher in transgenic mice where steatosis and signs of regenerative tissue and apoptotic nuclei were numerous (E,F)



Publications

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2. Cuyàs E, Fernández-Arroyo S, Corominas-Faja B, **Rodríguez-Gallego E**, Bosch J, Martín B, Llorens R, Joven J, Menéndez J. Oncometabolic mutation IDH1 R132H confers a metformin-hypersensitive phenotype; Oncotarget. 2015 Mar 30. [Epub ahead of print]

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8. **Rodríguez-Gallego, E., Guirro, M., Riera-Borrull, M., Hernández-Aguilera, A., Mariné-Casadó, R., Fernández-Arroyo, S., Beltrán-Debón, R., Sabench, F., Hernández, M., del Castillo, D., Menendez, J.A., Camps, J., Ras, R., Arola, L., Joven, J.. Mapping of the circulating metabolome reveals a-ketoglutarate as a predictor of morbid obesity-associated non-alcoholic fatty liver disease. International Journal Of Obesity. 2014.**

9. Joven J, March I, Espinel E, Fernández-Arroyo S, **Rodríguez-Gallego E**, Aragonès G, Beltrán-Debón R, Alonso-Villaverde C, Rios L, Martin-Paredero V, Menendez JA, Micol V, Segura-Carretero A, Camps J. Hibiscus sabdariffa extract lowers blood pressure and improves endothelial function. Molecular Nutrition & Food Research. 2014. Volumen 58 1374-1378.

10. Oliveras-Ferraros, C., Vazquez-Martin, A., Cuyàs, E., Corominas-Faja, B., **Rodríguez-Gallego, E.**, Fernandez-Arroyo, S., Martin-Castillo, B., Joven, J., Menendez, J.A.. Acquired resistance to metformin in breast cancer cells triggers transcriptome reprogramming toward a degradome-related metastatic stem-like profile. Cell Cycle 2014. Volumen 13. 1132 - 1144.

11. Joven, J., Micol, V., Segura-Carretero, A., Alonso-Villaverde, C., Menéndez, J.A., Aragonès, G., Barrajón-Catalán, E., Beltrán-Debón, R., Camps, J., Cufí, S., Fernández-Arroyo, S., Fernández-Gutiérrez, A., Guillén, E., Herranz-López, M., Iswaldi, I., Lozano-Sánchez, J., Martin-Castillo, B., Oliveras-Ferraros, C., Pérez-Sánchez, A., **Rodríguez-Gallego, E.**, Rull, A., Saura, D., Vázquez-Martín, A.. Polyphenols and the Modulation of Gene Expression Pathways: Can We Eat Our Way Out of the Danger of Chronic Disease?. Critical Reviews In Food Science And Nutrition. 2014. Volumen 54. 985 - 1001.

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17. Menéndez JA, Joven J, Aragonès G, Barrajón-Catalán E, Beltrán-Debón R, Borrás-Linares I, Camps J, Corominas-Faja B, Cufí S, Fernández-Arroyo S, Garcia-Heredia A, Hernández-Aguilera A, Herranz-López M, Jiménez-Sánchez C, López-Bonet E, Lozano-Sánchez J, Luciano-Mateo F, Martin-Castillo B, Martin-Paredero V, Pérez-Sánchez A, Oliveras-Ferraros C, Riera-Borrull M, **Rodríguez-Gallego E**, Quirantes-Piné R, Rull A, Tomás-Menor L, Vazquez-Martin A, Alonso-Villaverde C, Micol V, Segura-Carretero A. Xenohormetic and anti-aging activity of secoiridoid polyphenols present in extra virgin olive oil: A new

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Intellectual and industrial property

1. **Diagnóstico de esteatosis hepática no alcohólica**. Nº Solicitud P2947ES00. Universitat Rovira i Virgili.

2. **Ratones Cisgénicos sobreporductores de MCP-1**. Nº Solicitud P201330418. Universitat Rovira i Virgili.

Congress attendance

1. 83rd European Atherosclerosis Society Congress, Glasgow, 2015. **Poster Oral presentation**
2. 82nd European Atherosclerosis Society Congress, Madrid, 2014. **Poster Oral presentation**
3. EASL The International Liver Congress. London, 2014. **Poster.**
4. 1er Congreso Médico-Quirúrgico de la Obesidad, Madrid, 2013. **Poster**
5. Fórum CEICS (Campus d'Excel·lència Internacional Cataluña Sur), Tarragona-Reus, 2013 CEICS (Campus de Excelencia Internacional Cataluña Sur). **Attendee**
6. 80th EAS Congress, Milan (Italy), 24-28 Maig, 2012. **Poster Oral presentation**
7. EASL The International Liver Congress, Barcelona (Spain), 18-22 April, 2012. **Poster**
8. Fórum CEICS (Campus de Excelencia Internacional Cataluña Sur), Tarragona-Reus, 2011 CEICS (Campus de Excelencia Internacional Cataluña Sur). **Attendee**
9. Sesiones Científicas del Centro Catalán de la Nutrición 2011: Alimentos funcionales. CCNIEC, Reus, 2011. CNIEC (Centre Català de la Nutrició de l'Institut d'Estudis Catalans). **Attendee**
10. XXIII Congrés de la Societat Catalana de Cardiologia, 2011. **Poster**
11. 4th International Conference on Paraoxonases, Vilaseca, 2010. **Organitzation**
12. 34th FEBS Congress, Praga, 2009. **Poster**
13. Barcelona Biomed Conference, Barcelona, 2009**. Attendee**
14. 5th Workshop on Biomedical Genomics and Proteomics, Badalona, 2008. **Attendee**
15. SEBBM, Bilbao, 2008. **Attendee**
16. III Congreso Interuniversitario de Biotecnología, León, 2008. **Attendee**
17. 33th FEBS Congres, Athens, **2008. Attendee**