UNIVERSITAT DE BARCELONA

Bariatric surgery and diet change in rats

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BARIATRIC SURGERY AND DIET CHANGE IN RATS

Dissertation submitted by Joana Rossell Rusiñol, to opt for a doctoral degree at the Universitat de Barcelona

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This work was performed at the Department of Biochemistry and Molecular Biomedicine at the Facultat of Biologia in the Universitat de Barcelona, under the supervision of Professor Julia Peinado and Dr. Eva Pardina in the Metabolisme Lipídic group

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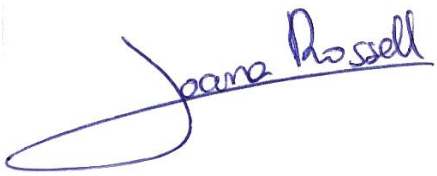

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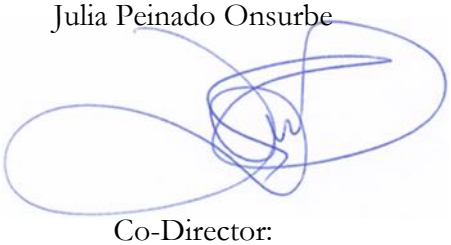

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University of Barcelona, Faculty of Biology

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Menjar molt i pair bé, no pot ser.

Quien bien come y mejor digiere, sólo de viejo se muere

Dis-moi ce que tu manges, je te dirai ce que tu es.

Der Mensch ist, was er ißt

Den som äter bra, arbetar bra

Mala digestio nula felicitas

ACKNOWLEDGEMENTS

Not all those who wander are lost. And life seldom works as you plan it.

Indeed, life is a succession of unexpected events, testing your capacity to adapt, react, and learn. And so is writing a thesis. People often say that you either win or lose, but I stay with you either you win or you learn… With every obstacle, you have an opportunity to adapt, rectify, or learn, and is not until the end of the process (in this case, the end of writing this doctoral thesis) that you realize how far you have arrived. It has not been easy, and I have many to thank for their collaboration and their help during this process.

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To my friends, here and around Europe. For all of them who have been waiting for some years for me to finish the thesis. Now it is almost done No one mentioned, no one forgotten.

To David, for his support this last year. You know I cannot wait to finish this project to start a new crazy one with you.

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ABBREVIATIONS

AA	Arachidonic acid
AGB	Adjustable gastric band
ALA	Alpha-linolenic acid
AMPs	Antimicrobial peptides
ANGPTL4	Angiopoietin-like protein 4
BMI	Body mass index
BS	Bariatric surgery
BW	Body weight
BWG	Body weight gain
CAF	Cafeteria diet
CVD	Cardiovascular diseases
C	Control
DC	Diet change
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DIO	Diet-induced obesity
DM2	Diabetes Mellitus type 2
DNL	De novo lipogenesis
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acids
FFA	Free fatty acids
GC-MS	Gas chromatography mass spectrometry

GIP	Glucose-dependent insulinotropic polypeptide
GLA	Gamma-linoleic acid
GLP-1	Glucagon-like peptide 1
GM	Gut microbiota
HFD	High-fat diet
HF	High fat
HFHS	High-fat high-sugar diet
HDL	High-density lipoproteins
HOMA-β	Homeostatic model assessment for β cell
HOMA-IR	Homeostatic model assessment for insulin resistance
IEC	Intestinal epithelial cells
IR	Insulin resistance
ISI	Insulin sensitivity index
LA	Linoleic acid
LIG	Lignoceric acid
LDL	Low-density lipoproteins
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MA	Myristic acid
MHO	Metabolically healthy obese
MUFA	Monounsaturated fatty acids
NAFLD	Non-alcoholic fatty liver disease
NCD	Noncommunicable diseases

OA	Oleic acid
PA	Palmitic acid
POA	Palmitoleic acid
PRRs	Pattern recognition receptors
PUFA	Polyunsaturated fatty acids
RYGB	Roux-en-Y gastric bypass
SA	Stearic acid
SCD1	Stearoyl-CoA desaturase 1
SCFA	Short chain fatty acids
SFA	Saturated fatty acids
SPM	Specialised Pro-resolving Mediators
TG	Triglycerides
TLRs	Toll-like receptors
VLDL	Very low-density lipoproteins
VSG	Vertical sleeve gastrectomy
VAC	Vaccenic acid
WAT	White adipose tissue
WD	Western diet
WHO	World health organization
Δ6D	delta-5 desaturase
Δ6D	delta-6 desaturase

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1. INTRODUCTION

Obesity has become a problem for most of the developed countries and is now also becoming a problem in developing countries, becoming a burden for the health systems in many areas. It is a multifactorial disease with a complex development, from simple fat mass accumulation to a series of comorbidities that worsens life quality and expectancy. Several factors act together in a complex interplay in the development of obesity: systemic inflammation, alterations in the gut microbiota, and modifications in lipid metabolism are factors appearing during obesity, and their role as causal, or consequence of obesity is still unclear.

A significant amount of research has been, is being, and will be done in order to cast some light on the etiology of obesity, to understand it and to learn how to fight it. Many studies are performed in humans, centered on the outcomes after bariatric surgery, which is the best available treatment at the moment. Many are performed on animal models, offering a controlled environment and accessibility to tissues, necessary for studying the molecular mechanisms behind the effects of bariatric surgery.

In this thesis, we offer the result of five years of research, studying the effect of a high-fat diet in a rat model, combined with bariatric surgery together, or not, with a change to a standard diet. We describe the subsequent anatomical changes, the modifications in liver composition, and in gut microbiota, all factors in the complexity of obesity.

1.1. OBESITY

In the 20th century, the development of modern medicine led to advances in the prevention and control of infectious diseases, decreasing mortality rates and increasing life expectancy in most of the developed countries (1,2). However, the decrease in mortality rates by infectious diseases have been followed by the rise of deaths by non-communicable diseases

(**NCD**), which are the first cause of death in the modern world. The most common NCD are cancer, cardiovascular diseases (**CVD**), and obesity, accounting for more than 70% of the global deaths (1,2).

1.1.1. *DEFINITION*

Obesity, together with overweight, is defined by the World Health Organization (**WHO**) as "the abnormal or excessive fat accumulation that may impair health" (3). Overweight and obesity are classified in adults by several methods, but the most commonly used is the Body Mass Index (**BMI**), also known as the Quetelet Index, which is the ratio of weight by height squared (kg/m^2) (4,5). The resulting number is classified in categories that define the nutritional status, with a BMI equal or over 25 an indicator of overweight, and a BMI equal or over 30 an indicator of obesity in to different degrees (**table 1**). A morbid obese subject is defined as someone with an obesity class III or with an obesity class II together with comorbidities (6).

Table 1. BMI and the corresponding nutritional status.

BMI	Nutritional status
< 18.5	Underweight
18.5–24.9	Normal weight
25.0–29.9	Pre-obesity
30.0–34.9	Obesity class I
35.0–39.9	Obesity class II
> 40	Obesity class III

The use of BMI is not as accurate as other anthropometric measurements, but its simplicity makes it a powerful tool; however, it fails in identifying the location of the fat accumulation, as it only in children correlates with the amount of visceral white adipose tissue (visceral **WAT**) (5,6). A common procedure in a risk population is to combine the measurements of BMI and waist circumference, as it offers a more accurate assessment of the risks of several comorbidities with obesity, such as CVD, stroke, blood lipid alterations, non-alcoholic fatty liver disease (**NAFLD**) and glucose homeostasis problems (7).

Obesity is one of the main health concerns as is linked to several diseases and comorbidities, and has replaced tobacco as the first life-style risk factor causing premature death; it is

estimated to reduce life expectancy between 5-20 years, mostly due to higher cardiovascular death, and entails an elevated cost for the health systems in many countries due to related endocrinological diseases and CVD (2,6,8,9). Obesity is a growing global problem that needs to be treated, as well as prevented.

1.1.2. *EPIDEMIOLOGY*

Obesity has triplicated its prevalence for the last 45 years, increasing to pandemic levels; the WHO estimated that in 2016 there were more than 1.9 billion adults in the world living with overweight, accounting for 39% of the adult population (**figure 1**). Of those, 650 million were obese (13% of the adult population) (2,3). Known for its supposed good habits and Mediterranean diet, Spain is surprisingly one of the countries with an elevated prevalence of weight problems (3). In 2012, more than half of the Spanish population (54%) was overweight, of which 17% were obese.

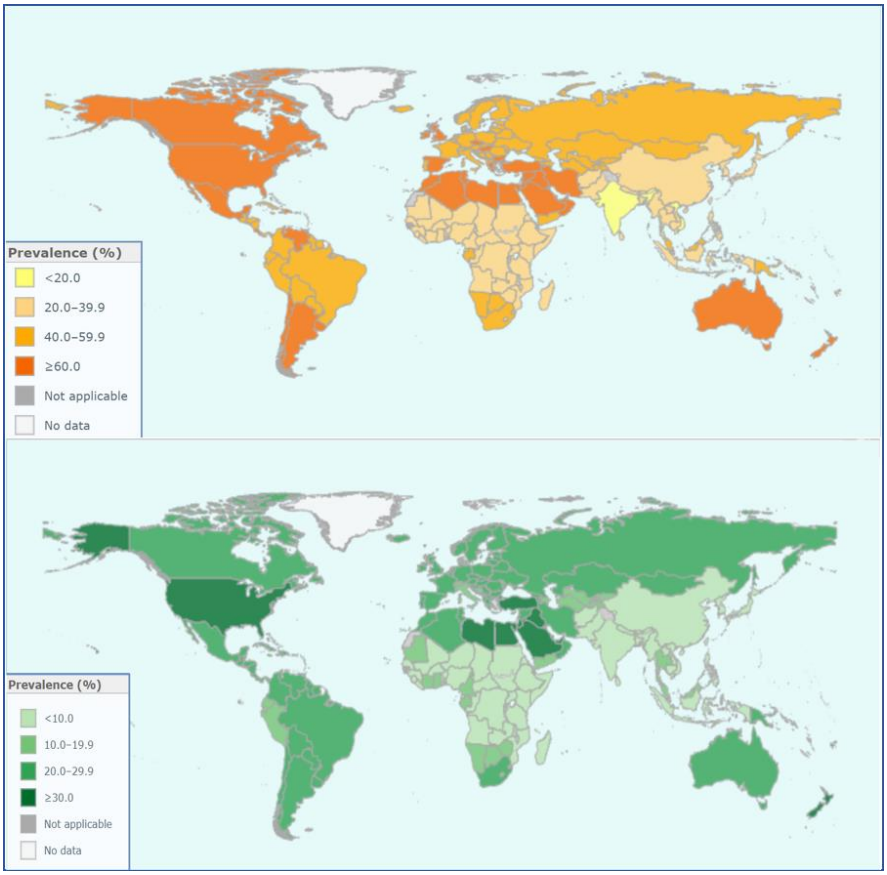

Figure 1. Overweight (upper) and obesity (lower) prevalence in the world, among adults, in 2016. Image from global health observatory, WHO (9).

In 2016 the prevalence of obesity increased with 4,6%, although the prevalence of overweight lowered to 39% (10). Far from being a solved problem, the WHO estimates that by 2030 obesity prevalence in Spain will increase up to 28% of the adult population (11).

Together with the increasing obesity rates in the adult population, developed societies are facing an added problem as younger populations are also being affected, with overweight appearing at earlier ages. Worldwide, obesity rates in children and teenage populations have increased dramatically, three-folding since the 70s' and with an estimated 126 million children living with obesity in 2016 (12,13). Spain was ranked as the second from top European countries with the highest overweight and obesity percentages in the last Children Obesity Surveillance Initiative (13) and the ratios seem to increase, as shown in a study from 2018 showing 54% of overweight in children under 9 years old (14). This is of special concern, as children and adolescents with weight problems have a higher risk of developing diabetes and depression, as well as becoming either overweight or obese when they reach adulthood. In a more recent study, Spain was placed as the 4th ranking European country with the highest rate of people under 19 years old living with obesity (13).

1.1.3. *CAUSES*

People with obesity have been stigmatized for years, viewed as lazy, lacking willpower, and unable to follow a diet, but the causes of the obesity pandemic do not rely on the choices and attributes of the individual alone (15,16). Although the fundamental cause of obesity is an imbalance between the calories ingested and the calories expended, this imbalance is heavily influenced by the surrounding environment, and by socio-economic and dietary factors that take part in a complex interplay with genetic and hormonal factors (**figure 2**) (2,16,17).

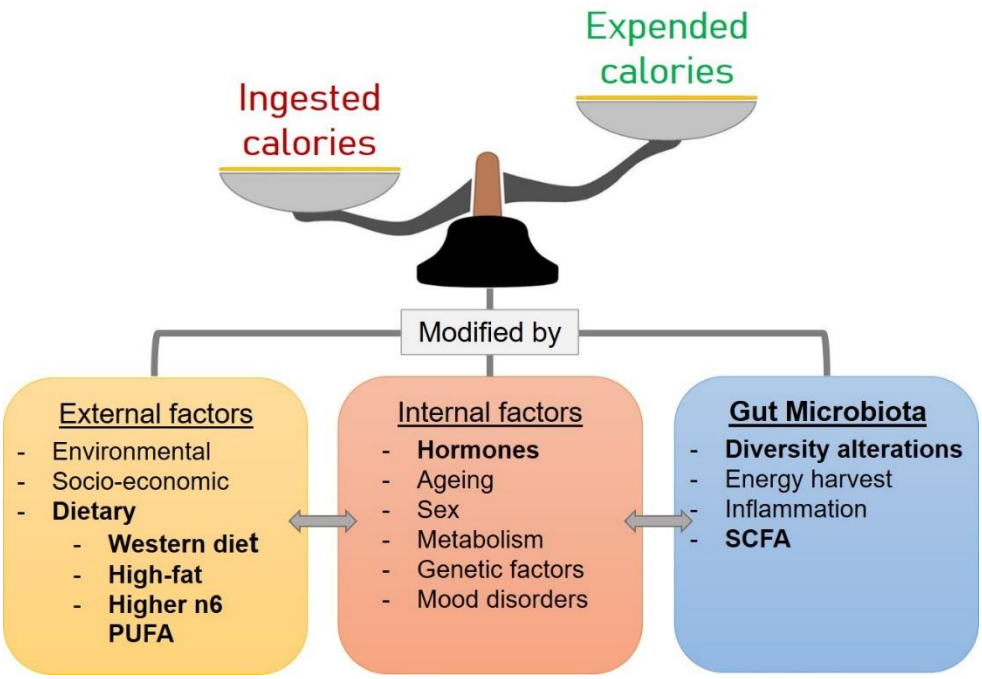

Figure 2. The energy imbalance leading to obesity and several factors that influence it. Marked in bold, factors discussed in this work. Abbreviations n6 PUFA stands for n6 polyunsaturated fatty acids; SCFA for short chain fatty acids. Adapted from Blüher 2019 (2).

1.1.3.a) **External factors**

ENVIRONMENTAL AND SOCIO-ECONOMIC

The obesity trends, far from being stopped, are increasing in the developed countries and spreading to developing countries, such as Africa and Asia, that are adopting westernized food habits and lifestyle (13,16). The obesogenic environments, defined as "the sum of influences that the surroundings, opportunities or conditions of life have on promoting obesity in individuals or populations", have an impact on the whole society but to a higher degree on lower-income families (17). Together with increased sedentarism -a common trait in our society- and genetic predisposition, they are the main drivers of the obesity pandemic that no country has yet succeeded to manage (2,6,7,17,18). In the past, obesity was linked to a wealthy status, being a disease of the rich, but the western diet (**WD**), described in the next section, has shifted the obesity ratios towards the socio-economically challenged population (13,19).

DIETARY

As commented above, in the last decades, developed societies have changed their food policies, as well as their alimentary habits, leading to an industrialized environment and food

production. This has increased the consumption of calorie-dense but nutrient-poor foods, such as fast food, as well as increased portion sizes and elevated consumption of sweet beverages, altogether leading to the apparition of obesogenic environments (5,17,20).

The WD is considered the single biggest risk factor for the development of obesity, offering cheap, hyper-caloric, highly palatable food with poor nutritional value (13,19). It is characterized by overconsumption of unhealthy foods consisting mainly of sweets, soft drinks, fat-rich foods, and red and processed meats, together with lower consumption of healthy foods such as fish, vegetables, whole grains, and fruit (16,19). Besides, the more well-known associated problems, such as insulin peaks after the intake of food with a high glycaemic index, the WD is also characterized by an imbalanced fatty acid intake (21). The higher intake of fried food together with the use of vegetable cooking oils from sunflower or canola, rich in linoleic acid (LA, 18:2, n-6), increase the ratio of n6:n3 polyunsaturated fatty acids (**PUFA**), which is associated with obesity and metabolic syndrome (22,23). This imbalance, together with an increased saturated fatty acid (**SFA**) intake and other components of the WD, contributes to the permanent low-grade inflammation present in obesity and other NCD (21).

1.1.3.b) Internal factors

HORMONAL

Obesity is also influenced by several hormones in our body and even though they are not a direct cause for it, they regulate how fat is distributed in the body or mediate in the satiety regulation. Some of the most studied are the following:

- Estrogens

Estrogens -popularly known as feminine hormones- seem to play an important role in determining where the fat is stored in the body, as estrogen receptors have been localized on the surface of adipocytes. They also play a role in protecting against obesity, as they are involved in the mechanisms regulating hunger and energy expenditure (24).

- Leptin

Leptin is an anorexigenic hormone mainly produced in the adipose tissue and the gastric mucosa, but also in salivary glands. Its effects were discovered as early as in the 1950s but the molecule was not identified until 1994. It is a key hormone maintaining weight stability as it regulates both satiety and energy expenditure by interacting with the hypothalamus (25–27). The release of gastric leptin is stimulated through several factors related to meal ingestion

and digestion, such as different intestinal neuropeptides and higher insulin levels in order to induce satiety after a meal and thus, decrease food intake (2). Obese people tend to be hyperlipidaemic, as leptin levels correlate with the amount of adiposity mass in the body, and a period of over-eating leads to increased circulating leptin levels which in turn lead to the development of leptin resistance. The effects are decreased satiety feeling after meals and lower energy expenditure, which worsens obesity prognosis and adds difficulty in losing weight with the help of traditional therapies such as dieting (2,9,25). On the other hand, genetic leptin deficiency also leads to obesity, but is a rare mutation in humans and not a common cause of obesity (26).

- Ghrelin

Discovered in 1999, ghrelin is a hormone produced mainly in the gastric fundus, but also in other parts of the intestinal tract, involved in weight homeostasis (25,28). It is an orexigenic hormone, stimulating appetite by interacting with the hypothalamus, and thus is considered the antagonist of leptin. Ghrelin contributes to obesity by promoting the maintenance of the actual body weight. Consequently, ghrelin levels increase while dieting or losing weight, promoting the hunger sensation even after meals and impedes long-term positive results and leads to regaining of weight (25,29). Genetic mutations related to ghrelin can also be found, like in patients with Prader-Willi syndrome, where ghrelin levels are higher and patients suffer from insatiable hunger and obesity (25).

- Adiponectin

The adipose tissue secretes adiponectin, a hormone participating in the regulation of glucose and lipid metabolism. Even though is not responsible for obesity itself, adiponectin plays a role in several of the comorbidities associated with obesity, as well as having the capacity to protect from chronic inflammation (30,31).

- Others

There are other hormones involved in the development of obesity and its comorbidities. Incretins are gut hormones involved in glucose regulation by stimulating insulin secretion after a meal. The most studied are glucose-dependent insulinotropic polypeptide (**GIP**) and glucagon-like peptide 1 (**GLP-1**), both secreted in the intestine (32,33). GLP-1 also acts in the attention and reward system of the brain, decreasing hunger (15).

GENETIC

Besides the environmental causes, other factors are contributing to high obesity rates. It is well accepted that genetics have a lesser role, but they increase the risk of weight gain by interacting with the obesogenic environment (5). There are several loci linked with obesity and they influence BMI, as shown in studies with twins, where monozygotic twins had a more similar adipose tissue distribution compared to dizygotic twins. Epigenetics also plays a strong role, as seen in experiments with rodents and in human observations where parents with a higher BMI had children with a higher risk of developing obesity. Still, the observed effect of the genes is mixed with the one exerted from the household environment as seen in studies with adoptive children, shading the true effect of genetics (34,35).

MOOD DISORDERS

Mood disorders are an often neglected factor in obesity, as an elevated percentage of obese subjects suffer from depression and anxiety, and a reciprocal link exists between them: obese individuals have a higher risk of being depressed, and depressed people have a higher risk of becoming obese (36,37). More obvious is the relationship between obesity and eating disorders, such as binge-eating disorder, or night-eating disorder, both related to psychiatric disorders (38). In light of this, psychosocial factors should also be taken into account in the etiology of obesity, as well as in the treatment (39).

1.1.3.c) **Gut microbiota**

In 2004, Bäckhed et al. found that gut microbiota (**GM**) was able to regulate the fat storage in mice in 2004 (40), suggesting a connection between GM and obesity. Much research has been done on this topic, trying to elucidate the relationship between microbiota and obesity, as GM has come on to the scene as a novel factor in the development of obesity. The gut microbiome is a highly adaptive system that is strongly influenced by diet, among other factors. Differences in the diet lead to substantial differences in the composition of the microbiota, which may favor the obesity outcome (33,41).

The GM and its relation with obesity will be properly introduced in chapter 1.4 .

1.1.4. *RELATED COMORBIDITIES*

Obesity, as mentioned before, is linked to several comorbidities that are responsible for the shortened life expectancy, many of which are clustered under the name of metabolic syndrome (**figure 3**) (5). They are the result of a long and progressive process in which the energy imbalance due to the higher calorie intake together with a low energy expenditure leads to an over-accumulation of adipose tissue and low-grade systemic inflammation (2,15). How fat is distributed in the body is metabolically relevant and influence the development of comorbidities: android obesity, with abdominal fat depositions surrounding the internal organs, is associated with most of the comorbidities and higher mortality-rate, while gynoid obesity, with subcutaneous fat deposition in parts like hips, is considered a less harmful or even protective type of obesity (5,6,42,43).

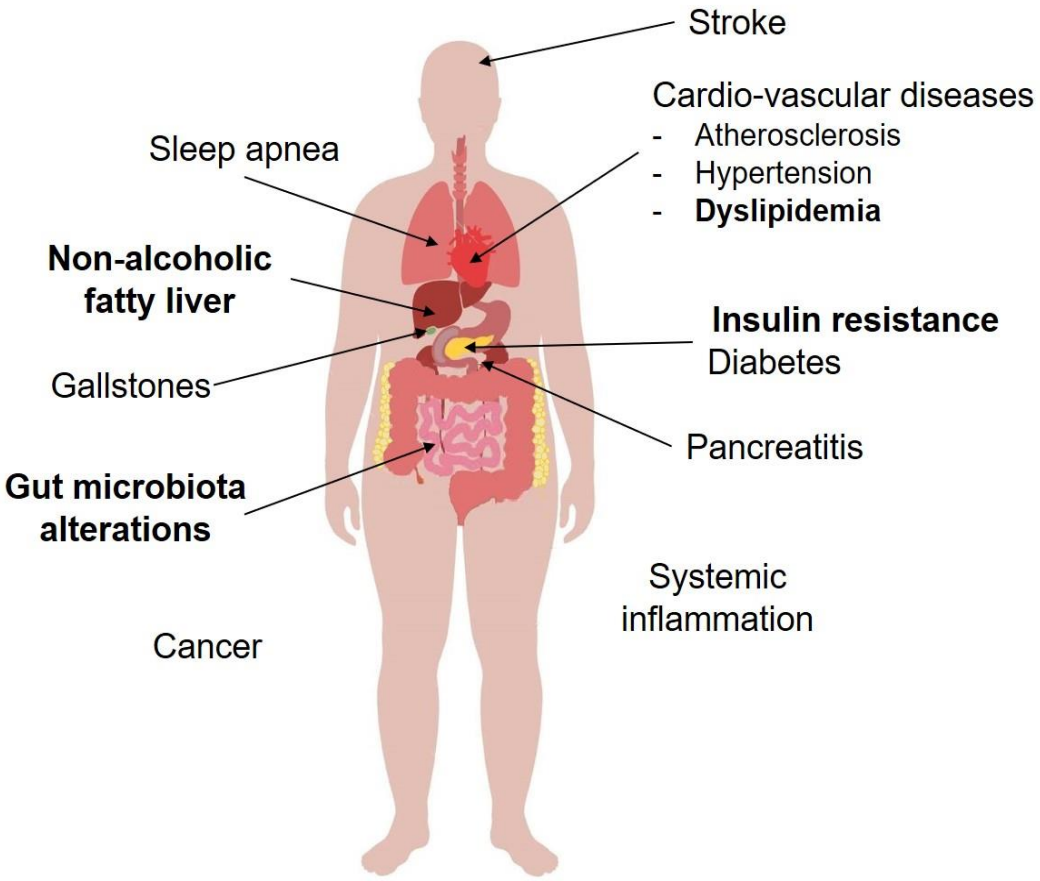

Figure 3. Comorbidities associated with obesity. In bold, comorbidities discussed in this work. Adapted from Dietz,2015 (7).

It is important to note that between 10 to 30% of obese people do not show the comorbidities associated with obesity and are thus considered metabolically healthy obese (**MHO**) subjects. MHO individuals have more subcutaneous fat deposition and a lower amount of fat deposited in visceral organs. Still, MHO subjects have a higher risk of developing diabetes and other comorbidities than lean subjects and are thought to be a transition stage towards becoming unhealthy (6,43,44).

Once a meal is ingested, digested and absorbed through the small intestine and carried to the liver through the portal vein, the remaining excess calories are transformed into triglycerides (**TG**), a highly energetic molecule that is hydrolyzed by the lipoprotein lipase (**LPL**) (45) and stored in the adipocytes of the WAT , which acts as a buffer avoiding that free fatty acids (**FFA**) causing lipotoxicity in the organism. The stored TG can later be transformed through lipolysis into fatty acids (**FA**) and glycerol and used as an energy source when needed (31,46). The adipocyte has a limited capacity to increase its volume in order to accumulate more TG, but a constant TG overflow surpasses the adipocyte capacity for hypertrophy, causing several internal malfunctions such as mitochondrial dystrophy and impaired glucose transport. Hypertrophic adipocytes lose their capacity of producing the functional amounts of adiponectin, inducing inflammation, a key factor determining the emergence of comorbidities (24,31).

1.1.4.a) **Low-grade inflammation**

The former mentioned leads to a permanent low-grade inflammation that characterizes obesity. Besides surpassing the buffer capacity of adipocytes, the constant higher calorie intake, accompanied by a lower expenditure, increases the ectopic fat accumulation in non-adipose tissue, such as the muscle or the liver, leading to lipotoxicity that damages the cells and causes inflammation. Although the order of apparition is not fully clear yet, the inflammation of the adipose tissue is accompanied by macrophage infiltration, which leads to further inflammation (47). The macrophage infiltration is also seen in the liver, with the apparition of a higher number of Kupffer cells (48). The inflamed adipose tissue and liver are responsible for the liberation of pro-inflammatory cytokines (49).

Besides the caloric excess, the diet composition may also contribute to the inflammatory process in the body. Inflammation and its resolution are mediated by peptides (cytokines), proteins, and lipid-derived mediators (50). The dietary imbalance in the n6:n3 PUFA ratio affects the production of specialized pro-resolving mediators (**SPM**), molecules responsible for the clearance and resolution of inflammation that derives from n6 and n3 PUFA

(21,50,51). The higher n6:n3 ratio lead to a lesser capacity of inflammation resolution, as well as increased pro-inflammatory lipid mediators that contribute to maintaining the permanent low-grade inflammation (21,52).

1.1.4.b) **Non-alcoholic fatty liver disease**

Non-alcoholic fatty liver disease is the most common liver disease in developed countries, affecting 25% of the adult population, up to 70% of the population with Diabetes Mellitus type 2 (**DM2**) in Europe, and 65% of people with obesity type I or II (53–55). NAFLD is a multifactorial disease, closely related to insulin resistance (**IR**) and metabolic syndrome (54). When the adipocyte ability to store TG fails, there is an increase in circulating FFA and TG. Together with TG from dietary sources and de novo lipogenesis (**DNL**), they accumulate in the liver, causing lipotoxicity, inflammation, and damage to the liver structure (53–55). Although NAFLD is a multifactorial disease, it is closely related to IR and metabolic syndrome (53,54).

1.1.4.c) **Insulin resistance and Diabetes Mellitus type 2**

Insulin resistance is one of the other main comorbidities of obesity. Lipotoxicity, increased visceral fat accumulation, and increased pro-inflammatory molecules and toxic metabolites derived from the adipose tissue directly affect glucose metabolism and contribute to the impairment of insulin signaling through several mechanisms (15,24,56). As a consequence of IR, the pancreas increases insulin secretion, raising the levels of circulating insulin, which also contributes to the inflammatory state, and is linked to the process of atherosclerosis and later to CVD (49,56). The combination of IR, higher levels of circulating glucose and insulin, and genetic factors contribute to β-cell dysfunction, ending up in DM2 (15).

1.1.4.d) **Dyslipidemia**

Obesity is also characterized by hypertriglyceridemia, elevated plasma FFA, increased small and dense low-density lipoproteins (**LDL**) with reduced cholesterol esters, leading to increased small dense LDL, and lower levels of the beneficial high-density lipoproteins (**HDL**) (57). The hepatic synthesis of TG and very low-density lipoproteins (**VLDL**) is increased due to the higher influx of FFA from the adipose tissue to the liver, together with the developing IR and a worsened FFA clearance. Dyslipidemia is one of the factors behind the elevated CVD risk in obesity (15,57).

1.2. TO TACKLE OBESITY

The high obesity rates have led to an elevated concern on how to manage this problem. Obesity is too often not considered as a progressive disease, so the initial overweight stages are many times ignored until obesity is consolidated (2). To manage obesity there are several approaches, including behavioral interventions and bariatric surgery.

1.2.1. *DIET*

Behavioral interventions involving diet and exercise are the most common approaches to treat obesity and the associated comorbidities. There are several types of diet, but most of them rely on a calorie restriction which can be more or less pronounced, from low-calorie diets (800-1500 kcal/day) to very-low calorie diets (less than 800 kcal/day) (58). Diets may also vary by composition, most of the diets reduce either carbohydrate (ketogenic diets) or fat as a way to reduce the calorie content, but some diets may also have increased healthy unsaturated fats (Mediterranean diet) (7,58–60).

Besides the weight loss itself, diet can be used to reduce the severity of several comorbidities and it is well known that even a modest weight loss is beneficial (7). Thus, a calorie-restricted diet is usually advised for NAFLD patients, which improves the plasmatic markers for liver alterations and the degree of hepatic steatosis. Another benefit of weight loss following dieting is the improvement of IR. Weight loss is more successful and long-lasting when it is accompanied by exercise (7,58,61).

Despite the benefits and the simplicity of losing weight through diet modifications, this approach is often directed only to people with overweight and is far from being an optimal solution when morbid obesity is well stablished. The achieved weight loss is usually modest and many patients show difficulties in following a diet, and even with success, patients tend to regain weight in the following 5 years (9,62).

1.2.2. *BARIATRIC SURGERY*

Bariatric surgery (**BS**), a surgical intervention against obesity, is the most effective treatment for higher degrees of obesity to lose weight and reduce comorbidities (9,63). It is recommended for patients that have tried to lose weight without success, or that had been unable to maintain the achieved weight loss for a prolonged period of time, or that has with a BMI equal or over 40 kg/m2 or between 35 and 40 kg/m2 while also presenting comorbidities (64).

1.2.2.a) **Origins**

Bariatric surgery, from the Greek baros -βάρος- meaning weight, were developed in the early 1950s as a metabolic surgery "an operative manipulation of a normal organ or organ system to achieve a biological result for a potential health gain" and is currently the most effective treatment against obesity (63). The jejunoileal bypass was the first surgery tested and consisted of excluding a large part of the small intestine from the nutrient flow, and thus, producing weight loss through malabsorption. Despite producing a significant weight loss it was later discarded due to the severe complications, but gave rise to new variations (9,63).

1.2.2.b) **Description**

The most common procedures currently used are the Roux-en-Y gastric bypass (**RYGB**), the adjustable gastric band (**AGB**), and the vertical sleeve gastrectomy (**VSG**) (**figure 4**). They have substantial differences and can be categorized as malabsorptive, restrictive, or both,

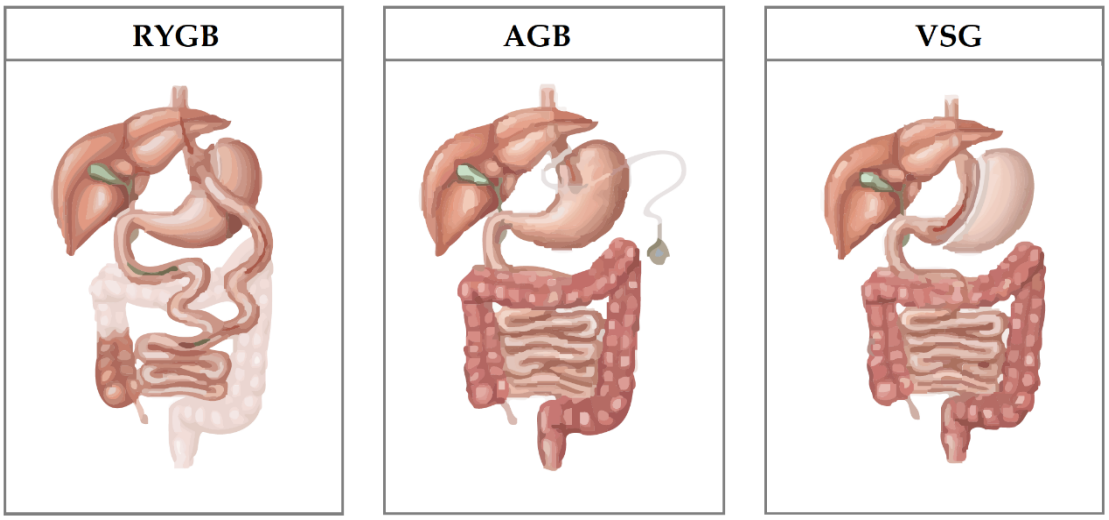

Figure 4. The most common types of bariatric surgery. The restrictive and malabsorptive Roux-en-Y gastric by-pass (RYGB), and the restrictive adjustable gastric band (AGB) and vertical sleeve gastrectomy (VSG). From Stefater, 2012 (9).

depending on the mechanism used to lose weight, although all share a mechanistic trait which is a physical restriction of the stomach (65). The stomach is an organ directly involved with satiation. The proximal part dilates before a meal in order to increase its capacity as food storage, while the distal part is responsible for triturating the food into small pieces so that they can pass the pylorus. Satiety after a meal is regulated by the capacity of the stomach and how fast it empties, among other factors (62).

ROUX-EN-Y GASTRIC BYPASS

The RYGB has been the most performed BS for many years and is both a malabsorptive and restrictive technique. It consists of creating a small gastric pouch, thus restricting the stomach capacity, where the jejunum is connected, bypassing the duodenum and part of the jejunum, to create malabsorption (9,65). It removes the pylorus, increasing the gastric emptying (62).

ADJUSTABLE GASTRIC BAND

The AGB is a restrictive procedure where a silicon band filled with saline is placed on the upper part of the stomach, reducing its capacity. It's surgically simpler but the effects are much humbler than the other two procedures, which is leading to a decreased popularity (9).

VERTICAL SLEEVE GASTRECTOMY

The VSG is also thought of as a restrictive procedure, up to 80% of the stomach is resected, leaving a sleeve-like pouch with severely reduced capacity without altering the intestinal nutrient flow. Although the pylorus is left intact, it is also associated with accelerated gastric emptying (9,62). Despite being the newest technique of the three, it has quickly gained popularity worldwide, as is surgically simpler than RYGB, requires less convalescence time. In addition, VSG does not show the malabsorption problems associated with RYGB, providing a minimal risk for vitamin deficiency or malnutrition and becoming the most performed BS in 2017 in the United States (63,66–68).

1.2.2.c) **Effects**

Obese patients who undergo BS experience improvements soon after the surgery. Visible effects are observed in the first months as there is an elevated decrease in body weight, especially in RYGB and VSG, which is also more rapid and sustained than in AGB patients (69). After one year of BS, anthropometrical parameters are also improved as can be seen in reduced waist circumference and more importantly, many comorbidities are improved, such

as several metabolic parameters related to metabolic syndrome, improved NAFLD lesions (70,71), and a less atherogenic profile of the plasmatic lipids (72). Interestingly, VSG patients show similar results to RYGB patients, indicating that much of the improvements seen after BS are not related to the associated malabsorption (67,73).

The mechanisms behind the positive effects of VSG are still largely unknown, but there are several potential mechanisms involved:

- Energy-balance related: VSG is a restrictive surgery leading to reduced intake, which can not alone account for the weight loss, as studies with calorie restriction do not achieve the same results (74). As other BS, VSG patients have an increased gastric emptying, which is associated with increased satiety (75).

- Regulation of gut hormones: Following VSG, there is a reorganization of several gut hormones such as reduced ghrelin, reduced Peptide YY, or increased GLP-1. They have an impact on hunger and satiety, although the exact mechanism remains unknown as there are contradictory data (76,77).

- Reorganization of the vagal nerve-brain axis: The vagal nerve is responsible for much of the gut-brain communication and is coupled to the stomach to sense the distension and engage satiation signals crucial for the appetite regulation (78,79). VSG largely damages the connections with the stomach, uncoupling recompense signals and increasing satiety, altering the complex network between the central nervous system and peripheral signals that regulate hunger and satiation (76).

- Gut microbiota: In recent years, the GM has been proposed as a potential mediator of the positive effects after VSG. Its role in obesity will be discussed in the next chapters.

1.2.3. *DIET AFTER BARIATRIC SURGERY*

One of the common features of the three most common BS types is the reduction of stomach capacity, which determines the amount of food ingested during meals. For patients undergoing BS, food is gradually introduced during the first four weeks adding textures and new foods each week until food intake is normalized by the end of the first month after surgery (80). The dietary recommendations after BS consist of eating a high-protein, low-

carbohydrate and low-fat diet of 1500 kcal, consisting of a 25% protein, 45% carbohydrates, and 30% fat (81). At the same time, a turn to healthier habits is strongly advised, with recommendations for a higher intake of fruit and vegetables, and reduction of calorie-dense foods such as sweets and fried foods. Maintaining a correct diet seems to be crucial for achieving a satisfactory weight loss after BS, as well as for maintaining the desired weight. Higher reductions of calories in the first year correlate with higher weight loss in the following years (80–82). Non-adherence to a correct diet is one of the causes of weight regain after BS (83,84) which affects up to 30% of patients during the 5 years after BS (84,85).

1.3. RODENTS AS AN ANIMAL MODEL FOR OBESITY

Animal models are an indispensable tool in research, but also their use is a source of debate and criticism in our society. The limitation of the *in vitro* techniques and the ethical restrictions in obtaining human samples still make animal models necessary for research, as humans and animals, especially mammals, share many molecular pathways and diseases (86).

The most commonly animal model used for the study of obesity and its comorbidities are rodents, as they are economical, easy to manipulate, and a well-characterized mammalian model. Several available modified strains are used as models for both obesity and DM2, such as the hyperphagic obese and Zucker and Koletsky rat strains, both with mutations on the Leptin receptor, but obesity can also be induced, through mechanically, chemically, or dietary methods in non-modified strains (35).

1.3.1. *DIET-INDUCED OBESITY*

Diet-induced obesity (**DIO**) is a widely used method consisting on the administration of high-calorie diets to animal models to increase their body weight and induce the apparition of obesity-related comorbidities. It resembles the developmental process of obesity in humans, and thus, allow researchers to study the effect of diet on genes and metabolism during the increasing weight phase and the apparition of comorbidities (19,35). There are

several DIO diets with different macronutrient composition, which can be enriched with sugars, fats, or both. The high-fat diet (**HFD**), the cafeteria diet (**CAF**), and the high-fat high-sugar diet (**HFHS**) are the most commonly used, with their respective ingredient variations (87,88).

The HFD is a fat-enriched diet, with 30 to 80% of the calorie content coming from fat. Rats have a nutritional requirement of 5% of fat content in their diet, and the administration of an HFD lead to increased body weight and the following apparition of obesity related conditions, such as IR (**figure 5**) (87,88). HFD can induce obesity more rapidly and efficiently than diets based on carbohydrates and low in fats, and thus, is a very popular diet used in the study of obesity (87).

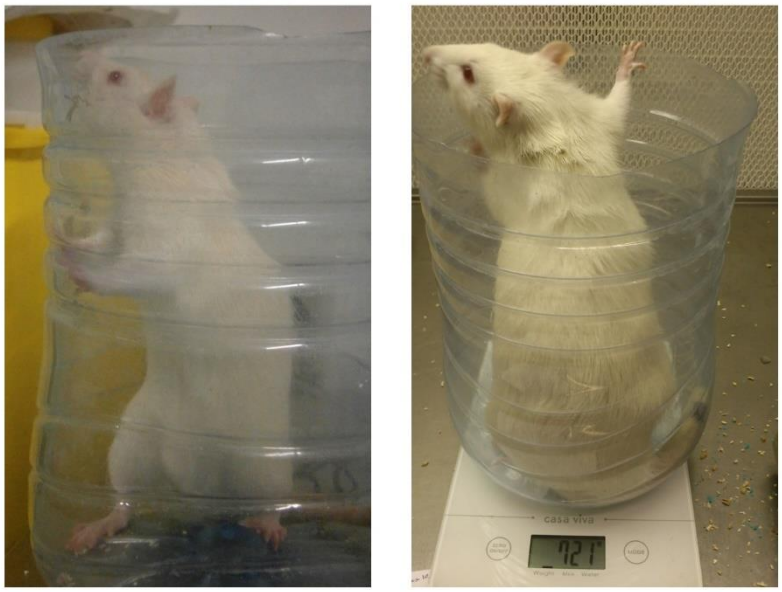

Figure 5. Comparison between a control and a DIO rat. Right, a rat fed with a standard chow diet. Left, a DIO rat in our study, after 11 weeks of a HFD.

The type of fat used in HFD is an important source of variability between studies. Usually, diets are enriched with mammal-derived fats (lard, or beef tallow) rich in SFA, with more capacity of inducing obesity than diets enriched with vegetable fats (soybean, olive, and coconut oil) richer in monounsaturated fatty acids (**MUFA**) and PUFA (35,87–89). The FA in the diet exert different effects: SFA are more obesogenic as they end up mainly stored in the adipose tissue, while MUFA and PUFA are prioritized as an energy source and stored to a lesser degree (87,88); an elevated concentration of PUFA can actually help reduce the weight gain as they increase satiety, as seen in HFD enriched with fish oil (87,90); SFA from

animal origin, with long-chained FA, are more effective inducing obesity and IR than vegetal SFA, composed mainly by medium-chain FA (35,88).

Animals on an HFD show a rapid increase in body weight during the first two weeks of diet administration, but this increase tends to reduce and stabilize in the following weeks as the food intake decreases. This reduction is explained by two mechanisms, a complex, and a simple, that may work together: an auto-compensation of the high caloric density of the HFD (88,91,92), and a loss of interest for the HFD after getting used to the new, more palatable, taste (35).

1.3.2. *BARIATRIC SURGERY EXPERIMENTS IN RODENTS*

As mentioned earlier, bariatric surgery has become the most effective and durable treatment against obesity. However, despite its popularity, most of the underlying mechanisms behind the improvements seen in body weight and metabolic parameters remain unknown (93). Although much research is performed in subjects undergoing bariatric surgery, many factors that are key for the understanding of the ongoing molecular processes after BS cannot be evaluated in individuals/humans (94) and thus, most of the surgical procedures have been established in rodent models, the most common being RYGB and VSG (95). The use of rodents in the study of BS has multiple advantages, as it allows us to observe the effects of BS in controlled situations of diet and environment that can be modified (93,95).

Rodent models offer (93,95):

- The opportunity of having detailed information about the feeding patterns after BS, which is usually a tricky point in many human studies.
- The possibility of adding controlled changes in diet in order to compare the effects combined with BS, as well as having diet controls.
- The use of sham-operated controls to compare with animals undergoing BS.
- The use of knock-out rodents for obesity related genes, and thus observe the differences in the BS outcome.

VSG in rats was established in 2007, with the procedure resembling the intervention performed in humans (96). A 60-70% proportion of the stomach is resected, removing

mainly the fundus which is responsible for ghrelin production and leaving a gastric tube without altering the pylorus or the rest of the gastrointestinal tract (**figure 6**) (96,97). The effects are similar to the ones in humans: A rapid reduction in body weight in the first two weeks (mainly from the adipose tissue), the maintenance of lower body weight, and the improvement of glucose homeostasis (93,95,98).

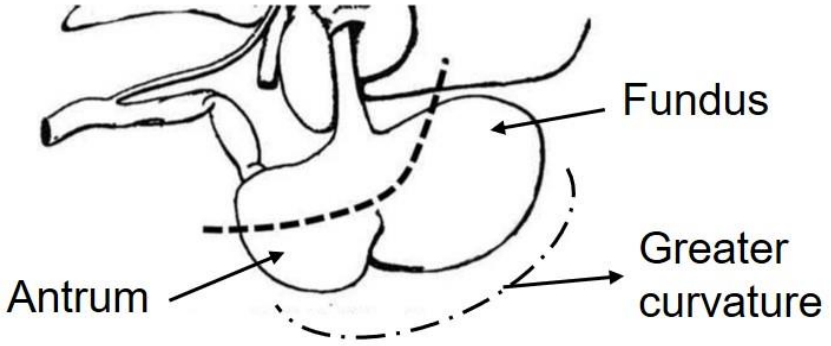

Figure 6. Vertical sleeve gastrectomy in a rat stomach. The dashed line indicates the resection line performed in VSG. From Lopez et al. 2010 (97).

Despite the initial weight loss, rats start to recover weight in the third week after BS, although without reaching the pre-surgery weight, thus maintaining a lower weight compared to non-operated controls (98,99). It has been observed that rats change their meal patterns after BS, eating smaller amounts but increasing their meal frequency, as a way to compensate for the mechanical restriction imposed by the reduced stomach pouch (95,99). Experiments in pair-fed animals -eating the same amount as rats subjected to VSG do- also show an elevated weight reduction, as well as some improvements in glucose metabolism, showing that eating less is crucial in the benefits seen after VSG (93). Interestingly, a study performed on VSG-rats exposed to food-restriction situations showed that VSG rats still had the capacity to overeat. When the restriction was over, rats ate bigger amounts to compensate for the weight loss, in the same way non-operated rats did. This shows that although the capacity for overeating is intact after VSG, the drive for doing it is suppressed in normal conditions (99). This fact argues against the initial ideas that the benefits of VSG arise only from the mechanical restriction, and that there are other factors involved, as the rest of the digestive tract remains unaltered (93,99).

1.4. GUT MICROBIOTA

With the technological advances in the last years, the GM –a highly plastic microbial community living in our intestines- has become the center of interest for many researchers. The possibility to analyze a bacterial community by the analysis of the ribosomal 16S, without being dependent on culture-methods, has led to the discovery of a new world inside us. Commensal bacteria, once seen as a potential threat to the host system, are now being seen as a necessary counterpart in several processes regulating the host homeostasis (100–102).

1.4.1. *DESCRIPTION*

The GM is the collection of living microorganisms inside our body, a population that exceeds 100 trillion, and that outnumber by a factor of 10 the number of host cells. It is composed of archaea, bacteria, fungi, and virus, although most research is often centered on the bacterial part (100,103).

1.4.1.a) **Composition**

The GM is a diverse, and dynamic community that has co-evolved with the host, ending up in constituting a more or less common microbial set. In humans, two major phyla dominate the GM: the Bacteroidetes and the Firmicutes, accounting for more than 60% of the microbiota (104–106). The phylum Actinobacteria is sometimes also counted as one of the main phyla, but with fewer numbers than the other two. The rest of the microbiota is composed of the phyla Proteobacteria, Verrucomicrobia, and others (**figure 7**) (102,104–107). The most common taxa found in each phylum are *Enterococcus* for Firmicutes, *Bacteroides* for the Bacteroidetes, *Bifidobacteria* for Actinobacteria, *Lactobacillus*, *Clostridium,* and *Escherichia coli* for Proteobacteria and *Akkermansia Mucinphilla* for Verrucomicrobia (101,108).

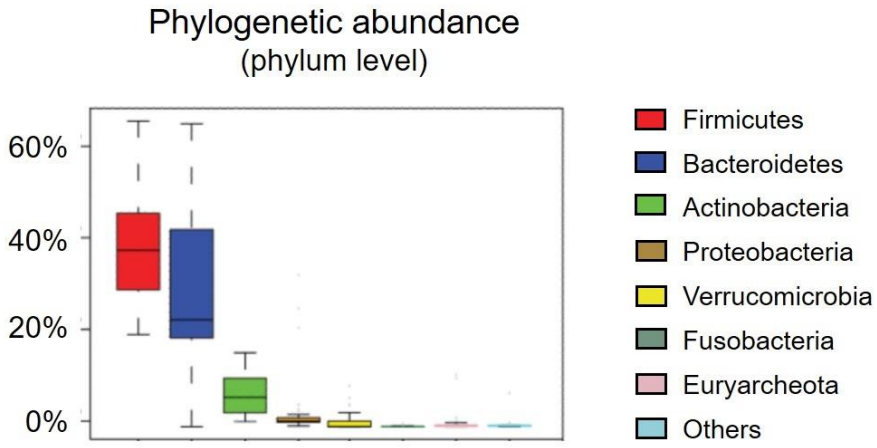

Figure 7. The most common genera of bacteria living in the mammalian gut. Adapted from Arumugam 2011. (106).

The microbiota is in constant change, as it is easily modified by environmental non-host dependent factors, such as child delivery methods, family microbiota composition, genetics or aging; or directly influenced by the host, such as diet, antibiotic use, habitat, etc. (102,107). The intestine is also able to regulate the microbiota, secreting antimicrobial peptides (**AMPs**) that modulate its composition (101).

Although many of the pathways are redundant between species, ensuring that the metabolic functions are maintained and stable, it is important to mention that the most abundant species do not necessarily correspond to the most common functions. Non or low abundant species can account for specific beneficial functions for both the host and the microbiota itself that are crucial, even though their numbers are small (106,109). This adds some difficulty in understanding the role that different bacteria have. Metagenomics, the study of the genes present in the GM environment, is thus often necessary to fully understand the exact functions and adjudicate a role for determinate bacteria (106). Categorizing the GM at lower taxonomical levels can be a powerful tool for diagnostics and thus attracts a big interest. However, despite the many studies being performed, a consensus for a clear characterization remains elusive. It has been observed that some taxa tend to cluster together in what is called enterotypes, which might represent optimal functional groups of bacteria. Still, more research and standardization need to be done to understand the full potential of bacterial compositional clusters (106,110).

1.4.1.b) **Diversity**

As in all ecosystems, diversity is an important and desirable factor in the GM. At an early age of an individual, the microbiota has a reduced diversity but it rapidly evolves, adapts, and stabilizes with time after exposure to the environmental factors mentioned above (102,110). A diverse microbiota offers more adaptability and better stress responses, as well as redundancy for key functions, ensuring a well-functioning microbiota even when there are modifications in composition (100).

1.4.2. *THE FUNCTION OF THE GUT MICROBIOTA*

The complex ecosystem that is the GM is often regarded as a whole metabolic organ, able to change and adapt to the host. With 100 times more genes than their human hosts, they provide for different advantages in what is in effect a symbiotic relationship with the host, regulating several processes. The host and the microbiota have a complex relationship in which they work together to maintain the homeostasis and the correct functioning of immunity and epithelial integrity, which contributes to the protection against pathogens (**figure 8**) (100,102,111).

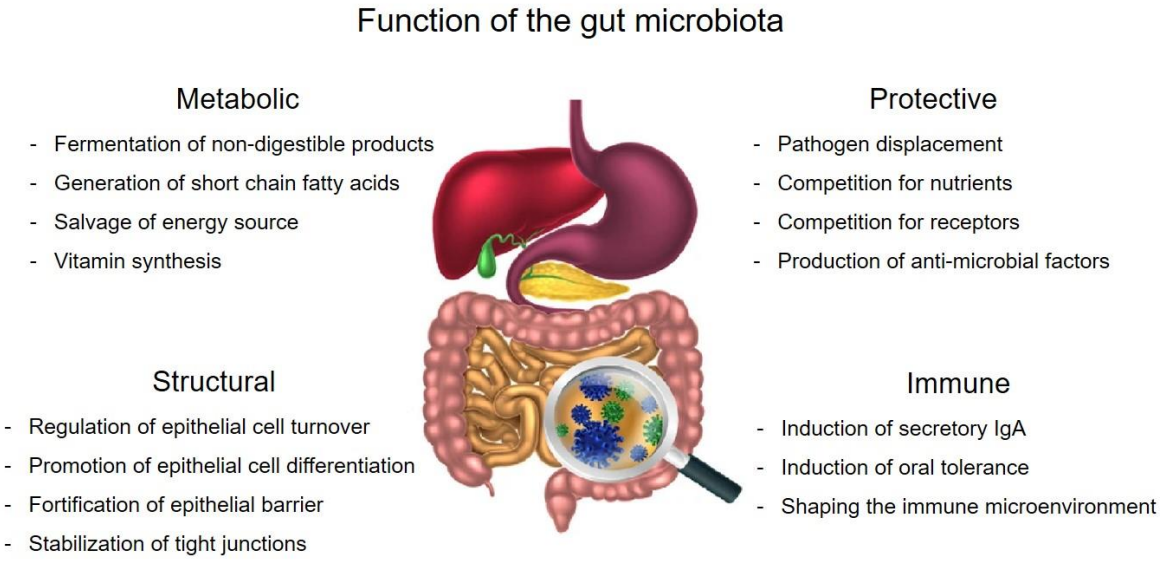

Figure 8. Functions of the GM. Text adapted from Yu, 2012 (101).

1.4.2.a) **Anaerobic reactor**

The GM colonizes the whole of our gastrointestinal tract but is in the intestine where it gains its most well-known functions, working as an anaerobic bioreactor. Our diet contains, or should optimally contain, a big portion of complex plant polysaccharides -such as cellulose and pectin- and resistant starch that mammals are unable to digest, offering substrate for microbial processes (104).

It is thought that the colonization of the mammalian intestine occurred early in the evolution, developing a symbiotic relationship in which the host provided for food and environment to the microbiota and, in exchange, the microbiota could digest the otherwise undigestible products, providing this additional function to mammals without the necessity to develop genes for it (100).

Besides energy, the metabolic process offered by the GM produces vitamins such as ascorbate, biotin, cobalamin, folate, pantothenate, thiamine, riboflavin, and vitamin K, beneficial for the host (106,111,112).

1.4.2.b) **Short chain fatty acids**

The undigested dietary fibers pass the small intestine and reach the large intestine where they are anaerobically fermented by the GM, mainly in the caecum and the ascending colon. The fermentation process produces energy and short chain fatty acids (**SCFA**), mainly acetate, propionate, and butyrate, that are used in the intestine, or absorbed and carried through the portal vein (109,113). The SCFA are one of the main energy sources for the enterocytes, and butyrate also has an important role in colonocyte differentiation and colonic cancer prevention (101,109).

1.4.2.c) **The gut barrier**

The gastrointestinal tract is the largest surface in the body that is in constant contact with the external environment. The gut epithelial is formed by a single layer of intestinal epithelial cells (**IEC**) and acts as a physical defense barrier against pathogens (101,111). The IEC, with their characteristic brush-like microvilli, are united by tight junctions, which allows the diffusion of small molecules and water through paracellular permeability. For larger molecules such as peptides, the transcellular permeability allows passage by means of transporters (101).

The GM is located on the microvilli, separated by a mucus layer that avoids the physical contact between the two and thus, prevents possible translocation of microbial products from the lumen to the interior. The mucus is a net-like structure formed by glycans (mainly mucin-2) secreted by specialized IEC called goblet cells (101,112,114). Commensal microbiota helps to maintain the integrity and well-functioning of the gut barrier: they stimulate the mucus secretion through the produced SCFA, which stimulates the secretion of mucin and facilitate the assembly of tight-junctions between the IEC, as well as degrade shed elements of the mucus layer and exfoliated epithelial lining cells (100,111,114).

The intestinal epithelial has a high turnover -it can be renovated in one week- helping to keep the homeostasis and the correct permeability of the gut barrier. Studies in germ-free animals have shown a decreased turnover rate, an altered microvilli formation, and a higher sensibility to the toxicity of dextran sodium sulfate, a compound used to cause damage to colonic cells, indicating the importance of the commensal microbiota in maintaining a healthy gut barrier (101,111,112).

1.4.2.d) **Immunity**

Apart from the physical defenses, the gut barrier is immunologically reinforced by phagocytes and lymphocytes, that are infiltrated in the lamina propria under the IEC, forming lymphoid structures. They produce cytokines, chemokines, and antimicrobial products into the mucus layer that are able to modulate the composition of GM, as well as being beneficial for the maintenance of the gut barrier, and at the same time, the GM plays a role in the development of the lymphoid structures (101,112,114).

The GM is, in fact, essential for the well-functioning of the innate immunity. Studies in germ-free mice shown a weakened immune system and young mice under antibiotic treatment had an increase in pro-inflammatory responses, as well as a higher risk of inflammatory disease (111). The pattern recognition receptors (**PRRs**) of the intestinal immune system, such as the toll-like receptors (**TLRs**), interact with the microbial products from the commensal microbiota, such as lipopolysaccharide (**LPS**). These interactions are necessary for the good development of the intestinal immune system: they activate the innate immunity, help with the recognition of multiple bacterial components, increase tolerance against microbial products, and are necessary for the TLRs to be able to protect the epithelial from injury, among other beneficial effects (111,112,115,116).

At the same time, the microbiota also offers a possible threat and can trigger diseases in circumstances where the immunity is weakened (114). The production of AMPs helps to control the population of commensal bacteria and changes along the intestinal tract, being higher in the ileum and lower in the colon, where a higher concentration of bacteria is needed for the fermentation process (117).

1.4.3. *MICROBIOTA IN OBESITY*

The increasing knowledge about the composition and the role of the GM led to further investigations attempting to elucidate its connections with several diseases, obesity being one of the most studied. It has been shown that the microbiota is different in obese subjects, whether humans or rodents, which led to an important question: Is the GM one of the causes of obesity? Or are GM modifications a side effect of obesity?

The identification of the role of the GM in the development of obesity can suppose a new target for fighting it.

1.4.3.a) **Dysbiosis**

The ingestion of a HFD has clear effects on the microbiota, especially when compared to control counterparts. The alterations in taxonomical composition and bacterial diversity and richness –a loss in species and in number- lead to dysbiosis, an imbalance in the regular microbiota (**figure 9**) (118). Dysbiosis results in the loss of homeostasis of the gut barrier which affects the intestinal mucosa (112). At the molecular level, these alterations lead to a disruption in the tight-junctions, resulting in a damaged intestinal epithelial (119). In normal situations, a well-functioning gut barrier does not allow the crossing of bacterial components, such as LPS, from the lumen to the inside (101), but a weakened barrier integrity has increased permeability, allowing the translocation of bacterial products (119). In fact, genetically obese mice and lean mice consuming an HFD showed elevated plasmatic levels of LPS, which correlated to increased adiposity and inflammation markers (120,121). Interestingly, those effects can be partially reversed by the administration of probiotics or antibiotics (121,122).

In humans, obese patients also present dysbiosis, together with an elevated basal level of activated TLR4 and LPS, which is also seen in DM2 patients. Interestingly, an elevation in circulating LPS after a meal rich in fat has been observed (122,123). Still, observations in human patients are complex, as feeding conditions (time, meal composition) are less standardized, and subjects present a higher variability (122).

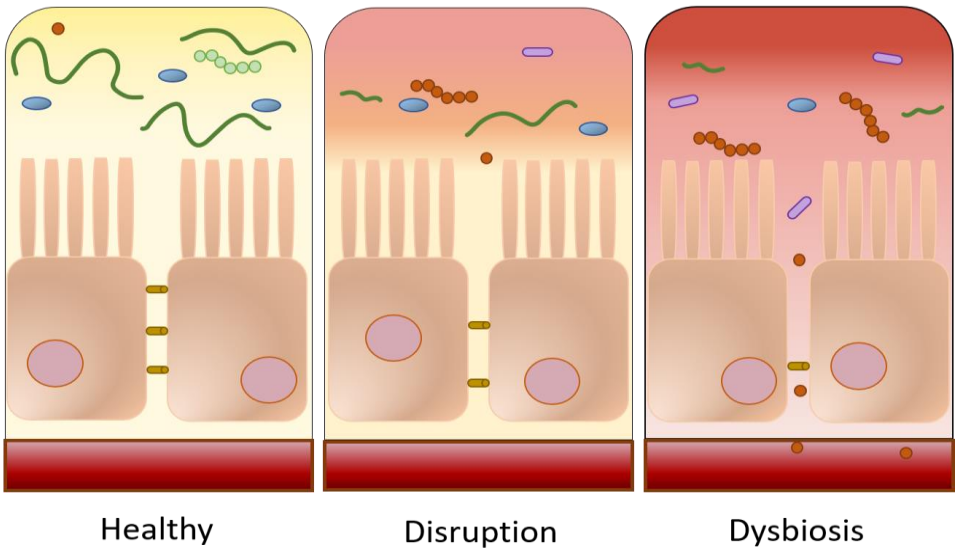

Figure 9. Progression from healthy intestine barrier to dysbiosis. In the healthy state, epithelial cells are hold together through the tight junctions, and commensal bacteria and the mucus layer contribute to the gut barrier, protecting the intestinal epithelial from other bacteria. In the disruption state, there is some disruption of the balance, with the mucus layer and the tight junctions uniting the IEC are decreased. In the dysbiosis state, the intestinal barrier is damaged, there is no protection on the intestinal epithelia and bacterial products are translocated into the blood capillary (101, 112, 118, 119).

1.4.3.b) **Modulation of the energy balance**

The microbiota has been proposed to be a key player in obesity through its capacity to regulate energy intake and expenditure. In 2004, Bäckhed et al. published a study where it was shown that germ-free mice had less weight gain and less fat deposition compared to normal mice. Not only that, but germ-free mice receiving a microbiota transplant also had a higher body weight and higher fat deposition despite having a reduced chow intake (40). Other studies also showed that germ-free mice were also unaffected from a high-caloric WD (124,125) and genetically obese mice were identified as having a microbiota with an increased capacity for energy harvesting (126). Despite the promising results, later studies challenged the former data, showing no association between shifts in the microbiota and markers of

energy harvesting (127) and indicated that germ-free mice were not protected from the effects of a HFD (128).

More research is still needed to fully understand how the microbiota can affect the energy balance, in more standardized conditions. At the moment, there are several suggested mechanisms in which the microbiota may modulate the energy balance:

ANGIOPOIETIN-LIKE PROTEIN 4

Angiopoietin-like protein 4 (**ANGPTL4**) is a protein inhibitor of the LPL. Components of the GM are inhibitors of ANGPTL4 in the intestinal epithelium, increasing levels of LPL and resulting in higher deposition of TGs in the adipocytes (40,100,129).

SHORT CHAIN FATTY ACIDS

As products of fermentation, SCFA have been proposed to increase the energy content in food (126). Also, SCFA are seen as mediators in the crosstalk between gut and peripheral tissues (130). The role of SCFA during energy modulation is unclear, and data can be contradictory, pointing to a complicated relationship between SCFA and obesity. On one hand, SCFA are increased in obese subjects, but the values tend to normalize with time (127,131), on the other, the supplementation of SCFA in mice protected them from obesity and DM2 (102). SCFA may have a role in appetite regulation, as they affect gut hormones such as GLP-1, leptin, ghrelin, as well as affecting lipogenesis, but more research is needed to elucidate the mechanism (33,41,102,132).

INCREASED LIPOGENESIS

The produced SCFA enter the blood circulation and acetate and butyrate are used as substrate for DNL giving rise to more newly synthesized TG, while propionate inhibit it (133).

1.4.3.c) **An obese phenotype?**

One of the objectives of many studies has been to identify a determinate microbiota composition during obesity. Initial studies saw that obese mice had a higher ratio of Firmicutes: Bacteroidetes (due to decrease in the Bacteroidetes portion) when compared to their lean counterparts, establishing a so-called obese-phenotype (105,126,134) that was also seen in humans (105). However, this ratio was challenged by several other studies observing the inverse shift and thus contradicting the stated ratio (119,135,136) and the observed taxonomical shifts have been found to be more dependent on a HFD than on obesity itself

(127,137,138). Also, a statistical analysis of several microbiota data found no significant relationship between obesity and taxonomic composition, with higher variation between studies than between each lean vs obese cohort (139).

The elevated number of factors influencing the microbiota studies makes it difficult to determinate of a common phenotype or a consensus in the taxonomical shifts seen in obesity, but despite this, it is clear that a HFD markedly alters the composition of the GM.

1.4.4. *GUT MICROBIOTA AND BARIATRIC SURGERY*

The GM is heavily affected by BS, as it physically modifies parts of the gastrointestinal tract. Many studies have been performed in the last years, investigating the modifications exerted by BS on GM, as it has been hypothesized that it could play a major role in the benefits following BS. Indeed, BS leads to many taxonomical shifts, affecting several taxa, but many of the taxa modifications are study-dependent and not consistent between studies, which difficult the identification of a common component (140).

Despite the difficulties in identifying key taxa modifications after BS, it has been observed that many of the post-BS modifications are linked to the improvements seen after BS, such as improved glucose homeostasis, decreased adiposity, and decreased inflammatory state (123,140). It is thought that those improvements are related to the GM, as BS has proven to increase microbial restoration and to contribute to partial recovery from the dysbiotic state seen in obesity (123). This suggests that GM can act as a key regulator during the metabolic recovery after BS.

2. HYPOTHESIS AND OBJECTIVES

As introduced in the previous chapter, an unbalanced, unhealthy diet is one of the main causes leading to obesity and the apparition of comorbidities. Bariatric surgery is currently the most effective and durable treatment against obesity. Despite being a commonly performed technique is still much investigated, as the mechanisms by which the weight is reduced and the comorbidities are improved are still largely unknown. Implementing healthier dietary habits after BS appears to be decisive to maintain the obtained benefits, besides the mechanistic restriction of the BS. On the other hand, dieting alone is also described to have beneficial effects if well implemented. Based on this information, we established the following **hypothesis**:

> A high-fat diet will induce modifications on a rodent model, such as increasing adiposity, unbalancing the gut microbiota, and modifying the fatty acid composition of several organs. The deleterious effects produced by the high-fat diet will be partly improved by either vertical sleeve gastrectomy or by a change of diet, while the combination of both actions will have a synergistic effect and a better outcome than both actions alone.

To explore this hypothesis the following **objectives** were formulated:

- Identify the major modifications caused by the HFD on adiposity, gut microbiota composition, and fatty acid composition in tissues.
- Study the effects caused by VSG when the high-fat diet is continued.
- Study the effects caused by a change of diet alone.
- Study the effects caused by a combination of VSG and a change of diet.

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3. ARTICLES

This dissertation is presented as the compilation of two published articles, as well as a third unpublished article, prepared for submission. All three manuscripts have the PhD student, Joana Rossell, as the first author. Also, non-published data is added in this section.

The results presented here, in form of article or with the unpublished material, are based on a set of 54 Sprague Dawley rats that were subjected under two main variables: diet and surgery. In turn, each main variable was divided into 3 sub-variables, resulting in 9 final groups that combine different diets and surgical approaches. During the process of elaborating the articles and as a suggestion from referees, there has been some changes in the nomenclature to define the different groups. To avoid confusions, we will briefly explain the study design and the different names for the groups (figure 1, article 2; figure 1, article 3): Animals were separated in three diet groups: One fed a standard chow diet, becoming the Control (C) group. The second fed a HFD during the whole experiment, becoming the DIO group (named D in article 2, and HF in article 3). The last was fed a HFD during the first 8 weeks, and then changed to standard chow for the last 4 weeks, becoming the diet-change (DC) group (named DIO+C and D+C in article 1, D+C in article 2, and DC in article 3). At week 8, animals on each group were divided in three further groups (n=6) and were subjected to no surgery (NS), simulated surgery (Sham) or VSG. The final groups, as will be named in the discussion, are the following: C-NS, C-sham, C-VSG; DIO-NS, DIO-sham, DIO-VSG; DC-NS, DC-sham, and DC-VSG.

3.1. DIRECTOR'S IMPACT FACTOR REPORT

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La Dra. Julia Peinado Onsurbe, Catedrática del Departamento de Bioquímica y Biomedicina Molecular de la Facultad de Biología de la Universidad de Barcelona, desea hacer constar que Joana Rossell Rusiñol ha sido doctoranda y colaboradora en nuestro grupo de investigación desde el año 2012.

El trabajo que ha realizado durante el desarrollo de su tesis ha estado enmarcado dentro de proyectos de obesidad mórbida, en los cuales figuro como IP, subvencionado por el Fondo de Investigación Sanitaria del Instituto de Saludo Carlos III.

El modelo de tesis por artículos no permite poner de manifiesto todo el trabajo realizado previamente a la publicación de los artículos, pero es muchísimo más amplio de lo que en dichos artículos se puede presentar.

El **primer artículo** se publicó en la prestigiosa revista *Obesity Surgery*, especializada en cirugía de la obesidad, situada en el primer decil dentro del área de *Surgery* y cuyo índice de impacto (2017) es de 3.607. El título del artículo es *"Diet Change After Sleeve Gastrectomy Is More Effective for Weight Loss Than Surgery Only"* y Joana Rossell figura como primera autora, siendo el último firmante el Dr. Baena-Fustegueras, el cirujano que llevó a cabo la cirugía bariátrica en las ratas. En este artículo se hacen constar los cambios antropométricos que tienen lugar en las ratas obesas y controles durante un determinado periodo de tiempo. Se ponen de manifiesto también, en dicho artículo, los cambios en el peso de todos los tejidos del animal. Todo el trabajo que consta en este trabajo fue llevado a cabo por Joana Rossell, excepto en el momento del sacrificio de los animales en que fue ayudada por los colaboradores del grupo de investigación.

El **segundo artículo** se ha publicado en la revista *European Journal of Nutrition* especializada en nutrición, situada en el primer cuartil dentro del área de *Nutrition and Dietetics* y cuyo índice de impacto (2019) es de 4.664. El título del artículo es *"Diet change affects intestinal microbiota restoration and improves vertical sleeve gastrectomy outcome in diet-induced obese rats"* y Joana Rossell figura como primera autora, siendo el último firmante el Dr. Klas I. Udekwu del Departmento de Biociencia Molecular del Instituto Wenner-Gren de la Universidad de Estocolmo en Suecia, donde Joana Rossell se desplazó durante unos meses para realizar un estudio pormenorizado de la composición bacteriana de los ciegos de las ratas que habíamos operado en Barcelona.

El tercer artículo que se presenta en esta tesis tiene por título *"Combination of diet and bariatric surgery promotes healthier changes in fatty acid profiles in the livers of obese rats".* Al igual que en los otros dos artículos, Joana Rossell figura como primera firmante y como segundo firmante está el Dr. Domingo que nos ha prestado su inestimable ayuda en el análisis de los ácidos grasos del hígado de las ratas que operamos para el primer artículo. El artículo todavía no se ha publicado, pero está preparado para enviar a la revista *Journal of Lipid Research*, situada en el primer cuartil dentro del área de Bioquímica y con un índice de impacto de 4.560 (2019).

Por lo que se refiere a todo el trabajo que ha realizado Joana Rossell en el laboratorio, no sólo ha demostrado su gran disponibilidad e interés sino también su capacidad para el poner a punto diferentes técnicas que nos seguirán siendo de gran utilidad en nuestro trabajo de investigación.

Por otra parte, ha adquirido una gran experiencia en la valoración de diferentes parámetros bioquímicos y ha demostrado su gran valía y su capacidad para acometer cualquier tipo de tarea relacionada con su campo de trabajo.

Tanto nuestro grupo de investigación como yo misma, estamos plenamente satisfechos del rendimiento de Joana Rossell y del trabajo de investigación que ha desarrollado, considerándola altamente cualificada para llevar a cabo cualquier tipo de trabajo de investigación.

Por otra parte, desearía hacer constar, que nuestro Departamento ha potenciado siempre los estudios bioquímicos, y de biología molecular aplicados a la biomedicina y actualmente figura como uno de los más prestigiosos del país. A pesar de estar formado por más de trescientos colaboradores entre profesores, doctorandos y becarios españoles y extranjeros, los criterios de selección son bastante estrictos en cuando a la formación de nuestros colaboradores, méritos que creemos reúne suficientemente Joana Rossell.

Barcelona a 1 de Octubre de 2020

Dra. Julia Peinado Onsurbe

3.2. ARTICLE 1

Title: Diet Change After Sleeve Gastrectomy Is More Effective for Weight Loss Than Surgery Only.

Authors: Joana Rossell [1], Marta González [2], Núria Mestres[2], Eva Pardina[1], David Ricart-Jané [1], Julia Peinado-Onsurbe [1], Juan Antonio Baena-Fustegueras[2]

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ORIGINAL CONTRIBUTIONS

Diet Change After Sleeve Gastrectomy Is More Effective for Weight Loss Than Surgery Only

Joana Rossell[1] · Marta González[2] · Núria Mestres[2] · Eva Pardina[1] · David Ricart-Jané[1] · Julia Peinado-Onsurbe[1] · Juan Antonio Baena-Fustegueras[2]

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Abstract
Background Bariatric surgery with or without diet change has become one of the most effective treatments for obesity. The objective of this study was to observe the effects of vertical sleeve gastrectomy (VSG) and diet change in Sprague-Dawley rats on both body and tissue weights.
Methods Eighteen rats were fed with a standard chow diet (SCD) (C group), and 36 rats were fed with a high-fat diet (HFD) (diet-induced obesity (DIO) group). After 8 weeks, the animals underwent VSG, sham surgery or no surgery (NS). After surgery, a third of the rats fed with the HFD changed to the SCD (DIO + C group). Body weight, food and energy intake were recorded daily during the experiment (12 weeks). Food efficiency (%) (FE) was determined from weekly weight gain and weekly kilocalorie consumed measurements.
Results The DIO group had higher and significant weight gain than the C group at the time of surgery ($p < 0.001$). The major weight loss (WL) was observed in the DIO + C-VSG group, during the 4 weeks after surgery. Adipose tissues in the DIO + C-VSG group were drastically reduced and had a weight similar to those in the C-VSG group.
Conclusion VSG and the diet change combination led to a greater WL, which was maintained during the 4 weeks post-surgery, leading to a normalization of body weight. VSG and diet change also affected most of the tissues, not only adipose, showing a global change in whole body composition.

Keywords Sleeve gastrectomy · Experimental models · Diet-induced obesity · DIO rats

Abbreviations

SCD	Standard chow diet
VSG	Vertical sleeve gastrectomy
HFD	High-fat diet
DIO	Diet-induced obesity
WL	Weight loss
FE	Food efficiency
BAT, eWAT and pWAT	Brown adipose tissue, epididymal white adipose tissue, and perirenal white adipose tissue

Introduction

Obesity is one of the major health problems in our society, leading to increased morbidity and mortality rates. Although many attempt a lifestyle modification [1, 2], bariatric surgery (BS) is the most effective and durable treatment for obesity and its co-morbidities [3–5], especially when combined with nutritional education and therapy [6–8].

Vertical sleeve gastrectomy (VSG) is a restrictive, non-reversible technique in which a resection of the stomach is performed, leaving a sleeve-like gastric pouch. VSG has increased its popularity, becoming the second most common procedure, due to a lesser surgical complexity [9, 10]. The effects of VSG are maintained weight loss, reduced hypertension and improved insulin sensitivity and diabetes mellitus,

Juan Antonio Baena-Fustegueras and Julia Peinado-Onsurbe share senior authorship.

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among others [11, 12]. However, the exact mechanism of these effects is still unknown.

Animal models have been widely used in the study of obesity, using genetic- or diet-induced obese animals [13–18], and VSG in rodents is described [19–22].

Obese patients change their lifestyle after VSG to improve the effects of BS. We designed an experiment where rats fed with a high-fat diet (HFD) changed to a standard chow diet (SCD) after SG. Our objective was to document and describe the VSG performed and the changes observed in the body and tissue weights, changes that are difficult to observe in obese patients.

Materials and Methods

Animals

The animal protocol was approved by the Ethical Committee for Animal Experimentation of the University of Lleida (CEEA. 04-05/12). Male Sprague-Dawley rats (9 weeks old, weight 315.7 ± 5.4 g) from the breeding house of the University of Lleida were housed in pairs in polypropylene cages under controlled conditions (22 °C, 12/12-h day-night cycle, 40–78% humidity). Eighteen animals (C group) were fed a SCD with a calorie composition of 20% protein, 13% fat and 67% carbohydrate (Tekland Global, 2014C, Envigo), and 36 animals (DIO group), in order to induce obesity, were fed a HFD with a calorie composition of 18% protein, 21% carbohydrate and 60% fat (fatty acid profile—37% saturated, 47% monounsaturated and 16% polyunsaturated) (TD.06414, adjusted calories 60/fat, Envigo). Food and water were given ad libitum, and the consumption and animal body weight were measured three times a week at the same time (08:30 to 09:30).

Study Design

After 8 weeks of diet (SCD or HFD), animals were divided into three groups with six C animals and 12 DIO animals and underwent VSG, simulated surgery (sham) or no surgery (NS). Six animals of each DIO group were switched to the SCD (DIO + C) to mimic the dietary modification after BS in humans. Thus, the following subgroups were established: C-NS, C-sham, C-VSG, DIO-NS, DIO-sham, DIO-VSG, DIO + C-NS, DIO + C-sham and DIO + C-VSG. All animals were sacrificed after 12 weeks.

Surgery

Animals under sham or VSG were housed in metabolic cages at 48 h pre-surgery and had 12 h of preoperative fasting, keeping water ad libitum until 1 h before the operation. Enrofloxacina was given 48 h before surgery as an antibiotic prophylaxis. Anaesthesia was induced by isoflurane, combined with O_2, 0.3–0.5 L/min cage, and maintained with xilacina (Rompun® 2%, 2 g/mL) and ketamina (Imalgene® 10%, 10 g/mL). The surgical area was shaven and sterilized prior to a laparotomic supraumbilical incision. The liberation of gastrosplenic adherences and the ligation and section of gastro-omental vessels was conducted at the greater curvature and pyloric antrum (Fig. 1). Delimitation of the gastric tube by two vascular clamps at the fundus and pyloric antrum and a third at 0.5 cm from the others covered the whole stomach. Gastric resection involved 70–80% of the whole stomach (upper curvature and fundus), resecting along the third clamp and leaving the edges to suture by the two other clamps. The first suture was performed from the fundus to the antrum; the second suture was performed with invaginating stitches; the third suture closed the midline laparotomy incision via continuous suture (Fig. 1). The sham surgery consisted of the same procedure, but the stomach remained intact. After surgery, 10 mL of saline was administered subcutaneously to prevent dehydration. Analgesia (buprenorfina) was administered during 24 h post-surgery and given orally with antibiotics (enrofloxacina) for the following 3 days. Food reintroduction after surgery was performed progressively, with the introduction of normal food on the fifth day. The surgery mortality rate was 11.1%. All applicable institutional and national guidelines for the care and use of animals were followed.

Sample Collection

Animals were sacrificed by decapitation at week 12 after a 12-h fast. Blood samples were collected in tubes containing EDTA. Plasma was obtained through centrifugation (2500 rpm, 15 min, 4 °C). Tissues were collected, weighed, frozen and stored at −80 °C.

Data Analysis

Weight data are expressed as the mean ± SEM. Data were analysed by two-way ANOVA followed by Bonferroni post-test using Prism 5.0 (GraphPad Corp., San Diego, CA, USA). A $p < 0.05$ was considered statistically significant.

Results

Food Intake and Weight Evolution Before Surgery

The DIO group had a higher weight gain (Fig. 2), and significant differences between groups appeared from day 4 ($p < 0.05$) and were maintained ($p < 0.001$) until week 8. The C weights tended to stabilize at week 6, reducing daily weight gain.

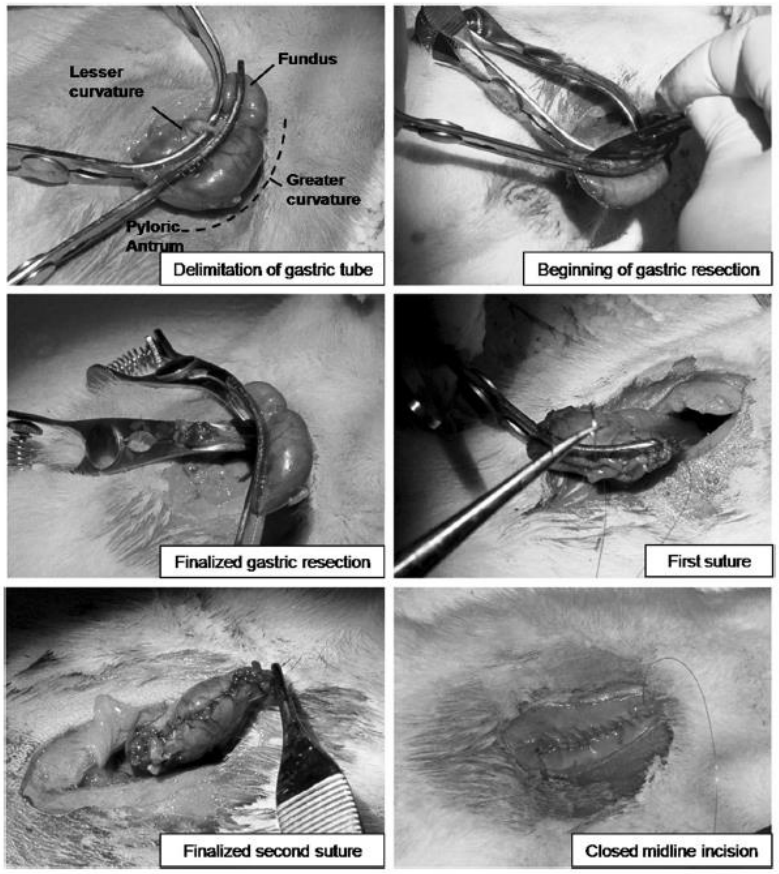

Fig. 1 Vertical sleeve gastrectomy procedure in rats

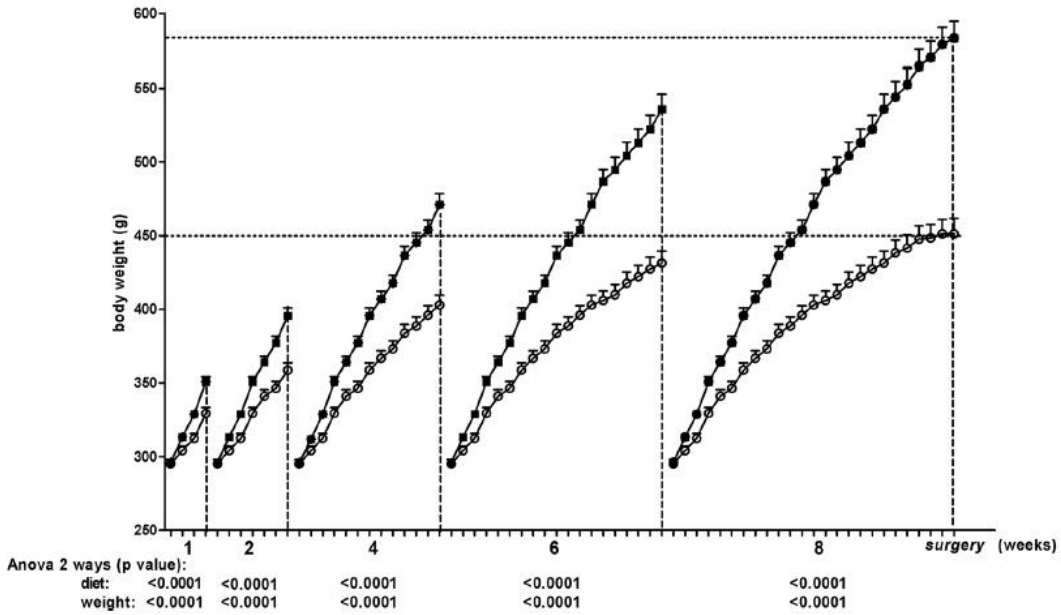

Fig. 2 Body weight curves for the C and DIO groups from week 1 to week 8, prior to surgery. The data are expressed in grammes as the mean ± SEM. Two-way ANOVA (anova-2). *White dots* indicate the C group; *black squares* indicate the DIO group

Energy intake (kcal/animal/day) was significantly different between C and DIO groups ($p < 0.001$). FE showed no significant differences (Table 1).

Food Intake and Weight Evolution in the DIO and C Groups After Surgery

Figure 3 shows the weight loss evolution after surgery. The NS rats continued to gain weight during the next 4 weeks (from 8 to 12 weeks). Energy intake range was significantly different between the C and DIO groups ($p < 0.001$). FE was similar in the DIO (3.10%) and C (2.82%) groups (Table 1).

The C-sham group (Fig. 3) lost 11% (week 9, $p < 0.001$) and 5% (week 10) but increased 1% (week 12) compared to the week 8 values. The DIO-sham group lost less weight than the C-sham group and started gaining weight at week 10 (4% vs. week 8), up to 10% (week 12). The DIO-sham group restored the initial food intake (Table 1) slower than the C-sham group but ingested more kilocalorie ($p < 0.001$, except at week 10). The C-sham group recovered FE (Table 1) a week before (week 10) the DIO-sham group (week 11).

The C-VSG group (Fig. 3) lost 12, 8 and 3% (weeks 9, 10 and 11, respectively) after surgery. The DIO-VSG group lost 16, 15, 8 and 5% at weeks 9 through 12. Between weeks 8 and 10, the food and energy intake between the groups was significantly different. FE began to equilibrate at week 12 (Table 1).

Food Intake and Weight Evolution in the DIO + C Group After Surgery

The DIO + C-NS group gained 3% while the DIO-NS group gained 11% at week 12 (Fig. 3). Although higher food intake (Table 1), energy intake was lower than the DIO-NS group. FE (Table 1) was significantly lower than the DIO groups ($p < 0.001$).

The DIO + C-sham group (Fig. 3) had a greater WL than C-sham or DIO-sham groups up to week 12. Food intake was significantly higher than the C and DIO groups at week 12 and was similar to the DIO + C-NS group.

Figure 3 shows that the DIO + C-VSG group had the highest WL during all weeks, being 50% higher than the DIO-VSG group at week 12. The DIO + C-VSG group had a WL of 23, 20, 13 and 10% at weeks 9, 10, 11 and 12, respectively ($p < 0.001$), with significant differences when compared to the other groups. Food intake (Table 1) was less than the DIO + C-NS, C-VSG (except week 12) and DIO-VSG (except week 10) groups. From week10, the energy intake was similar to the C-VSG group, but only at week 11 did we find significant differences between the DIO + C and DIO-VSG groups. At week 12, there were no FE differences between the VSG groups (Table 1).

Effect on Tissues in the DIO and C Groups After Surgery

Sham and VSG surgeries caused a decrease in both body and tissue weights (Table 2). The C-sham group showed a significant reduction of 23, 40 and 36% in brown adipose tissue (BAT) ($p < 0.05$), epididymal white adipose tissue (eWAT) ($p < 0.01$) and perirenal white adipose tissue (pWAT) ($p < 0.01$), respectively, compared to the C-NS group. VSG in the C group caused a weight reduction in most of the tissues, e.g. liver (8%, p = ns), thymus (33%, $p < 0.05$), eWAT (70%, $p < 0.01$) and pWAT (74%, $p < 0.001$).

The DIO-NS group had increased liver ($p < 0.001$), heart ($p < 0.001$), eWAT ($p < 0.001$) and pWAT ($p < 0.001$), among others, compared to the C-NS group. The DIO-sham group had some significantly increased lung ($p < 0.001$) and brain ($p < 0.05$) weights when compared to the DIO-NS group. The DIO-VSG group compared to the DIO-NS group had decreased liver (15%, $p < 0.01$), heart (17%, $p < 0.001$), BAT (70%, $p < 0.001$) and pWAT (36%, $p < 0.01$) weights, among others. Four tissues had increased weights: spleen (41%, $p < 0.01$), stomach (30%, $p < 0.05$), thymus (3%, p = ns) and adrenal glands (32%, $p < 0.001$).

Effect on Weight Loss in Tissues and Organs in the DIO + C Group After Surgery

In the DIO + C-sham group, we observed a significant decrease in lung ($p < 0.001$), thymus ($p < 0.05$), BAT (19%), eWAT (16%) and pWAT (31%) weights. Some of the tissues in DIO + C-VSG group that decreased compared to the DIO + C-NS group were liver (40%, $p < 0.001$), heart (32%, $p < 0.001$), brain (13%, $p < 0.05$), BAT 65% ($p < 0.001$), eWAT (84%, $p < 0.001$) and pWAT (85%, $p < 0.001$).

Discussion

Overweight and obesity may be prevented by dietary modifications. Diets rich in fat not only induce obesity in humans [23, 24] but also in animals [25–27]. In this work, we studied rats fed with a HFD that underwent VSG and how VSG affects the body and tissue weight when combined with a dietary change, as it is usually performed in humans.

The most recent review [25] about the amount of fat required to induce obesity in animals concluded that the best method to induce obesity in animals was to use semi-purified HFD containing 40% of kilocalorie from animal fat.

In both rats [17] and mice [27], a positive relationship has been found between the amount of fat in diet and body or fat weight. In the animals we used in our experiments, both in C and DIO, we also have observed this correlation, which at week 12 was very high in VSG rats

Table 1 Food intake and food efficiency in different rat groups (no surgery, sham or VSG) before or after surgery

	Week	C-NS	C-sham	C-VSG	DIO-NS	DIO-sham	DIO-VSG	DIO + C-NS	DIO + C-sham	DIO + C-VSG
g/animal/day	*8*	*21.3 ± 0.7*			*21.5 ± 0.6*					
	9	24.6 ± 0.8	12.3 ± 0.9 ^{ooo}	6.1 ± 0.5 ^{ooo,***}	20.4 ± 0.3 $^{+}$	3.2 ± 0.2 $^{ooo,+++}$	3.7 ± 0.5 ^{ooo}	21.8 ± 0.9	2.5 ± 0.5 $^{ooo,+++}$	2.3 ± 0.7 ^{ooo}
	10	24.2 ± 0.7	24.6 ± 1.4	20.2 ± 0.6	25.0 ± 0.9	15.0 ± 0.4 $^{ooo,+++}$	8.5 ± 0.8 $^{ooo,***,+++}$	23.5 ± 0.3	16.3 ± 1.3 $^{oo,+++}$	17.3 ± 0.8 ^{xxx}
	11	20.1 ± 0.5	21.5 ± 0.0	23.9 ± 1.2	21.1 ± 0.5	20.3 ± 0.3	20.8 ± 1.6	26.4 ± 0.3 $^{+}$	13.3 ± 0.5 $^{ooo,++,xx}$	18.1 ± 1.8 ^{oo}
	12	22.1 ± 0.4	22.8 ± 0.1	19.4 ± 1.0	21.4 ± 0.8	23.0 ± 0.2	14.7 ± 1.2 ^{ooo,***}	24.9 ± 0.6	27.5 ± 0.4	22.7 ± 0.4 ^{xx}
kcal/animal/day	*8*	*61.9 ± 2.0*			*109.9 ± 3.0* ^{ooo}					
	9	71.3 ± 2.2	35.8 ± 1.6 ^{ooo}	17.7 ± 1.5 ^{ooo,***}	104.0 ± 1.5 $^{+++}$	16.3 ± 0.9 ^{ooo}	18.6 ± 2.5 ^{ooo}	63.3 ± 2.7 ^{xxx}	7.3 ± 1.5 $^{ooo,+}$	6.8 ± 2.0 ^{ooo}
	10	70.1 ± 2.0	71.3 ± 4.2	58.5 ± 1.7 ^{o}	127.5 ± 4.5 $^{+++}$	76.3 ± 2.3 ^{ooo}	43.4 ± 4.0 ^{ooo,***}	68.1 ± 1.0 ^{xxx}	47.2 ± 3.9 $^{+xx}$	50.3 ± 2.3
	11	58.4 ± 1.3	62.3 ± 0.1	69.2 ± 3.6	107.6 ± 2.7 $^{+++}$	103.7 ± 1.7 $^{+++}$	106.0 ± 8.4 $^{++}$	76.4 ± 1.0 ^{xx}	38.5 ± 1.4 ^{oo,xxx}	52.4 ± 5.3 ^{xxx}
	12	64.0 ± 1.1	66.0 ± 0.4	56.4 ± 2.8	109.3 ± 4.2 $^{+++}$	117.4 ± 1.2 $^{+++}$	75.1 ± 6.1 ^{ooo,***}	72.1 ± 1.6 ^{xxx}	79.7 ± 1.3 ^{xxx}	65.8 ± 1.3
Food efficiency (%)	*8*	*4.9 ± 0.5*			*5.2 ± 0.6*					
	9	2.41 ± 0.36	−20.3 ± 4.8 ^{ooo}	−55.4 ± 0.3 ^{ooo,***}	3.3 ± 0.3	−8.0 ± 6.0 $^{+++}$	−89.6 ± 14.8 $^{ooo,***,+++}$	3.5 ± 1.1	−93.1 ± 0.2 $^{ooo,+++,xxx}$	−304.4 ± 164.6 $^{ooo,+++,***,xxx}$
	10	−0.1 ± 0.4	4.4 ± 2.6	1.1 ± 1.7	0.5 ± 0.5	−1.0 ± 1.0	0.37 ± 3.1	0.3 ± 0.1	5.1 ± 1.4 ^{x}	8.4 ± 1.7
	11	2.2 ± 0.2	4.3 ± 0.4	9.5 ± 2.4 ^{oo}	2.4 ± 0.2	5.25 ± 0.6	4.6 ± 0.7	2.2 ± 0.4	7.6 ± 1.7	4.6 ± 0.6
	12	3.1 ± 0.6	3.7 ± 0.5	3.7 ± 0.4	2.7 ± 0.5	4.7 ± 0, 1	3.6 ± 0.7	−1.3 ± 0.9 ^{xxx}	1.0 ± 1.6	3.1 ± 0.8

The data are expressed as the mean ± SEM. Two-way ANOVA (anova-2) and Bonferroni post-tests were used to study the interactions between the effects of diet ($p < 0.0001$) and surgery ($p < 0.0001$) vs. weeks. The items in italics indicate pre-surgery values. The symbol (°) indicates the differences between sham or VSG for each group (control, DIO or DIO + C) and NS; the symbol (*) indicates the differences between VSG and sham for each group; the symbol (+) indicates the differences between NS, sham or VSG for DIO and the same group for C; the symbol (x) indicates the differences between NS, sham or VSG for DIO + C and the same group for DIO. One symbol, $p < 0.05$; two symbols, $p < 0.01$; three symbols, $p < 0.001$

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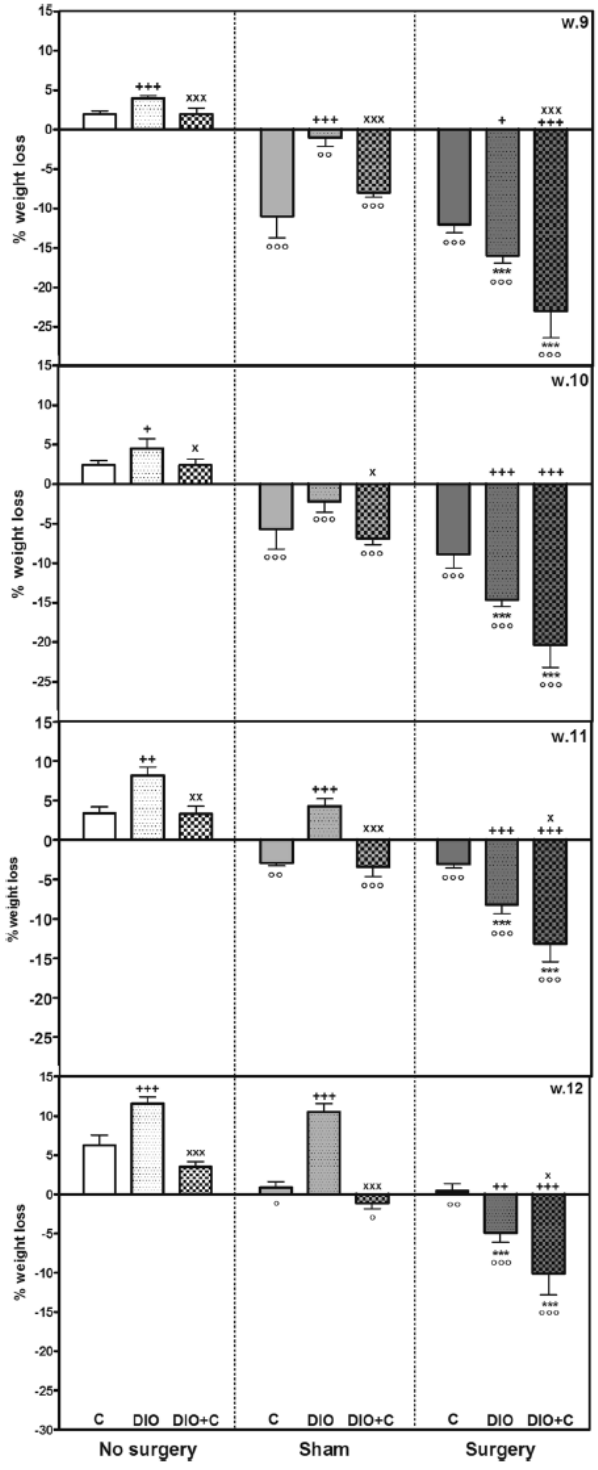

◀ **Fig. 3** Weight loss evolution at weeks 9, 10, 11 and 12. Data are expressed in %, difference between pre-surgery weight minus post-surgery weight and pre-surgery weight. Two-way ANOVA (anova-2) and Bonferroni post-tests were used to study the interactions between the effects of diet and the effects of surgery. In all cases, the surgery and diet effect was $p < 0.0001$. *Clear bars* stand for C, *light dotted bars* for DIO, and *grid* for DIO + C. The symbol (°) indicates the differences between sham or VSG for each group and NS; the symbol (*) indicates the differences between VSG and sham; the symbol (+) indicates the differences between C vs. DIO and DIO + C; the symbol (x) indicates the differences between DIO vs. DIO + C. One symbol, $p < 0.05$; two symbols, $p < 0.01$; three symbols, $p < 0.001$. *ns* non-significant

vs. body weight ($r = 0.725$, $p < 0.001$) or vs. fat pad ($r = 0.861$, $p < 0.001$), in sham rats was less vs. body weight ($r = 0.504$, $p = 0.046$) or fat pad ($r = 0.588$, $p < 0.05$) and did not exist in NS rats.

Some studies have indicated that the development of obesity is prevented in humans [28] and rats [29] when the increase in dietary fat is accompanied by an increase in protein (high protein/carbohydrate (P/CH) and low carbohydrate/fat (CH/F) ratios). Once obesity is established, as for example in our DIO + C animals that have been fed with HFD (ratio P/CH = 0.30 and CH/F = 12) and switched to standard diet at week 8, despite the change in ratio (ratio P/CH = 0.86 CH:F = 0.80), they had less weight during the following weeks than the rest of DIO group, which contrasts with that described by other authors [30].

The D + C-sham group had the highest food intake at week 12, and the D + C-VSG group was the only VSG group that had a higher intake than at pre-surgery, surprising because 80% of the stomach was resected. Despite this, the DIO + C groups had a considerable reduction in calorie intake due to the dietary change, comparable to the reduction observed in the DIO-VSG group. It is possible that the DIO + C groups try to compensate for the lower caloric density of the food by increasing their intake amount [31–33].

Perhaps, a limitation of our study was the length of time taken to induce obesity in DIO (12 weeks, 8 weeks for DIO + C) and thus be able to check whether as other authors have observed [34] that rats that switched from HFD to standard chow after 17 weeks returned to control levels of body weight and composition, while rats that switched after 30 weeks did not. It has also been observed that animals that were fed with HFD during 12 weeks underwent BS and then changed to standard diet, and they started to regain weight after 12 days [35, 36]. We also observed that D + C-VSG group behaved similarly to the C groups at week 12, especially when compared to the C-VSG group, having a similar body (415 vs. 393 g) and tissue weight. They differed completely from the DIO groups, having much lower body and tissue weights despite having been on the same diet for 8 weeks. D + C-VSG group started to regain weight after 12 days.

There are controversial data in the literature about the time the rats begin to gain weight after VSG. Thus, although our C-VSG animals start at the fourth week, as described by other authors in Wistar rats [19], other authors observed that after VSG, animals fed with a HFD

Table 2 Tissue weight in different groups (NS, sham or VSG) after 12 weeks of diet

Weight (g)	Weeks	Control			DIO			Anova 2 ways (*p* value): diet effect/surgery effect on C vs. DIO	DIO + C			Anova 2 ways (*p* value): diet effect/surgery effect on DIO + C vs. C or DIO
		No surgery	Sham	Sleeve	No surgery	Sham	Sleeve		No surgery	Sham	Sleeve	
BW	10	508.6 ± 4.4	463.0 ± 21.0	362.8 ± 21.0 °°°-**	614.3 ± 24.0	585.5 ± 18.6	503.0 ± 28.4 °°°-*	0.001/0.001				0.001/0.001
	12	489.2 ± 14.3	447.3 ± 5.9	393.4 ± 12, 7 °°	655.2 ± 24.0	658.5 ± 35.3 +	517.5 ± 17.0 °°°-***		648.1 ± 32.0	639.0 ± 21.3	415.0 ± 32.7 °°°-***	
Fetge	10	11.05 ± 0.16	9.63 ± 0.35	8.07 ± 048 °	14.57 ± 0.94	12.51 ± 0.60	11.33 ± 1.0 °°	0.001/0.001				0.001/0.001
	12	10.36 ± 0.37	10.02 ± 0.32	9.58 ± 0.41	14.10 ± 0.77	14.74 ± 1.07	10.60 ± 0.88 °°-***		14.93 ± 0.89	11.97 ± 1.45	9.00 ± 0.94 °°°	
Kidney	10	2.66 ± 0.07	2.44 ± 0.12	1.92 ± 0.11 °°°-*	3.26 ± 0.16	2.90 ± 0.05 °	2.56 ± 0.14 °°°-*	0.001/0.001				0.001/0.001
	12	2.40 ± 0.04	2.30 ± 0.04	2.16 ± 0.09	3.44 ± 0.03	3.51 ± 0.08 +++	2.71 ± 0.16 °°°-***		3.26 ± 0.20	3.34 ± 0.11	2.19 ± 0.16 °°°-***	
Spleen	10	1.06 ± 0.09	0.92 ± 0.02	0.83 ± 0.04	0.88 ± 0.03	0.86 ± 0.02	0.96 ± 0.05	ns/ns				ns/ns
	12	1.05 ± 0.09	1.14 ± 0.12	0.89 ± 0.04	0.94 ± 0.04	0.98 ± 0.07	1.33 ± 0.14 °°-** _ ++		0.92 ± 0.09	0.98 ± 0.16	1.02 ± 0.07	
Lung	10	1.66 ± 0.08	1.61 ± 0.18	1.79 ± 0.14	1.91 ± 0.13	1.83 ± 0.66	1.88 ± 0.16	0.05/ns				0.001/0.001
	12	1.57 ± 0.09	1.59 ± 0.12	1.56 ± 0.03	1.85 ± 0.09	2.43 ± 0.06	1.76 ± 0.13 ***		1.82 ± 0.08	1.87 ± 0.06	1.53 ± 0.08	
Heart	10	1.49 ± 0.03	1.43 ± 0.08	1.12 ± 0.01 °°°-**	1.55 ± 0.04	1.58 ± 0.03	1.44 ± 0.05	0.001/0.001				0.001/0.001
	12	1.29 ± 0.06 +	1.19 ± 0.04 +	1.16 ± 0.06	1.64 ± 0.04	1.63 ± 0.06	1.36 ± 0.06 °°°-***		1.64 ± 0.09	1.70 ± 0.04	1.11 ± 0.06 °°°-***	
Brain	10	2.18 ± 0.06	2.00 ± 0.03	1.81 ± 0.06 °°°	2.16 ± 0.07	1.99 ± 0.04	2.02 ± 0.08	ns/0.001				ns/0.001
	12	2.07 ± 0.05	2.01 ± 0.08	1.87 ± 0.07	2.03 ± 0.05	2.21 ± 0.04 +	1.93 ± 0.07 **		2.05 ± 0.06	2.05 ± 0.09	1.78 ± 0.05 °-*	
Stomach	10	1.93 ± 0.06	1.65 ± 0.04	1.73 ± 0.11	1.88 ± 0.08	1.78 ± 0.09	2.14 ± 0.19	ns/0.01				ns/ns
	12	1.84 ± 0.09	1.81 ± 0.04	2.29 ± 0.35	2.03 ± 0.10	2.04 ± 0.08	2.64 ± 0.31 °-*		2.05 ± 0.1	2.44 ± 0.26	2.04 ± 0.48	
Thymus	10	0.28 ± 0.03	0.30 ± 0.04	0.34 ± 0.03	0.37 ± 0.05	0.50 ± 0.05	0.35 ± 0.03 *	0.001/ns				0.01/ns
	12	0.33 ± 0.04	0.24 ± 0.04	0.22 ± 0.02	0.37 ± 0.04	0.52 ± 0.04	0.38 ± 0.08		0.37 ± 0.05	0.34 ± 0.04	0.26 ± 0.06	
Pancreas	10	0.64 ± 0.06	0.80 ± 0.05	0.76 ± 0.09	0.59 ± 0.04	0.74 ± 0.04	0.69 ± 0.05	ns/ns				0.05/ns
	12	0.71 ± 0.04	0.66 ± 0.05	0.75 ± 0.03	0.79 ± 0.09	0.81 ± 0.04	0.79 ± 0.07		0.91 ± 0.06	0.81 ± 0.08	0.85 ± 0.12	
Adrenal glands	10	0.057 ± 0.002	0.055 ± 0.005	0.057 ± 0.003	0.057 ± 0.001	0.054 ± 0.001	0.062 ± 0.004	0.001/0.05				0.001/0.001
	12	0.053 ± 0.003	0.046 ± 0.002	0.051 ± 0.005	0.056 ± 0.003	0.058 ± 0.002	0.074 ± 0.004 °°°-*** _ +		0.057 ± 0.005	0.059 ± 0.000	0.065 ± 0.001	
BAT	10	0.39 ± 0.03	0.38 ± 0.02	0.21 ± 0.03 °	0.75 ± 0.04	0.55 ± 0.03 °°	0.28 ± 0.03 °°°-***	0.001/0.001				0.05/0.001
	12	0.48 ± 0.03	0.37 ± 0.02	0.20 ± 0.01 °°°	0.60 ± 0.07	0.69 ± 0.08	0.18 ± 0.03 °°°-***		0.72 ± 0.12	0.58 ± 0.07	0.25 ± 0.04 °°°-**	
eWAT	10	4.98 ± 0.57	2.72 ± 0.66	1.27 ± 0.38 °	9.17 ± 0.75	11.55 ± 0.96	7.93 ± 1.35 °	0.001/0.001				0.001/0.001
	12	6.75 ± 0.76	4.05 ± 0.11	2.02 ± 0.20 °°	11.28 ± 1.20	11.57 ± 1.33	6.85 ± 1.20 °°-**		12.69 ± 2.39	10.61 ± 0.89	2.01 ± 0.46 °°°-***	
lWAT	10	9.61 ± 0.76	7.84 ± 0.76	3.86 ± 1.85 °°°	22.72 ± 2.19	26.94 ± 1.89	12.82 ± 2.79 °°°	0.001/0.001				0.001/0.001
	12	13.16 ± 1.09	8.43 ± 0.70	3.36 ± 0.27 °°°	23.05 ± 2.88	24.72 ± 2.56	14.85 ± 1.68 °°-***		28.77 ± 2.42	19.75 ± 2.01 °°	4.22 ± 1.27 °°°-***	

The data are expressed in grammes as the mean ± SEM. Two-way ANOVA (anova-2) and Bonferroni post-tests were used to study the interactions between the effects of diet and the effect of surgery. In the middle column are the results from the DIO vs. C groups, and in the last column are the results from the DIO + C vs. C or DIO groups. The symbol (^{o}) indicates the differences between sham or VSG for each group and NS; the symbol (*) indicates the differences between VSG and sham; the symbol ($^{+}$) indicates the differences between NS, sham or VSG for DIO and the same group for C; the symbol (^{x}) indicates the differences between NS, sham or VSG for DIO + C and the same group for DIO. One symbol, $p < 0.05$; two symbols, $p < 0.01$; three symbols, $p < 0.001$; *ns*, non-significant

and switched to a SCD began to recover at the second week (as our animals did) [36]. Other strains such as Zucker [37] or Long-Evan recovered from the first week [38].

In an experiment similar to ours but carried out in Wistar [39], the rats reached a final weight of 572 ± 19 g after 12 weeks of diet with 59% of the calories from fat (our highest DIO weight was 655 ± 24 g). They obtained higher eWAT in DIO-NS (21.7 ± 1.9 vs. 11.3 ± 1.2 g in our rats), in DIO-sham (18.9 ± 0.9 vs. 11.6 ± 1.3 g) and in DIO-VSG groups as well (14.0 ± 1.1 vs. 6.9 ± 1.2), even if the WL compared to the DIO-NS group was lower (35%) than in our rats (39%). However, our data from pWAT were 1.2 times higher than the data described in another article from the same group [40]. These differences can be attributed both to the different ratio of P/CH or P/F or because different strains can have a variable response to HFD, resulting in animals gaining excess weight more rapidly than others [14].

In the DIO + C-VSG group, adipose tissues return to values similar to the C-VSG group, which shows that the diet change combined with surgery is more effective than surgery alone, as we do not see that decrease in the DIO-VSG group. Dietary change alone is not sufficient either, as the DIO + C-NS group gains less weight than the NS groups, but adipose tissues are maintained. Liver weight in the DIO-VSG group falls below the value described by other authors [39].

We found it interesting that the stomach after VSG (a resection of almost 80%) had a higher weight than NS in the C and DIO groups. We observed abdominal adhesions and scar tissue, but not in the DIO + C-VSG group. The stomach is reported to suffer macroscopic modifications after VSG [36], although other authors did not find differences [41].

Other organs also changed weight after VSG in the DIO or DIO + C groups. Increased adrenal weight (normalized by body weight) coupled with reduced thymus weight has been described in RYGB-treated rats, suggesting an elevated hypothalamo-pituitary-adrenocortical (HPA) axis tone, but not in VSG-treated rats [38]. Additionally, HFD-induced obesity has been described to increase the HPA axis response to acute stress [38]. Other authors [22] observe a heart hypertrophy in VSG rats but we observed a decrease.

Conclusions

VSG and a diet change combination leads to a major decrease in body weight. Effects are observed not only in adipose tissues, as is expected, but also in other tissues not related to obesity, also contributing to a greater maintained WL during 4 weeks. These changes lead to a normalization of both body weight and adipose tissues, up to C-NS levels, showing the importance of a nutritional change after VSG.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures were performed in accordance with the internal protocols of our laboratory, which were authorized by the University of Barcelona's Ethical Committee for Animal Experimentation and ratified, in accordance with current Spanish legislation, by the Departament de Medi Ambient i Habitatge of the Catalan Government (Generalitat de Catalunya). The animal protocol was approved by the Ethical Committee for Animal Experimentation of the University of Lleida (CEEA. 04-05/12).

Informed Consent Does not apply.

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3.3. ARTICLE 2

Title: Diet change affects intestinal microbiota restoration and improves vertical sleeve gastrectomy outcome in diet-induced obese rats.

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ORIGINAL CONTRIBUTION

Diet change affects intestinal microbiota restoration and improves vertical sleeve gastrectomy outcome in diet-induced obese rats

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Abstract

Purpose Obesity, a worldwide health problem, is linked to an abnormal gut microbiota and is currently most effectively treated by bariatric surgery. Our aim was to characterize the microbiota of high-fat fed Sprague–Dawley rats when subjected to bariatric surgery (i.e., vertical sleeve gastrectomy) and posterior refeeding with either a high-fat or control diet. We hypothesized that bariatric surgery followed by the control diet was more effective in reverting the microbiota modifications caused by the high-fat diet when compared to either of the two factors alone.

Methods Using next-generation sequencing of ribosomal RNA amplicons, we analyzed and compared the composition of the cecal microbiota after vertical sleeve gastrectomy with control groups representing non-operated rats, control fed, high-fat fed, and post-operative diet-switched animals. Rats were fed either a high-fat or control low-fat diet and were separated into three comparison groups after eight weeks comprising no surgery, sham surgery, and vertical sleeve gastrectomy. Half of the rats were then moved from the HFD to the control diet. Using next-generation sequencing of ribosomal RNA amplicons, we analyzed the composition of the cecal microbiota of rats allocated to the vertical sleeve gastrectomy group and compared it to that of the non-surgical, control fed, high-fat fed, and post-operative diet-switched groups. Additionally, we correlated different biological parameters with the genera exhibiting the highest variation in abundance between the groups.

Results The high-fat diet was the strongest driver of altered taxonomic composition, relative microbial abundance, and diversity in the cecum. These effects were partially reversed in the diet-switched cohort, especially when combined with sleeve gastrectomy, resulting in increased diversity and shifting relative abundances. Several highly-affected genera were correlated with obesity-related parameters.

Conclusions The dysbiotic state caused by high-fat diet was improved by the change to the lower fat, higher fiber control diet. Bariatric surgery contributed significantly and additively to the diet in restoring microbiome diversity and complexity. These results highlight the importance of dietary intervention following bariatric surgery for improved restoration of cecal diversity, as neither surgery nor change of diet alone had the same effects as when combined.

Keywords Bariatric surgery · High-fat diet · Rat models · Microbiota

Abbreviations

SCD	Standard chow diet
HFD	High-fat diet
D	Diet induced obesity
BS	Bariatric surgery
VSG	Vertical sleeve gastrectomy
NS	No surgery

Introduction

The gut microbiota is considered a metabolic organ consisting of more than 500 species of bacteria, viruses, and other organisms living in our intestines, involved in the host

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intestinal immunity and integrity [1]. Additionally, the gut microbiota has metabolic functions, regulating homeostasis and modifying the capacity for energy harvesting and has thus been proposed as a contributor to the development of obesity [2–4].

Along with producing dietary-induced obesity (D) in rodents [5], the high-fat diet (HFD) causes alterations in the microbial community assemblage when compared to control animals [6–8]. Obese animals have lower microbial diversity and perturbed abundances of the major gut phyla, *Firmicutes* and *Bacteroidetes*. It has been demonstrated that these changes in diversity are more dependent on diet than on weight gain or obesity itself [7, 9, 10] and can be reversed with calorie/fat restricted diets [11, 12]. The ratio of *Bacteroidetes:Firmicutes* was previously correlated to obesity [2, 6] but new analyses claims that no exact relationship can be established [13, 14]. Nevertheless, these alterations lead to a dysbiotic state, resulting in leaky gut and metabolic endotoxemia (i.e., low grade elevation of plasma LPS), potential drivers of the inflammatory response characteristic of obesity [9, 15, 16].

Bariatric surgery (BS), mainly Roux-en-Y gastric bypass (RYGB) and vertical sleeve gastrectomy (VSG), is presently the most effective treatment for obesity. Both procedures have similarly successful results despite being anatomically different: VSG consists of stomach resection and unchanged intestinal tract, and RYGB consists of stomach resection and modified intestinal tract, the first part of the small intestine being bypassed causing also malabsorption [17-21]. The main benefits associated with BS are a significant and sustained weight loss and improved insulin resistance [22]. However, BS is also associated with several potential complications depending on the specific surgery: RYGB is associated with increased risk of malnutrition and blood glucose fluctuations, as well as being a more complicated surgery; VSG patients have higher risk of developing gastroesophageal reflux disease [23–25]. Due to the similar benefits with less severe complications VSG popularity as the preferred BS is increasing [26]. Studies in both animal models and humans have shown that BS causes changes in the microbial community, several of which show apparent correlation with the health improvements seen following BS [8, 27–30].

This is the continuation of a previous article, where rats after being fed either a control diet or HFD for 8 weeks underwent either no surgery, simulated (Sham) surgery, or VSG [18]. Half of the HFD-fed rats were then changed to the control diet for the remaining 4 weeks, emulating dietary recommendations for weight loss (increased fruit and vegetables, reduced fat) after BS [31, 32]. We previously found that the combination of diet change and VSG in rats exerted a major effect on the weight of body and organs, reducing them to control levels. Due to the relationship between obesity and gut microbiota previously described, we decided to analyze the effects that diet and surgery had on the gut microbiome itself. The aim of the current study was to analyze the cecal microbiota using 16S RNA analysis, and describe what effect the experimental parameters—HFD, dietary switch, surgery, or combinations of the above—had on the gut microbiome.

Materials and methods

Animals

The animal protocol was approved by the Ethical Committee for Animal Experimentation of the University of Lleida (CEEA. 04–05/12). Male Sprague–Dawley rats (9 weeks old, weight 315.7 ± 5.4 g) from the breeding house of the University of Lleida were pair-housed in polypropylene cages under controlled conditions (22 °C, 12/12-h day-night cycle, 40–78% humidity).

Study design

Eighteen animals were fed a control diet (C group) with a calorie composition of 20% protein, 67% carbohydrate, and 13% fat (Tekland Global, 2014C, Envigo), and 36 animals were fed a HFD (D and posterior D + C group) with a calorie composition of 18% protein, 21% carbohydrate, and 60% fat (TD.06414, adjusted calories 60/fat, Envigo). Detailed composition of both diets is found in Online Resources 1. Food and water were given ad libitum. After 8 weeks, animals were divided into three groups (six C and 12 D animals per group) and underwent no surgery (NS), Sham, or VSG. Animals continued on their designated diet for four more weeks, apart from six animals of each D group that were then switched back to control diet (D + C), establishing the following subgroups: C-NS, C-Sham, C-VSG, D-NS, D-Sham, D-VSG, D + C-NS, D + C-Sham, and D + C-VSG (Fig. 1). VSG and sham interventions were performed according to previously described procedures [18]. In brief, VSG animals had 70–80% of their stomach removed under anesthesia while Sham animals underwent the same operative procedure but their stomach remained intact. Both Sham and VSG animals received antibiotic treatment (Enrofloxacina, 10 mg/kg every 12 h) for 5 days (2 days pre-surgery as a prophylactic treatment, and the following three days post-surgery). Surgery had a mortality rate of 9.25% during the first two days post-surgery.

Sample collection

Animals were sacrificed by decapitation at week 12 after a 12 h fast. Blood samples were collected in tubes containing

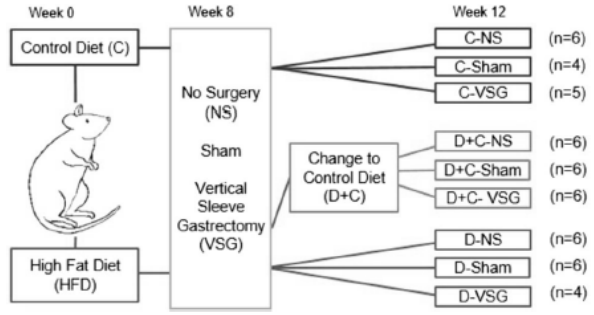

Fig. 1 Experiment design and group distribution. Rats were fed either the control diet (red line) or HFD (blue line) for 8 weeks. At week eight, each diet group was then divided in three ($n=6$), and subjected to one of the three surgical situations: No Surgery, Sham surgery or VSG. Half of the HFD-fed rats were then switched to the control diet (green line). Rats continued the allocated diet until week 12

EDTA and plasma was obtained through centrifugation (2500 rpm, 15 min, 4 °C). Caeca were collected, snap-frozen in liquid nitrogen and stored at − 20 °C. Epididymal and perirenal adipose tissues were collected, weighed and stored at − 20 °C.

DNA extraction

DNA was extracted from cecum samples using either the QIAamp Fast DNA Stool Mini kit or the DNA easy Power Soil Kit (QIAGEN, Hilden, Germany). The concentration of DNA was assessed using the Quant-iT PicoGreen dsDNA Assay kit (ThermoFisher, Massachusetts, USA). DNA samples were stored at − 20℃.

Sequencing and analysis of 16S amplicon data

Isolated DNA was amplified with forward primer 341F: (CCTACGGGNGGCWGCAG) and reverse primer 805R: (GGACTACHVGGGTWTCTAAT) targeting the V3—V4 hypervariable region of the coding sequence for the 16S small ribosomal RNA, rRNA. Amplified DNA was sequenced on an Illumina MiSeq machine, using the MiSeq v3.0 reagent kit leading to 2 × 300 bp paired-end reads. Initial demultiplexing was done with the default Illumina MiSeq Control Software (2.6.2.1). Down-stream quality control, trimming, filtering, merging of forward and reverse reads, chimera removal and identification of amplicon sequence variants (ASV) was performed with the R software version 3.4.2 (https://www.R-project.org), using the DADA2 R package version 1.6 [33, 34]. Metabolic reconstruction from 16 s amplicon data of KEGG pathways ko04973 (carbohydrate digestion and absorption) and ko00071 (fatty acid degradation) was done from normalized to even depth ASV abundance tables for the top 100 most abundant taxa created using the above described method, followed by submission to the Piphillin server using the KEGG database, release of October 2018, and a cut-off sequence similarity value of 95% [35].

Biochemical parameters

Glucose was enzymatically analyzed in a METROLAB 2300 auto-analyzer (RAL, Laboratory Techniques, Spain); glucagon and leptin were measured using an ELISA kit (R&D, USA), insulin and unacylated ghrelin were measured using an ELISA kit (Bertin Bioreagent, France), all according to the manufacturer's protocols.

Glucose homeostasis indicators

Glucose homeostasis was measured by the insulin sensitivity index (ISI), the homeostatic model assessment of insulin resistance (HOMA-IR) and the homeostatic model assessment of β cell function (HOMA-β) calculated according the formulas: ISI = 1/fasting glucose fasting insulin; HOMA-IR = fasting insulin fasting glucose/22.5; HOMA-β = 20 × fasting insulin/(fasting glucose − 3.5).

Data analysis and statistics

Body weight gain (BWG) was calculated by subtracting initial body weight from measured body weight at posterior time. The adiposity index was calculated as the sum of epididymal and perirenal adipose tissues/body weight × 100. Body weight values were expressed as mean ± SEM. Differences in BWG were analyzed by a repeated measure ANOVA with Mauchly's sphericity test followed by GG corrections, using the R software. Body weight gain at week 12 and biochemical parameter differences were analyzed by a two-way ANOVA followed by a Tukey post-test. Graphics and statistical analysis of gut microbiota were done with the Phyloseq package version 1.20.0 [36]. Taxa were expressed as relative abundances with expressed values as the mean of each group. The Alpha diversity was determined using Shannon and Simpson indices and differences were analyzed by a two-way ANOVA followed by a Tukey post-test. Beta diversity was estimated by Non-Metric Multidimensional Scaling (NMDS) and differences were analyzed by PERMANOVA. The DeSeq2 R package [37] was used to analyze differentially abundant taxa on genus level (false discovery rate (FDR) < 0.01 and $\log_2$-fold change ($\log_2$ FC) > |10|) associated with the various combinations of diet and surgery. The correlation analysis between differentially abundant genera and biochemical parameters was assessed by Spearman's correlation method using the R software, coefficients were plotted on a heatmap using the

corrplot package. Differences were considered significant when P values < 0.05.

Results

General parameters

Results on body and organ weight were previously published [18] and are thus not described in detail here. Body weight gain was significantly higher after 1 week of HFD in the D group ($P < 0.001$) and continued to be so during the whole experiment (except D-VSG and D + C-VSG). At the end of the experiment, the BWG for D-VSG was close to C-NS, while D + C-VSG was similar to both C-Sham and C-NS (Fig. 2a). Adiposity index was similar for all the NS groups, D + C-Sham, D-Sham, and D-VSG, higher than in C-VSG and D + C-VSG. Leptin decreased significantly in C-VSG and D + C-VSG groups, while Ghrelin was lower in all the D groups. The maintained HFD tended to increase insulin levels and thus HOMA-IR but was not significant. Surgery had some effect only for the C and the D + C groups, especially with respect to ISI, were D + C-VSG showed the best insulin sensitivity (Fig. 2b).

Diversity

Alpha diversity—diversity in each group, calculated by Shannon and Simpson Indices—ranked the D groups as the least diverse, and the control C-NS as the most diverse (Fig. 3a). Sham and VSG surgery negatively affected diversity in C and D-groups, but combined with the dietary switch, increased diversity for D + C. Taken as a whole, diet was the main factor affecting alpha diversity, together with the combination of diet and surgery, while surgery alone had less of an effect (and no effect in the Simpson Index) (Two-way ANOVA, $P < 0.01$).

Non-Metric Multidimensional Scaling (NMDS), based on Bray–Curtis dissimilarities, was used to assess the beta-diversity—differences in taxonomic abundances between samples—(Fig. 3b) showing that diet and VSG had a very significant impact ($P < 0.001$, PERMANOVA). Constrained ordination showed diet as the strongest factor separating samples on the first, most explanatory axis, and thus, the strongest factor driving the separation of populations between C and D groups. The D samples formed a distinct, and more defined, group compared to the other two diets. Interestingly, the D + C groups showed less defined clustering, with samples scattered intermediately between clusters representing the D and C cohorts, most evident for D + C-Sham and D + C-VSG, which overlapped with the C-VSG group. Surgery also had a significant impact, resulting in a tight clustering for C-VSG when compared to the respective NS and Sham.

Modifications in relative abundances of cecal microbes

Diet and surgery induced substantial differences between groups. The microbiota was dominated by the phyla *Bacteroidetes* and *Firmicutes*, which accounted for more than 80% of the microbiome in all groups (Fig. 4a).

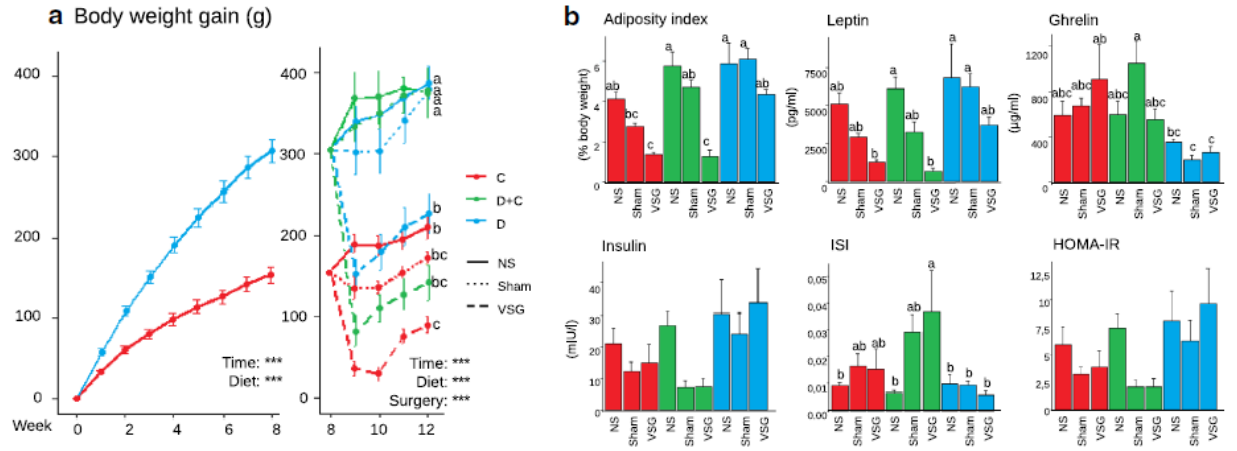

Fig. 2 **a** The D group had a higher body weight gain from week one ($P < 0.001$). At week eight, half of the D animals switched from the HFD to the control diet, and all were divided into surgery groups NS, Sham or VSG. At week 12, VSG had a significantly lower BWG than Sham or NS in the same diet group, especially for D + C ($P < 0.001$). **b** Different parameters at week 12. The adiposity index was lowered by the combination of VSG and diet switch. Leptin was affected by VSG. Ghrelin was reduced in D groups. Insulin Sensitivity Index increased in D + C-VSG. No significant changes were seen in Insulin or HOMA-IR. P values < 0.001 (***) and **a–c** correspond for significantly different groups (Tukey post-test)

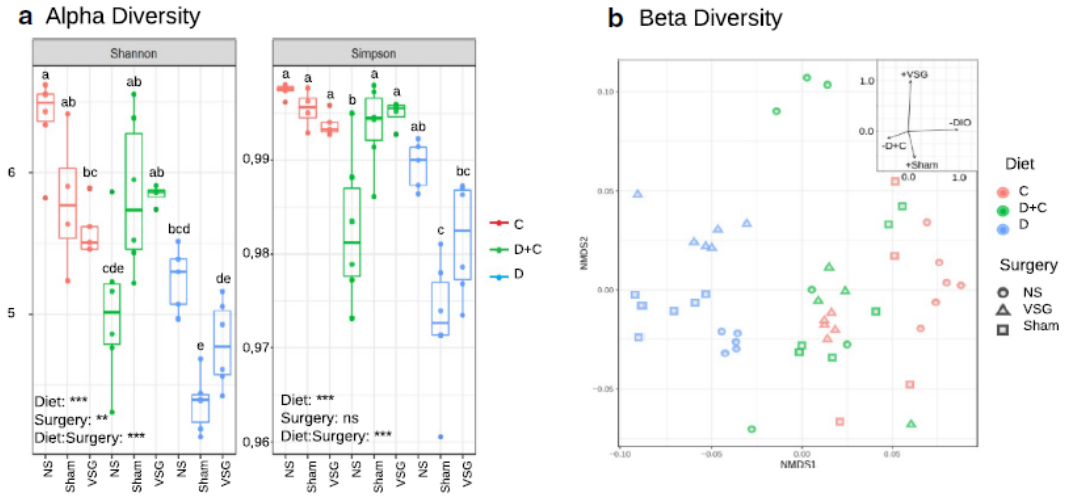

Fig. 3 Diversity measures. **a** Shannon and Simpson indices showing sample alpha diversity. The bottom and top of the boxplot indicate the first and third quartile, whilst the line inside the box show the median. Diversity was reduced by both Sham surgery and VSG in all groups. D samples had the lowest diversity except for D + C-NS in the NS situation. *P* values < 0.001 (***), *P* values < 0.01 (**) and **a–c** correspond for significantly different groups (Tukey post-test). **b** Beta diversity. The non-metric Multidimensinal Scaling (NMDS) plot for the bacterial communities in our samples based on Bray–Curtis dissimilarities. D groups, C-NS and C-VSG formed distinct clusters. PERMANOVA analysis: Surgery ($P < 0.001$), Diet ($P < 0.001$). D + C samples formed less defined clusters, but were very distinct from D samples and were overlapping the C-VSG and C-Sham groups

On average, the maintained HFD in D increased the *Bacteroidetes* fraction, but this fraction was normalized after the switch to the control diet in D + C groups. The diet change also increased the relative abundance of *Verrucomicrobia*, up to 16.31%, in the D + C-NS, but not in the antibiotic treated D + C-Sham and D + C-VSG groups. Importantly, these two groups showed a strong similarity at the phyla level to the C-NS and C-Sham groups. On the other hand, surgery increased the relative abundance of *Firmicutes* in D-VSG compared with D-NS (42.43% vs 37.10%, resp.), while reducing *Proteobacteria* (0.87% vs 4.05%, resp.). In the C groups, no substantial differences were seen between C-NS and C-Sham, but VSG reduced the *Firmicutes* levels (51.41%). At the family level (Fig. 4b), the D groups had higher *Bacteroidaceae* abundance, drastic reductions in *Bacteroidales_S24-7* (less than 5%) and reduced *Ruminococcaceae* abundance (also observed in D + C-NS) compared with C. The family *Rikenellaceae* was reduced after Sham or VSG, but only in HFD-fed groups. D + C-NS and D-VSG similarly had higher levels of *Erysipelotrichaceae* (2.65% and 3.92%). VSG reduced the amount of several families in the *Firmicutes* phylum, such as *Christensenellaceae*, *Clostridiaceae*, *Clostridiales*, and *Defluviitalaceae*. Diet and surgery significantly increased or decreased some genera (mainly belonging to the *Firmicutes* phylum) when compared to their respective C group (Online Resource 2). Several *Ruminococcaceae* decreased in both D and D + C in the NS groups, as well as several genera belonging to *Lachnospiraceae* (*Acetifactor*, *Cellulosilyticum*, and *Lachnospiraceae_NK4A136_group*) which also increased in D + C-Sham. The groups D + C-VSG, D-Sham and D-VSG had fewer significantly different genera with respect to their control matching groups. The *Lachnospiraceae_NK4A136_group* was common in all groups but responded differently to each experimental situation.

Correlation between cecal bacteria and other parameters

To observe possible associations between the 30 genera showing highest change in the dataset showing significant change and diverse biological parameters, we performed a correlation analysis between them. Figure 5 shows a plot of Spearman's correlation coefficient for significant correlations. We observe that the genus *Akkermansia* strongly correlated to the adiposity index and to the carbohydrate digestion pathway related to the formation of short chain fatty acids. *Erysipelatoclostridium* had similar correlations in addition to *Blautia*. *Bacteroides*, *Faecalitalea*, and *Terrisporobacter* being inversely correlated with ghrelin.

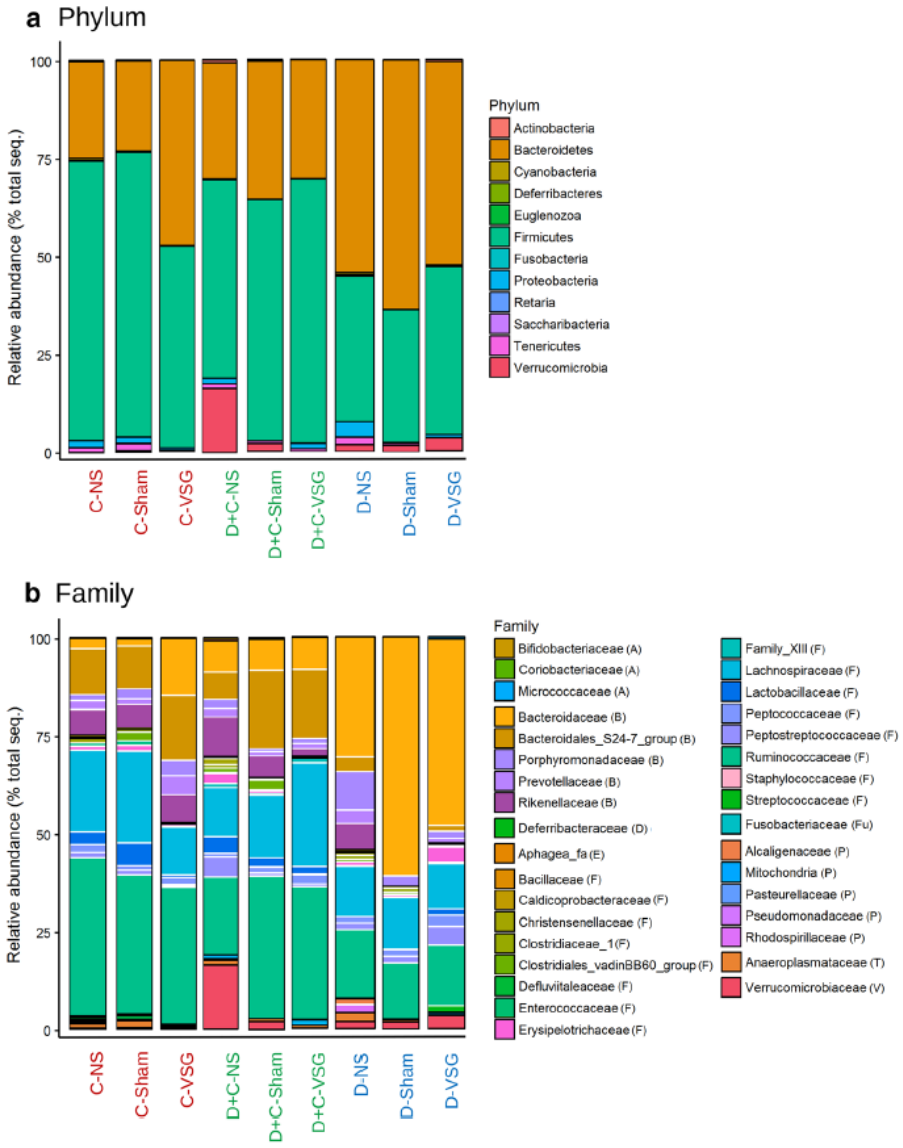

Fig. 4 Relative abundances of bacterial composition, **a** at the phylum level, dominated by the *Bacteroidetes* and the *Firmicutes* phyla. Relative abundances of bacterial composition, **b** at the family level, with phyla separations marked with black lines and family separations marked with white lines. Family names in legend grouped by phyla (phylums initial letter). Group labels were marked according the diet: Red for C, Blue for D, Green for D+C

Discussion

In this work we investigated the effect of HFD, VSG (accompanied by dietary switch or not) and change of diet alone on the Sprague–Dawley rat cecal microbiota, a follow-up of our earlier work reporting on weight loss results [18]. The combined effect of diet and surgery had distinctive effects on the microbiota in line with previous reports [8, 28, 30]. In addition to this, an obvious effect attributable to pre-operative antibiotic prophylaxis is noted, reflected in the effects observed in the Sham-operated cohort of this study. This reasoning is entirely in line with the ecological theory where, at the species level, functional redundancy is prevalent in such complex communities. However, the significance is downplayed by therapy-oriented efforts in attributing pathophysiology to individual microbes, such as in the absence of a direct effect (e.g., toxin production by the causative pathogen) where the community function (host phenotype) may not be reliant on a singular organism. We have thus exclusively approached how surgery (VSG, Sham surgery or, No Surgery) combined with dietary variables (control diet, HFD, or HFD switched to control diet post-operatively) affect the gut microbiota.

The relative abundance of the main phyla was strongly affected by diet, and high levels of *Bacteroidetes* accompanied by concomitant low levels of *Firmicutes* were noted for the D cohort (Fig. 4a) as shown in previous

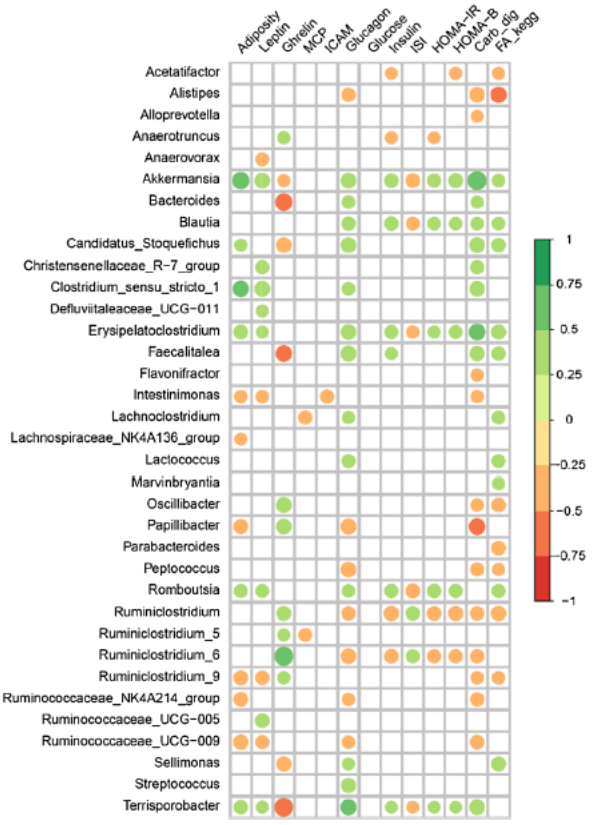

Fig. 5 Graphic representation of the Spearman correlation coefficients between the significantly altered genera obtained by DESeq and different parameters such as adiposity index, biochemical parameters, and KEGG pathways. Positive correlations are shown in green color and negative correlations in red color. The color intensity and the circle size are proportional to the correlation coefficients. Only genera with significant correlations (<0.05) are shown

studies [10, 38]. The maintained HFD also altered diversity, separating the D populations from both C and D + C, no matter the surgical approach (Fig. 3b), and lowered the alpha diversity (Fig. 3a), an indication of dysbiosis [39]. The HFD is said to have a lesser negative influence on the alpha diversity than other obesogenic diets (such as the one mimicking the western diet used by Bortolin et al. [40]. Nevertheless, we observed a pronounced reduction that persisted after the switch of diet alone. Alpha diversity was also affected by VSG, but to a lesser degree than by the change of diet, although VSG only removes the glandular part of the stomach and leaves the intestine intact [28]. Nonetheless, both C and D groups subjected to VSG had reduced alpha diversity, similar to what has been seen in humans and animals [41, 42], but also to Sham surgery, suggesting the effects of the antibiotic Enrofloxacina were the primary cause rather than the surgery itself. Interestingly, the combination of antibiotic and diet change increased alpha diversity for D + C-Sham and D + C-VSG. This could imply that a wipeout effect from the antibiotic was needed to achieve a more beneficial microbiota restoration after the change of diet. This highlights the strong, but often-neglected effect of pre-operative antibiotics on the microbiome (highlighted in a recent review [43]). More research is thus warranted towards further understanding of how antibiotic choices may be amenable pre-operatively in the re-establishment of a healthy microbiota.

The D + C groups were of particular interest in this study as the diet switch to the control diet, even in the absence of other factors, resulted in major compositional differences in the HFD-fed rats showing a partial restoration of the original microbiota. This is in line with other studies [4] where a significant reduction in the relative abundance of *Bacteroidetes* and an increase in *Firmicutes* compared to D was reported. As we showed previously [18], 'recovery' (i.e., net weight and adipose tissue reduction) was better achieved in the group combining diet switch and VSG. In this study, we observed improvements in BWG, adiposity index, leptin, ghrelin and ISI, returning to control values in the D + C-VSG group (Fig. 2a, b). Furthermore, D + C-VSG rat microbiomes had a closer resemblance to the C groups on the phylum level (Fig. 4a) but still differed at family level (Fig. 4b), proving the difficulties of proper restoration of the microbiota after a diet-induced perturbation, especially at lower taxonomy levels, as seen in other studies with HFD-fed rodents [6, 15, 44]. Once more, this remains a strong indicator of community-level synergistic effects, and argues against single microbial species causality.

While not disregarding the possibility of redundant species function, changes in abundance of specific taxa warrants attention; *Akkermansia muciniphila*, the only member of *Verrucomicrobiaceae*, is described as a marker of improved host health [45]. This species was elevated in D + C-NS (Fig. 4b) and seems to be directly affected by antibiotic administration, as levels were not increased in D + C-Sham or D + C-VSG. *A. mucinphila* was positively associated with several parameters related to obesity (Fig. 5) but also with increased adiposity. Several of the genera found in Fig. 5 contain species that produce short chain fatty acid (SCFA). The SCFAs are involved in the maintenance of the intestinal epithelium and have been associated with obesity and its comorbidities, alongside with improvements in intestinal inflammation [46–50]. Indeed, we found correlations—both negative and positive—between significantly changed genera and the chosen parameters, and we observed similar correlation patterns for the following taxa; (*Akkermansia*, *Blautia* and *Erysipelatoclostridium*, or *Ruminiclostridium* and *Ruminiclostridium-6*). Again, this may highlight the synergistic effects at the community level, instead of pin-pointing single species as causative agents.

To maximize the weight loss associated with VSG and stabilize the microbiome, a diet-switch combined with probiotic administration may maximize health gains by counteracting HFD effects and reduce body weight [39, 51]. The aforementioned *A. mucinphila* is a proposed probiotic, implicated in combating obesity and metabolic syndrome [45, 51]. Accordingly, any prospective probiotic cocktail devised to facilitate weight loss would likely require extensive testing in multiple dietary backgrounds. Another line of reasoning regarding optimal weight loss after BS is the effect of VSG on SCFA-producers, resulting in reductions of butyrate and decreased LPS translocation (leaky gut) relevant to tight junction regulation in the intestinal epithelium [40]. Clearly, more work is needed to clarify the nature of probiotics, SCFA-producers and several other organisms in host physiology, but also and perhaps more importantly, the intricate structures and relationships between and among taxa at the community level. Approaching exact numbers and true abundances will require more in-depth analyses combining deep sequencing and reverse transcriptomics to identify actively dividing populations stimulated by each intervention.

The difficulties associated with extending results from animal models to human interventions are diverse and widely acknowledged. Rodents allow for the inclusion of controls necessary for the optimal evaluation of multiple variables and their effects on gut microbiota. However, to facilitate easier comparisons in such studies, a need exists to standardize diets. Such improved diets are currently being developed in several instances [13], as the standard HFD is an inadequate representation of western food habits, believed to be responsible to some extent for the obesity pandemic. Also, an additional no-surgery antibiotic-treated group would have been useful in evaluating this effect, and opens up avenues for further investigation on future studies.

In conclusion, this study provides evidence that a controlled diet following VSG is key to increasing alpha diversity and restoring the HFD-perturbed taxonomomic composition of the microbiota. It highlights the effect of antibiotic exposure in the pre-operative stage, hinting at its importance in microbiome modulation caused by the procedure. Gut microbiota alterations may be beneficial during recovery of healthy body weight after bariatric surgery. Further studies are needed to elucidate the intricate relationship that gut microbiota, diet, weight loss, antibiotics and BS have. Modulation may represent a new plausible target in improving the outcome of interventions against obesity.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval The authors declare that all the animal studies have been approved by the Ethical Committee for Animal Experimentation of the University of Lleida (CEEA. 04–05/12).

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3.4. ARTICLE 3 (READY FOR SUBMISSION)

Article

Combination of diet and bariatric surgery promotes healthier changes in fatty acid profiles in the livers of obese rats

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Abstract: Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease and is associated with obesity and metabolic syndrome. Calorie-restricted diets are often advised as a treatment, but bariatric surgery is the best option to treat both obesity and NAFLD in an effective and durable way. We aimed to assess the effects of diet, bariatric surgery, or a combination of diet and bariatric surgery on fatty acid compositions in the livers of obese rats. Sprague Dawley rats were fed a high-fat diet to induce obesity. They were then subjected to a control diet, a vertical sleeve gastrectomy (VSG), or both, before the analysis of their hepatic fatty acid compositions. Obese rats had lower saturated fatty acid levels and higher monounsaturated and polyunsaturated fatty acid amounts. Changing diet reduced the amount of hepatic fat without affecting most of the fatty acid composition, while VSG alone elicited few changes. The combination of dietary change and VSG reversed most of the effects of the high-fat diet, thus demonstrating that it was the most effective way of reversing the hepatic changes seen in obesity.

Keywords: High-fat diet; fatty acid; lipid metabolism; obesity; fatty liver; sleeve gastrectomy; bariatric surgery

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease and one of the several comorbidities associated with obesity. It is also closely associated with insulin resistance and metabolic syndrome (1-3). The accumulation of visceral fat in obese patients is linked to a higher influx of triacylglycerides (TG) to the liver, which leads to the increased hepatic TAG levels seen in NAFLD (3-5). Elevated levels of non-esterified fatty acids (NEFA) and other derivatives of fatty acid (FA) metabolism cause hepatic inflammation, contributing to the later stages of NAFLD such as non-alcoholic steatohepatitis and irreversible hepatic damage (2, 3).

Dietary fat is a source of essential (EFA) and non-essential FAs. Fatty acids have important structural and molecular roles, participate in cell signaling, and are involved in the resolution of inflammatory processes (6, 7). Unbalanced fat-enriched diets are one of the main factors leading to obesity and NAFLD (3, 8). In rodents, a prolonged high-fat diet (HFD) is traditionally used to induce obesity, increasing body weight and inducing several metabolic changes (9).

NAFLD is often improved with calorie-restricted diets (2, 10), although bariatric surgery (BS) is considered the most effective treatment of both NAFLD and obesity, significantly reducing the hepatic fat content (5, 11). Vertical sleeve gastrectomy (VSG) is a restrictive procedure that has recently become the most widely performed BS, promoting weight loss and improving insulin resistance and blood lipid profiles. Furthermore, it involves less surgical complexity and fewer drawbacks, such as risk for nutritional deficiencies, compared to other types of BS (11-13).

We designed an experiment in which diet-induced obese rats were subjected to VSG, a dietary intervention (i.e, a change to control diet), or a combination of both. Our objective was to study how HFD, VSG, and diet

change affect the liver and alter its FA profile and metabolism. Due to sampling limitations, these changes are difficult to assess in obese patients. In this study, we observed that the HFD increased adiposity in the whole body and liver, also affecting hepatic FA profiles. These modifications were mostly reversed only when VSG was combined with a change of diet.

2. Materials and Methods

Animals

Male Sprague Dawley rats (9 weeks old, weighing 315 ± 5 g) from the breeding house of the University of Lleida were maintained in an environmentally controlled animal facility. The animals were divided into three main groups (n = 18). The control (**C**) group was fed a standard chow diet (CD) that provided 20% of the calories from protein, 67% from carbohydrates, and 13% from fat (Teklad Global, 2014C, Envigo) (see complete composition in Table 1).

Table 1. Diet composition.

	CD		**HFD**	
Diet composition	g/kg	% calories	g/kg	% calories
Protein	143	20	235	18.4
Carbohydrate	480	67	273	21.3
Fat	40	13	343	60.3
Fatty acid composition	g/kg	% fat	g/kg	% fat
Saturated	6	15.0	125	36.4
Monounsaturated	7	17.5	161	46.8
Polyunsaturated	21	52.5	54	15.8
MA (C14:0)	-	-	5	1.4
PA (C16:0)	5	12.5	80	23.4
POA (C16:1)	-	-	10	2.8
SA (C18:0)	1	2.5	39	11.5
OA (C18:1)	7	17.5	147	42.8
LA (C18:2)	20	50.0	47	13.7
ALA (C18:3n-3)	1	2.5	6	1.6

Detailed compositions of the control diet (CD) (Teklad Global 14% Protein Rodent Maintenance Diet, 2014) and high-fat diet (HFD) (TD.06414; Adjusted Calories Diet, 60 kcal from fat) obtained from Envigo (Indianapolis, USA). ALA, alpha-linolenic acid; LA, linoleic acid; MA, myristic acid; OA, oleic acid; PA, palmitic acid; POA, palmitoleic acid; SA, stearic acid.

The high-fat (**HF**) group was fed an HFD that provided 18% of the calories from protein, 21% from carbohydrates, and 60% from fat (TD.06414; adjusted calories, 60/fat, Envigo). The diet-change (**DC**) group was fed an HFD in the first eight weeks of the experiment, before switching to the CD in the last 4 weeks of the study. Food and water were given *ad libitum*, and intake and body weight were measured three times a week. In week 8, each group was further divided into three groups: one that received no surgery (NS), one in which surgery was simulated (Sham), and one that underwent a VSG. Thus, the following groups were established (figure 1): C-NS, C-Sham, C-VSG, HF-NS, HF-Sham, HF-VSG, DC-NS, DC-Sham, and DC-VSG. The protocol was approved by the Ethical Committee for Animal Experimentation of the University of Lleida (CEEA 04-05/12) and of the University of Barcelona (CEEA 8676).

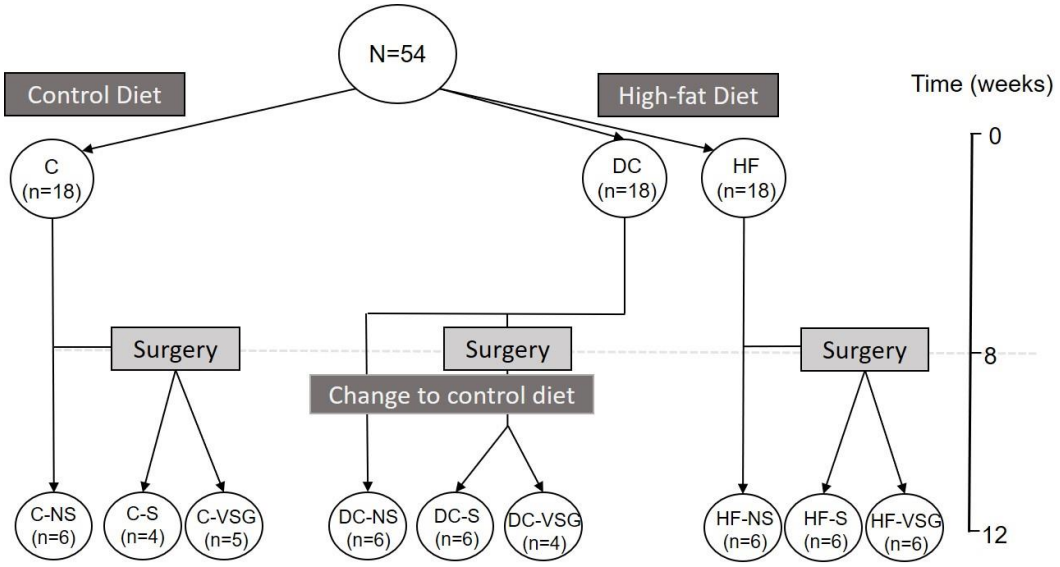

Figure 1. Study design showing the division into three main groups: C fed a control diet, and DC and HF fed a high-fat diet. At week 8, the DC group changed to control diet. All the groups were then divided into three further groups and subjected to Sham surgery (S), vertical sleeve gastrectomy (VSG) or no surgery (NS).

Surgery

Sham surgery and VSG were performed as described by Rossell et al. (14). Briefly, 80% of the stomach was removed in the VSG groups, while an incision into the abdominal cavity but no stomach resection was performed in the Sham groups. The mortality rate due to surgical complications was 9.25% during the two days post-surgery. Food was reintroduced progressively after surgery, with normalization on the fifth day. Animals were maintained for four more weeks in the new conditions until euthanasia.

Sample collection

Animals were euthanized by decapitation in week 12 after a 12-h fast. Blood samples were collected in tubes containing EDTA and plasma was obtained through centrifugation (500 *g* for 15 min at 4ºC). Liver and perirenal and epididymal white adipose tissue pads (counted together and referred to henceforth as "fat pads") were collected, weighed, frozen, and stored at -80ºC.

Plasma analysis

Triacylglycerides, NEFA, total cholesterol (Chol), HDL cholesterol (cHDL), LDL cholesterol (cLDL), and glucose levels in the plasma were enzymatically analyzed using a colorimetric method (RAL, Laboratory Techniques, Spain). Monocyte chemoattractant protein-1 (MCP-1), glucagon, and insulin levels were analyzed with commercial ELISA kits. Glucose homeostasis was calculated with the following formulas: insulin sensitivity index (ISI) = 1/(fasting glucose (mmol/l) × fasting insulin (mIU/l)); the homeostatic model assessment of insulin resistance (HOMA-IR) = (fasting glucose (mmol/l) × fasting insulin (mIU/l))/ 22.5; and the homeostatic model assessment of β cell function (HOMA-β) = 20 × fasting insulin/(fasting glucose – 3.5).

Hepatic lipid extraction and quantification

Total hepatic lipid extraction was performed using the hexane:isopropanol method. Hepatic samples (500 mg) were incubated with 2 mL of hexane:isopropanol (3:2, v/v) overnight at room temperature in an orbital. 0.3 mL of 0.47 M sodium sulfate was added to the sample before it was vortexed and centrifuged. The hexane (top phase) containing the lipid fraction was transferred to a new vial, dried, and weighed.

Lipid quantification was performed using thin-layer chromatography (TLC). Lipid extraction samples were dissolved in chloroform and spotted onto silica gel TLC plates (Merck). Plates were developed in hexane, ether, and formic acid solvent, before visualization with copper sulfate.

Quantification of liver fatty acid compositions

Liver fatty acid composition was determined as fatty acid methyl esters (FAME) after a methylation reaction, using the method of Lepage and Roy (15). Gas chromatography-mass spectroscopy (GCMS) was performed on a Shimadzu GCMS-QP2010 Plus gas chromatograph/mass spectrometer (Shimadzu Co., Kyoto, Japan), operated with a split/splitless injector, a Shimadzu AOC-20i autoinjector, and a Shimadzu AOC-20s autosampler. The SupraWAX-280 column was used (Teknokroma Analítica SA, Sant Cugat del Vallés, Barcelona, Spain), while the GCMS solution software was applied to process the acquired data. FAME peaks were identified through mass spectra and by comparing the elution pattern and relative retention times of the FAMEs using a reference FAME mixture (GLC-744 Nu-Chek Prep. Inc., Elysian, Minnesota, USA). Results are expressed in relative amounts (molar percentage of total fatty acids).

The FAs identified were (in alphabetical order): arachidonic acid (AA), C20:4n-6; alpha-linolenic acid (ALA), C18:3n-3; dihomo-γ-linolenic acid (DGLA), C20:3n-6; docosahexaenoic acid (DHA), C22:6n-3; docosapentaenoic acid (DPA), C22:5n-3 and C22:5n-6; eicosapentaenoic acid (EPA), C20:5n-3; gamma-linoleic acid (GLA), C18:3n-6; linoleic acid (LA), C18:2n-6; lignoceric acid (LIG), C24:0; myristic acid (MA), C14:0; oleic acid (OA), C18:1n-9; palmitic acid (PA), C16:0; palmitoleic acid (POA), C16:1n-7; stearic acid (SA), C18:0; and vaccenic acid (VAC), C18:1n-7.

The activities of the enzymes involved in FA metabolism were estimated as the product-to-precursor ratios of individual FAs as follows: stearoyl-CoA desaturase 1 (SCD1) activity as the ratio of 16:1n-7/16:0; stearoyl-CoA desaturase 18 (SCD18) activity as the ratio of 18:1n-9/18:0; elongase activity as the ratio of 18:1n-7/16:1n-7; delta-6 desaturase (Δ6D) activity as the ratio of 18:3n-6/18:2n-6; and delta-5 desaturase (Δ5D) activity as the ratio of 20:4n-6/20:3n-6. In addition, de novo lipogenesis (DNL) was estimated as the ratio of 16:0/18:2n-6 and the EFA status index (EFASTI) as the ratio of (∑n-3 +∑n-6)/(∑n-7+∑n-9), based on absolute fatty acid amounts (141).

Statistical analysis

Results are presented as mean ± SEM. Normally distributed quantitative variables were assessed by the Shapiro-Wilk test. Skewed data were logarithmically transformed. The effect of diet, surgery, and their interaction was analyzed with a two-way ANOVA. Significant results were analyzed with Tukey's test to identify statistically significant differences between the groups. Statistical comparisons were considered significant at $P < 0.05$. All statistical analyses were performed using the R program (www.R-project.org).

3. Results

3.1. General characteristics

The HFD increased body weight and fat depositions in the HF-NS group, which remained high after changing the diet in the DC-NS group (Table 2). VSG significantly reduced body weight and fat pad weight in the animals fed the HFD, especially when combined with dietary change in the DC-VSG group (36.4% and 86.4%, respectively, compared to the HF-NS group). The livers of the rats in the HF-NS group had three times the fat content of those in the animals of the C-NS group, with increased levels of esters and TGs, as well as alteration in liver appearance. Only dietary change significantly reduced the amount of hepatic fat. There were no differences between the Sham and NS groups (Table S1).

3.2. Fatty acid profile in liver tissues

Both surgery and diet led to several changes in liver FA profiles (Table 3). No differences were seen between the Sham groups and their respective NS groups (Table S2). The proportion of **saturated fatty acids (SFA)** decreased in animals on the HFD (mainly PA), but increased in the VSG groups (mainly LIG). **Monounsaturated fatty acids** (**MUFA**) showed the opposite pattern. The HFD significantly increased OA levels (the HF-NS group had 180% more OA levels than the C-NS group), which decreased with dietary change, especially when combined with surgery in the DC-VSG group. **Polyunsaturated fatty acid** (**PUFA**) levels decreased in rats fed the HFD, but increased in those that underwent VSG and a dietary change. The HFD lowered both n-3 and n-6 PUFA levels.

Table 2. General characteristics.

	Two-way ANOVA			C		HF		DC	
	Surgery NS/S/VSG	Diet C/HF/DC	S:D	NS	VSG	NS	VSG	NS	VSG
Body weight (g)	**	***	*	489 ± 14.3 bc	393 ± 12.7 c	652 ± 28.6 a	508 ± 14 b	648 ± 32.2 a	415 ± 32.7 bc
Fat pad weight (g)	***	**	***	19.9 ± 1.7 bcd	5.4 ± 0.5 d	40.3 ± 7.3 a	23 ± 1 abcd	38.4 ± 5.6 ab	5.5 ± 1.7 d
Hepatic fat (mg)	ns	*** c/a/b	ns	102 ± 20.9	107 ± 25.2	369 ± 40.6	334 ± 84.6	201 ± 13.5	155 ± 16.6
Hepatic fat (HF) (%)	ns	*** c/a/b	ns	0.94 ± 0.17	1.05 ± 0.23	2.8 ± 0.29	2.91 ± 0.63	1.43 ± 0.07	1.57 ± 0.05
TG (mg/dl)	***	***	*	119 ± 11 a	57.1 ± 4.8 c	63 ± 5.7 bc	65.1 ± 5.4 bc	107 ± 8.3 ab	48.1 ± 5.8 bc
NEFA (mg/dl)	ns	ns	ns	14.3 ± 1.3	13.27 ± 3	12.99 ± 0.4	12.63 ± 1.8	13.63 ± 0.7	10.38 ± 0.7
Chol (mg/dl)	*	ns	*	61.9 ± 2.4 bc	81.3 ± 4.9 ab	56 ± 4.5 c	56.1 ± 1.2 c	68.5 ± 4.4 bc	94.2 ± 10.4 a
HDL (mg/dl)	ns	ns	ns	39.2 ± 2.5	45.6 ± 1.2	33.7 ± 3.1	33.8 ± 1	38.7 ± 2.4	48.8 ± 4.9
LDL (mg/dl)	***	ns	***	10.5 ± 0.8 c	19.9 ± 2.9 ab	12.9 ± 1.2 c	11.2 ± 0.5 c	13.8 ± 2.8 bc	26.4 ± 2.5 a
Esters (µg/mg HF)	ns	**	ns	15.3 ± 4.7	6.1 ± 0.6	51.4 ± 12.3	35.3 ± 9.8	17.5 ± 0.5	6 ± NA
Liver TAG (µg/mg HF)	ns	* b/a/b	ns	45.8 ± 3.8	48.8 ± 9.8	187 ± 91.1	130 ± 21.2	75 ± 12.4	56.2 ± 27.2
Liver FAs (µg/mg HF)	ns	ns	ns	285 ± 120	157 ± 43.2	167 ± 56.6	66.5 ± 12	195 ± 15	99.1 ± 20.2
Liver cholesterol	ns	ns	ns	0.87 ± 0.29	1.3 ± 0.35	0.48 ± 0.11	0.39 ± 0.1	0.45 ± 0.04	1 ± 0.34
Liver DAG	ns	ns	***	1.12 ± 0.21 b	0.79 ± 0.04 bc	1.1 ± 0.11 b	0.84 ± 0.16 bc	0.83 ± 0.08 bc	2.33 ± NA a
Glucagon (ng/mL)	ns	** b/a/ab	ns	238 ± 51.8	301 ± 73.4	547 ± 102	461 ± 27.9	406 ± 45.2	282 ± 72.9
Glucose (mmol/L)	ns	ns	ns	6.32 ± 0.14	5.79 ± 0.19	6.03 ± 0.19	6.36 ± 0.14	6.18 ± 0.13	6.22 ± 0.14
Insulin (mIU/L)	ns	ns	*	21.1 ± 4.8 ab	18.8 ± 5.9 ab	30.3 ± 10.4 a	33.6 ± 10.8 a	26.7 ± 4.2 a	7.4 ± 2.7 b
ISI	ns	ns	*	0.009 ± 0.001 ab	0.015 ± 0.008 ab	0.01 ± 0.003 ab	0.006 ± 0.001 b	0.007 ± 0.001 b	0.037 ± 0.015 a
HOMA-IR	ns	ns	*	6 ± 1.5 ab	4.9 ± 1.5 ab	8.1 ± 2.8 ab	9.7 ± 3.2 a	7.4 ± 1.3 a	2.1 ± 0.8 b
HOMA-β	ns	ns	*	146 ± 27.2 ab	161 ± 55.1 ab	248 ± 91.7 a	228 ± 68 a	198 ± 24 a	51.9 ± 16.8 b
MCP-1	ns	ns	ns	560±37.3	573±72.9-	662±104-	537±76.1-	487±49.6-	498±79.5-

Data are expressed as means ± SEM. Data were analyzed by a two-way ANOVA (anova-2). *P < 0.05; **P < 0.01; ***P < 0.001. Tukey's test was performed for all the groups in which the Surgery:Diet (S:D) interaction was significant or for the main factors (surgery or diet) in which the factors were significant but their interaction was not. Group means with different letters are significantly different (p < 0.05) from other means in the same row. NS, no surgery; S, simulated surgery, VSG, vertical sleeve gastrectomy; C, control; HF, high-fat ; DC, diet change, HFD in the first 8 weeks before switching to the control diet in the last 4 weeks.

VSG significantly reduced body weight and fat pad weight in the animals fed the HFD, especially when combined with dietary change in the DC-VSG group (36.4% and 86.4%, respectively, compared to the HF-NS group). The livers of the rats in the HF-NS group had three times the fat content of those in the animals of the C-NS group, with increased levels of esters and TGs, as well as alteration in liver appearance. Only dietary change significantly reduced the amount of hepatic fat. There were no differences between the Sham and NS groups (Table S1).

3.3. Fatty acid profile in liver tissues

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3.4. Fatty acid ratios, estimated enzyme activities, and essential fatty acid status index

Several ratios and enzyme activities were estimated from the analyzed FA profiles (Table 4). No differences were seen between the Sham groups and their respective NS groups (Table S3). The AA/EPA ratio increased in all the groups after VSG, while the AA/DHA ratio decreased in the HF groups although there were few differences between the groups. Diet was the only factor that affected the MUFA/PUFA ratio, with higher values in the HF-NS group.

The HFD lowered SCD1 activity, but only in the HF groups, as the change of diet led to higher values. Δ5D activity was also lower in the HF groups, remaining so even with the change of diet in DC-NS. The HFD had the opposite effect on SCD18 activity, increasing it in the HF groups, especially when compared to the C groups.

The HFD decreased the EFASTI (59% in the HF-NS group). Changing back to the CD had no effect on the EFASTI, but increased it when combined with VSG in both the C-VSG and DC-VSG groups.

Table 3. Liver fatty acid profiles.

	Two-way ANOVA			C		HF		DC	
	Surgery NS/S/VSG	Diet C/HF/DC	S:D	NS	VSG	NS	VSG	NS	VSG
SFA	***** b/b/a**	*** a/b/a**	**ns**	**37 ± 0.85**	**43.2 ± 0.78**	**33 ± 1.16**	**35.4 ± 1.38**	**34.8 ± 1.63**	**43.5 ± 0.67**
MA	**	ns	*	0.46 ± 0.05 a	0.31 ± 0.02 ab	0.4 ± 0.04 a	0.33 ± 0.02 ab	0.46 ± 0.05 a	0.23 ± 0.02 b
PA	*	***	*	21.6 ± 0.24 a	21 ± 0.28 ab	19 ± 0.43 c	19.1 ± 0.33 c	19.6 ± 0.26 bc	20.3 ± 0.15 abc
SA	*** b/b/a	ns	ns	14.4 ± 0.99	21 ± 0.54	13.2 ± 1.22	15.5 ± 1.5	14.2 ± 1.47	22 ± 0.66
LIG	***	****	*	0.4 ± 0.05 bc	0.65 ± 0.03 a	0.19 ± 0.02 de	0.28 ± 0.04 cde	0.33 ± 0.03 cd	0.61 ± 0.04 a
UFA	***** a/a/b**	*** b/a/b**	**ns**	**63 ± 0.85**	**56.8 ± 0.78**	**67 ± 1.16**	**64.6 ± 1.38**	**65.2 ± 1.63**	**56.5 ± 0.67**
MUFA	*** a/a/b**	***** c/a/b**	**ns**	**16.1 ± 1.25**	**9.8 ± 0.58**	**30.3 ± 1.53**	**22.2 ± 1.79**	**23.3 ± 1.81**	**10.6 ± 0.72**
POA	***	***	*	1.94 ± 0.3 a	0.99 ± 0.09 bc	0.85 ± 0.14 bc	0.48 ± 0.03 c	1.34 ± 0.13 ab	0.93 ± 0.08 bc
VAC	***	***	*	4.4 ± 0.23 a	3.1 ± 0.15 bc	2.6 ± 0.1 cd	2 ± 0.02 d	3.4 ± 0.09 b	2.7 ± 0.28 bcd
OA	ns	***	*	9.4 ± 0.82 c	5.5 ± 0.45 c	26.3 ± 1.37 a	19.3 ± 1.74 b	18.1 ± 1.61 b	6.7 ± 0.4 c
PUFA	**ns**	**	**	**47 ± 0.71 a**	**46.9 ± 0.23 a**	**36.7 ± 1.4 c**	**42.4 ± 0.7 b**	**41.9 ± 0.46 b**	**45.9 ± 0.25 ab**
n-3	ns	** a/b/a	ns	3.7 ± 0.19	4.3 ± 0.15	3 ± 0.24	3.3 ± 0.12	4.1 ± 0.09	3.9 ± 0.42
ALA	***	ns	**	0.62 ± 0.08 a	0.19 ± 0.02 c	0.42 ± 0.06 abc	0.5 ± 0.07 ab	0.46 ± 0.08 abc	0.18 ± 0.01 c
EPA	** a/b/b	** a/ab/b	ns	0.15 ± 0.02	0.08 ± 0.01	0.08 ± 0.02	0.06 ± 0.01	0.11 ± 0.01	0.06 ± 0.01
DPA n-3	ns	ns	ns	0.49 ± 0.03	0.67 ± 0.06	0.4 ± 0.1	0.44 ± 0.04	0.55 ± 0.07	0.55 ± 0.07
DHA	* a/a/a	ns	ns	2.4 ± 0.21	3.3 ± 0.15	2.1 ± 0.21	2.3 ± 0.17	2.9 ± 0.24	3.1 ± 0.36
n-6	ns	***	***	43.3 ± 0.53 a	42.6 ± 0.3 ab	33.7 ± 1.26 d	39.1 ± 0.63 bc	37.9 ± 0.47 c	42 ± 0.48 abc
LA	***	***	***	22.9 ± 0.84 a	17.8 ± 0.37 bcd	18.3 ± 0.86 bcd	21.7 ± 0.9 ab	19.2 ± 0.89 abcd	17 ± 0.19d
GLA	ns	ns	ns	0.3 ± 0.03	0.16 ± 0.03	0.42 ± 0.09	0.29 ± 0.03	0.38 ± 0.04	0.2 ± 0.03
DGLA	***	*	*	0.45 ± 0.05 a	0.25 ± 0.03 b	0.32 ± 0.05 ab	0.33 ± 0.03 ab	0.36 ± 0.03 ab	0.3 ± 0.01 ab
AA	** b/b/a	*** a/b/a	ns	18.4 ± 1.09	23.3 ± 0.33	12 ± 0.96	14.4 ± 1.37	15.9 ± 1.32	23.2 ± 0.41
DPA n-6	ns	*** c/a/b	ns	0.37 ± 0.03	0.33 ± 0.03	0.95 ± 0.16	0.72 ± 0.04	0.56 ± 0.09	0.38 ± 0.08

Data are expressed in molar percentage (%) as means ± SEM. Data were analyzed by a two-way ANOVA (anova-2). *P < 0.05; **P < 0.01; ***P < 0.001. Tukey's test was performed for all the groups in which the Surgery:Diet (S:D) interaction was significant or for the main factors (surgery or diet) in which the factors were significant but their interaction was not. Group means with different letters are significantly different (p < 0.05) from other means in the same row, while common letters imply no differences between means.. NS, no surgery; S, simulated surgery; VSG, vertical sleeve gastrectomy; C, control; HF, high-fat ; DC, diet change, HFD in the first 8 weeks before switching to the control diet in the last 4 weeks. AA, arachidonic acid; ALA, alpha-linolenic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; GLA, gamma-linoleic acid; LA, linoleic acid; LIG, lignoceric acid; MA, myristic acid; MUFA, monounsaturated fatty acid; NA, nervonic acid; OA, oleic acid; PA, palmitic acid; POA, palmitoleic acid; PUFA, polyunsaturated fatty acid; SA, stearic acid; SFA, saturated fatty acid; UFA, unsaturated fatty acid; VAC, vaccenic acid.

Table 4. Indices, ratios and enzyme activities.

	Two-way ANOVA			C		HF		DC	
	Surgery NS/S/VSG	Diet C/HF/DC	S:D	NS	VSG	NS	VSG	NS	VSG
AA/EPA	* b/ab/a	ns	ns	135 ± 23.8	359 ± 60.3	258 ± 94.6	277 ± 57.5	169 ± 34	454 ± 77
AA/DHA	ns	***	*	7.8 ± 0.42 a	7 ± 0.28 ab	5.9 ± 0.5 ab	6.3 ± 0.31 ab	5.4 ± 0.2 b	7.8 ± 0.91 a
n-6/n-3	ns	* ab/a/b	ns	11.8 ± 0.5	11.7 ± 0.9	10 ± 0.4	11.7 ± 1	11.4 ± 0.5	12 ± 0.4
SFA/MUFA	***	***	***	2.4 ± 0.26 bc	4.5 ± 0.41 a	1.1 ± 0.09 d	1.7 ± 0.19 cd	1.6 ± 0.18 cd	4.2 ± 0.36 a
SFA/PUFA	*	ns	*	0.79 ± 0.02 a	0.92 ± 0.02 a	0.91 ± 0.05 a	0.84 ± 0.03 a	0.83 ± 0.04 a	0.95 ± 0.02 a
MUFA/PUFA	ns	*** c/a/b	ns	0.34 ± 0.03	0.21 ± 0.01	0.84 ± 0.07	0.53 ± 0.05	0.56 ± 0.05	0.23 ± 0.02
*Δ5*D	***	ns	**	43.6 ± 5.2 c	99.8 ± 11.1 a	41.3 ± 6.3 c	45.5 ± 7.4 c	46.5 ± 6.5 c	78.8 ± 2.6 ab
*Δ6*D	* a/a/a	ns	ns	0.02 ± 0.002	0.014 ± 0.001	0.017 ± 0.002	0.015 ± 0.001	0.019 ± 0.002	0.018 ± 0.001
SCD1	***	***	*	0.09 ± 0.01 a	0.05 ± 0.00 bc	0.04 ± 0.01 bc	0.02 ± 0.00 c	0.07 ± 0.01 ab	0.05 ± 0.00 bc
SCD18	***	***	*	0.69 ± 0.1 c	0.26 ± 0.03 d	2.09 ± 0.25 a	1.37 ± 0.28 ab	1.42 ± 0.29 ab	0.3 ± 0.03d
EFASTI	***	***	**	3 ± 0.29 cd	4.8 ± 0.31 a	1.2 ± 0.10 f	2 ± 0.17 ef	1.9 ± 0.15 ef	4.4 ± 0.33 ab
Elongase	ns	ns	**	2.5 ± 0.32 b	3.3 ± 0.28 ab	3.5 ± 0.53 ab	4.3 ± 0.22 a	2.6 ± 0.24 b	3 ± 0.37 ab
DNL	**	ns	***	0.95 ± 0.03 ab	1.2 ± 0.03 a	1.1 ± 0.05 ab	0.89 ± 0.03 b	1 ± 0.07 ab	1.2 ± 0.01 a

Data are expressed as means ± SEM. Data were analyzed by a two-way ANOVA. *P < 0.05; **P < 0.01; ***P < 0.001. Tukey's test was performed for all the groups in which the Surgery:Diet (S:D) interaction was significant or for the main factors (surgery or diet) in which the factors were significant but their interaction was not. group means with different letters are significantly different (p < 0.05) from other means in the same row, while common letters imply no differences between means. NS, no surgery; S, simulated surgery; VSG, vertical sleeve gastrectomy; C, control; HF, high-fat ; DC, diet change, HFD in the first 8 weeks before switching to the control diet in the last 4 weeks; SCD1, stearoyl-CoA desaturase 1 (16:1n-7/18:1n-9); SCD16, stearoyl-CoA desaturase 16 (16:1n-7/16:0); SCD18, stearoyl-CoA desaturase 18 (18:1n-9/18:0); Δ5D, delta-5 desaturase (20:4n-6/20:3n-6); Δ6D, delta-6 desaturase (18:3n-6/18:2n-6); DNL, de novo lipogenesis (16:0/18:2n-6); elongase (18:1n-7/16:1n-7); EFASTI, Essential Fatty-Acid Status Index (∑n-3 +∑n-6)/(∑n-7+∑n-9).

4. Discussion

In this study, we analyzed hepatic FA compositions and other biochemical parameters in rats fed an HFD. We also analyzed the changes in FA composition after changing to the CD, VSG, or after a combination of the two.

The HF groups presented increased hepatic fat content (mass and percentage), twice the amount of esters, three times the levels of TAG, higher MUFA concentrations, and a higher n-6/n-3 PUFA ratio, which are also seen in NAFLD (17-19). The higher influx of fat leading to hepatic accumulation was diet-related, as the fat intake in the HF groups was 700% higher than in the animals fed the CD. The variations in DNL indicated that this was not a source for the differences seen in the HF groups. We did not measure β-oxidation, but this may be another source of the increase in hepatic fat content; it has been reported to be reduced in animals fed an HFD due to modifications in the lipid membranes of peroxisomes (20, 21), decreasing FA consumption and increasing its accumulation. We also found that the HF-NS group showed increased AA/EPA and AA/DHA ratios compared to the C-NS group, in accordance with the pro-inflammatory FA profile previously observed in NAFLD (22). Interestingly, the levels of the inflammatory plasma marker MCP-1, an indicator of macrophage infiltration, remained similar between the groups, although it has been reported to be increased by HFDs (23).

The HF groups presented lower SFA and higher MUFA fractions, despite the high proportions of SFA and MUFA in the HFD (36% and 47%, respectively). This might be explained by the use of lard as the main source of fat, which, though rich in SFA, also has a high amount of OA. High OA intake is also associated with NAFLD and inflammation (18, 21, 24), but may reduce the parameters associated with metabolic syndrome such as hypertriglyceridemia (25). Indeed, we observed that plasma TAG levels were decreased by the HFD. The PUFA fraction was also decreased by the HFD, as described previously (19, 23). This is an interesting finding, given that the HFD contained more LA and ALA than the CD. Both n-3 and n-6 PUFAs have important roles in maintaining cell membrane fluidity and in cell signaling (6), and seem to be involved in obesity. A high PUFA intake can inhibit DNL, and n-3 PUFAs limit hepatic TAG accumulation (18). However, we did observe a higher TAG accumulation. Moreover, n-3 PUFAs are anti-inflammatory, as ALA is a precursor of long-chain PUFAs (LCPUFAs). Furthermore, both EPA and DHA are precursors of specialized pro-resolving molecules, such as protectins and resolvins, which are responsible for the resolution of inflammatory processes (7). Lower levels of n-3 PUFAs might impair this resolution, leading to the continued inflammatory state characteristic of obesity and NAFLD (26). Supplementation with EPA and DHA has been reported to improve steatosis and hepatic inflammation (23, 27).

We observed a positive correlation between SCD1 activity and the percentage of hepatic fat (Figure 1(a); Pearson correlation $R = -0.52$, $P < 0.01$) and also between SCD18 activity and the percentage of hepatic fat (Figure 1(b); Pearson correlation $R = 0.75$, $P < 0.001$), as previously reported (28, 29). The percentage of hepatic fat negatively correlated with the AA/DHA ratio (Figure 1(c); Pearson correlation $R = -0.42$, $P = 0.029$). SCD1 and SCD18 regulate the desaturation of SFA to MUFA. SCD18 desaturates the FAs synthesized through DNL to OA, which is stored as TAG (6). In our study, the HF groups had higher estimated SCD18 activity without changes in DNL. This may have been due to the higher OA content in the HFD, which may affect the estimation of SCD18 activity even though the estimation of desaturase activities correlates with mRNA expression (19).

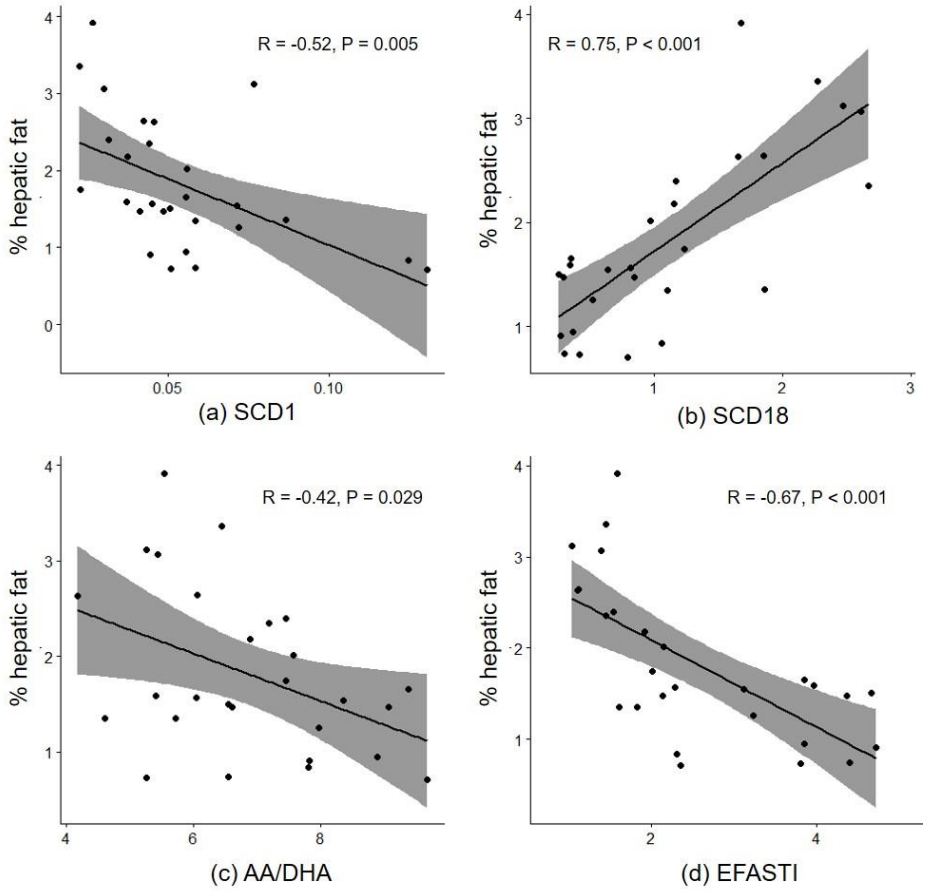

Figure 2. Pearson correlation between the percentage of hepatic fat and (a) SCD1 activity, (b) SCD18 activity, (c) the AA/DHA ratio, and (d) EFASTI.

Switching from the HFD to the CD in the DC groups mainly improved the hepatic fat content, as well as hepatic ester and TAG levels, which recovered (understanding recovery as returning to values similar to those of the C groups). The DC groups also showed normalized SFA and improved MUFA values. Although VSG also altered SFA and MUFA values, there was no combined effect of VSG and dietary change. However, only a few parameters were altered by VSG alone, since most of the data analyzed showed no differences between NS and VSG when the groups were kept on the HFD. The DC-VSG group showed improvements in many of the FA levels, ratios, and enzyme activities, several of which recovered to control values. This finding was particularly interesting as changing to a healthier diet (DC-NS) reduced hepatic fat content (as also observed in humans (30)), but did not reverse several of the molecular changes caused by the HFD. The combination of dietary change and VSG proved to be the best solution for NAFLD, as demonstrated by the recovery of several parameters such as the PUFA fraction, other FAs, SCD18 activity, Δ5D activity, and the EFASTI. The improvement in the EFASTI, which negatively correlated with the percentage of hepatic fat (Figure 1(d)), is interesting as it indicates the status of EFAs and, thus, the availability of FAs for the synthesis of specialized pro-resolving molecules in order to resolve inflammation (7). Another interesting finding is the reduction in LA and ALA, in the C-VSG and DC-VSG groups compared to their respective NS groups, despite a similar intake. This may have been due to their increased use as precursors to deal with the ongoing postsurgical inflammation, since the AA/EPA and AA/DHA ratios were also increased (30). In addition, the AA/DHA ratio negatively correlated with the percentage of hepatic fat (Figure 1(c)), indicating a higher basal inflammatory state in the obese animals. in the increases in these ratios tended to be lower in the HF-VSG than in the C-VSG, possibly indicating a deficient inflammatory response after surgery.

We stress that many of the recovered parameters had values that were closer to those of the C-VSG group than the C-NS group, as VSG also affected the non-obese C groups. It is now understood that the benefits of VSG are not only due to reduced intake: rats compensate for the effects of VSG by increasing the number of smaller meals (20, 31).We observed an almost normalized intake after 4 weeks (except in the HF-VSG group, where it

remained lower; Table S4). We recently saw a similar trend in gut microbiota (32), which was affected by VSG regardless of the diet. In the present study, combined treatment (the DC-VSG group) showed improvements in animals that had initially received the HFD, but the microbiome was not fully restored and its values were closer to those of the rats in the C-VSG group, indicating that there are still some unknown mechanisms underlying the effects of VSG.

This study presents some limitations. The HFD used did not have added sugar, and so was not representative of the Western diet in humans. Furthermore, the fact that the diets were not EFA-balanced may have affected some of our results, although we observed higher EFA levels in the CD-fed animals. We also analyzed the FAs without separating the different fractions, and enzyme activities were estimated rather than measured. Despite these limitations, however, our study also has important strengths, such as the complex design analyzing dietary change and VSG both as separate variables and in combination, using controls for each variable.

5. Conclusion

Liver FA compositions, as well as the activities of several enzymes associated with hepatic FA metabolism, were altered in an HFD-induced NAFLD model. The HFD led to obesity, higher fat accumulation, modified FA compositions, and a pro-inflammatory FA profile. The combination of dietary change and VSG was the most effective in reversing the hepatic consequences of HFDs. The changes in FA composition and the activities of enzymes involved in FA metabolism in HFD-induced fatty livers may dysregulate resolvins and protectins, contributing to the inflammatory state seen in NAFLD. Further studies are necessary to elucidate this; however what is clear is that these changes can be reversed by combining VSG and dietary change.

Supplementary Materials: Table S1. General characteristics (all surgical groups); Table S2. Fatty acid profile in liver tissues (all surgical groups); Table S3. Indices, ratios, and enzyme activities in the liver (all surgical groups); and Table S4. Intake (calories and nutrients) at week 12.

Author Contributions: Conceptualization, Julia Peinado and Joan Carles Domingo; methodology, Joana Rossell and Begoña Cordobilla; software, Joana Rossell; validation, Julia Peinado, Joan Carles Domingo, and Eva Pardina; formal analysis, Joana Rossell, Joan Carles Domingo, Julia Peinado-Onsurbe, and Eva Pardina; investigation, Joana Rossell, Julia Peinado-Onsurbe, and Eva Pardina; resources, David Ricart-Jané, Joan Carles Domingo, and Julia Peinado-Onsurbe; data curation, Joana Rossell and Joan Carles Domingo; writing — original draft preparation, Joana Rossell; writing — review and editing, Eva Pardina; visualization, David Ricart-Jané and Juan Antonio Baena-Fustegueras; supervision, Eva Pardina; project administration, Julia Peinado; funding acquisition, Julia Peinado, Joan Carles Domingo and Juan Antonio Baena-Fustegueras. All authors have read and agreed to the published version of the manuscript.

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Table S1. General characteristics (all surgical groups)

	2way ANOVA			C			HF			DC		
	Surgery NS/S/VSG	Diet C/HF/DC	S:D	NS	Sham	VSG	NS	Sham	VSG	NS	Sham	VSG
Body Weight (g)	**	***	*	489±14.3bc	447±5.9bc	393±12.7c	652±28.6a	659±35.3a	508±14b	648±32.2a	639±21.3a	415±32.7bc
Fat pads (g)	***	**	***	19.9±1.7bcd	12.5±0.7cd	5.4±0.5d	40.3±7.3a	40.9±5.5a	23±1abcd	38.4±5.6ab	30.1±3.1abc	5.5±1.7d
Hepatic fat (mg)	ns	*** c/a/b	ns	102±20.9c	104±20.3c	107±25.2c	369±40.6ab	443±81.6a	334±84.6ab	201±13.5bc	253±36.2abc	155±16.6bc
Hepatic fat (%)	ns	*** c/a/b	ns	0.94±0.17c	1.07±0.24c	1.05±0.23c	2.8±0.29ab	2.7±0.22ab	2.91±0.63a	1.43±0.07bc	1.89±0.21abc	1.57±0.05abc
TG (mg/dl)	***	***	*	119±11a	92.7±7abc	57.1±4.8c	63±5.7bc	71.3±18.6bc	65.1±5.4bc	107±8.3ab	86.4±14.8abc	48.1±5.8bc
NEFA (mg/dl)	ns	ns	ns	14.3±1.3a	12.9±0.8a	13.27±3a	12.99±0.4a	14.9±0.9a	12.63±1.8a	13.63±0.7a	16.53±0.8a	10.38±0.7a
Chol (mg/dl)	*	ns	*	61.9±2.4bc	66.8±6bc	81.3±4.9ab	56±4.5c	58.4±3.7c	56.1±1.2c	68.5±4.4bc	64.6±2.5bc	94.2±10.4a
HDL (mg/dl)	ns	ns	ns	39.2±2.5ab	38±2.6ab	45.6±1.2ab	33.7±3.1b	35.4±2.2b	33.8±1b	38.7±2.4ab	36.9±1.2ab	48.8±4.9a
LDL (mg/dl)	***	ns	***	10.5±0.8c	12.1±1.5c	19.9±2.9ab	12.9±1.2c	12.9±0.9c	11.2±0.5c	13.8±2.8bc	15.5±0.3bc	26.4±2.5a
Esters (µg/mg HF)	ns	**	ns	15.3±4.7b	8.4±1.5b	6.1±0.6b	51.4±12.3a	31.7±3.9ab	35.3±9.8ab	17.5±0.5ab	20.6±4.8ab	6±NAb
TAG liver (µg/mg HF)	ns	* b/a/b	ns	45.8±3.8a	92±16.7a	48.8±9.8a	187±91.1a	153±5.8a	130±21.2a	75±12.4a	71±21.8a	56.2±27.2a
FA liver (µg/mg HF)	ns	ns	ns	285±120a	159±18a	157±43.2a	167±56.6a	55.3±11.7a	66.5±12a	195±15a	144±5.4a	99.1±20.2a
Chol Liver	ns	ns	ns	0.87±0.29a	0.89±0.35a	1.3±0.35a	0.48±0.11a	0.31±0.01a	0.39±0.1a	0.45±0.04a	0.51±0.05a	1±0.34a
DAG liver	ns	ns	***	1.12±0.21b	0.78±0.02bc	0.79±0.04bc	1.1±0.11b	0.48±0c	0.84±0.16bc	0.83±0.08bc	0.61±0.04bc	2.33±NAa
Glucagon (ng/mL)	ns	** b/a/ab	ns	238±51.8-	264±18.1-	301±73.4-	547±102-	409±52.8-	461±27.9-	406±45.2-	362±66.3-	282±72.9-
Glucose (mmol/L)	ns	ns	ns	6.32±0.14-	6.03±0.13-	5.79±0.19-	6.03±0.19-	5.86±0.2-	6.36±0.14-	6.18±0.13-	5.74±0.14-	6.22±0.14-
Insulin (mIU/L)	ns	ns	*	21.1±4.8ab	12.3±2.8ab	18.8±5.9ab	30.3±10.4a	24±6.6a	33.6±10.8a	26.7±4.2a	7.1±2.1b	7.4±2.7b
ISI	ns	ns	*	0.009±0.001 ab	0.016±0.005 ab	0.015±0.008 ab	0.01±0.003 ab	0.009±0.002 ab	0.006±0.001 b	0.007±0.001 b	0.029±0.007a	0.037±0.015a
HOMA-IR	ns	ns	*	6±1.5ab	3.3±0.7ab	4.9±1.5ab	8.1±2.8ab	6.3±1.8ab	9.7±3.2a	7.4±1.3a	2.1±0.6b	2.1±0.8b
HOMA-B	ns	ns	*	146±27.2ab	101±27.7ab	161±55.1ab	248±91.7a	202±52.4a	228±68a	198±24a	71.7±19.6ab	51.9±16.8b
MCP1 (pg/mL)	ns	ns	ns	560±37.3-	708±98.9-	573±72.9-	662±104-	705±104-	537±76.1-	487±49.6-	632±94.4-	498±79.5-

Data are expressed as means ± SEM. Data were analyzed by a two-way ANOVA (anova-2). *P < 0.05; **P < 0.01; ***P < 0.001. Tukey's test was performed for all the groups in which the Surgery:Diet (S:D) interaction was significant or for the main factors (surgery or diet) in which the factors were significant but their interaction was not. Group means with different letters are significantly different (p < 0.05) from other means in the same row. NS, no surgery; S and Sham, simulated surgery, VSG, vertical sleeve gastrectomy; C, control; HF, high-fat ; DC, diet change, HFD in the first 8 weeks before switching to the control diet in the last 4 weeks.

Table S2. Fatty acid profile in liver tissue (all surgical groups)

	2way ANOVA			C			HF			DC		
	Surgery NS/S/VSG	Diet C/HF/DC	S:D	NS	Sham	VSG	NS	Sham	VSG	NS	Sham	VSG
SFA	*** b/b/a	* a/b/a	ns	37±0.85	37.8±0.78	43.2±0.78	33±1.16	32.9±1.14	35.4±1.38	34.8±1.63	38.1±0.72	43.5±0.67
MA	**	ns	*	0.46±0.05a	0.3±0.02ab	0.31±0.02ab	0.4±0.04a	0.43±0.03a	0.33±0.02ab	0.46±0.05a	0.34±0.02ab	0.23±0.02b
PA	*	***	*	21.6±0.24a	20.1±0.15abc	21±0.28ab	19±0.43c	19.4±0.34c	19.1±0.33c	19.6±0.26bc	19.3±0.19c	20.3±0.15abc
SA	*** b/b/a	ns	ns	14.4±0.99	16.6±0.65	21±0.54	13.2±1.22	12.8±0.96	15.5±1.5	14.2±1.47	17.9±0.8	22±0.66
LIG	***	****	*	0.4±0.05bc	0.54±0.01ab	0.65±0.03a	0.19±0.02de	0.16±0.02e	0.28±0.04cde	0.33±0.03cd	0.39±0.03bc	0.61±0.04a
UFA	*** a/a/b	* b/a/b	ns	63±0.85	62.2±0.78	56.8±0.78	67±1.16	67.1±1.14	64.6±1.38	65.2±1.63	61.9±0.72	56.5±0.67
MUFA	* a/a/b	*** c/a/b	ns	16.1±1.25	13.5±0.7	9.8±0.58	30.3±1.53	30.8±1.83	22.2±1.79	23.3±1.81	19.3±0.72	10.6±0.72
POA	***	***	*	1.94±0.3a	1.21±0.08abc	0.99±0.09bc	0.85±0.14bc	1.01±0.17bc	0.48±0.03c	1.34±0.13ab	0.79±0.08bc	0.93±0.08bc
VAC	***	***	*	4.4±0.23a	4.6±0.25a	3.1±0.15bc	2.6±0.1cd	2.8±0.15bc	2±0.02d	3.4±0.09b	3±0.13bc	2.7±0.28bcd
OA	ns	***	*	9.4±0.82c	7.5±0.72c	5.5±0.45c	26.3±1.37a	26.5±1.58a	19.3±1.74b	18.1±1.61b	15.1±0.69b	6.7±0.4c
PUFA	ns	**	**	47±0.71a	48.7±0.33a	46.9±0.23a	36.7±1.4c	36.3±1.73c	42.4±0.7b	41.9±0.46b	42.6±0.3b	45.9±0.25ab
n3	ns	** a/b/a	ns	3.7±0.19	3.9±0.31	4.3±0.15	3±0.24	3±0.21	3.3±0.12	4.1±0.09	3.8±0.29	3.9±0.42
ALA	***	ns	**	0.62±0.08a	0.44±0.04abc	0.19±0.02c	0.42±0.06abc	0.5±0.07ab	0.5±0.07ab	0.46±0.08abc	0.31±0.04bc	0.18±0.01c
EPA	** a/b/b	** a/ab/b	ns	0.15±0.02	0.14±0.02	0.08±0.01	0.08±0.02	0.08±0.02	0.06±0.01	0.11±0.01	0.07±0.01	0.06±0.01
DPA n3	ns	ns	ns	0.49±0.03	0.45±0.04	0.67±0.06	0.4±0.1	0.41±0.13	0.44±0.04	0.55±0.07	0.41±0.04	0.55±0.07
DHA	* a/a/a	ns	ns	2.4±0.21	2.8±0.38	3.3±0.15	2.1±0.21	2±0.18	2.3±0.17	2.9±0.24	3±0.34	3.1±0.36
n6	ns	***	***	43.3±0.53a	44.8±0.57a	42.6±0.3ab	33.7±1.26d	33.4±1.55d	39.1±0.63bc	37.9±0.47c	38.8±0.41bc	42±0.48abc
LA	***	***	***	22.9±0.84a	21.7±0.84abc	17.8±0.37bcd	18.3±0.86bcd	18.3±0.83bcd	21.7±0.9ab	19.2±0.89abcd	17.7±1.08cd	17±0.19d
GLA	ns	ns	ns	0.3±0.03	0.32±0.07	0.16±0.03	0.42±0.09	0.32±0.02	0.29±0.03	0.38±0.04	0.41±0.06	0.2±0.03
DGLA	***	*	*	0.45±0.05a	0.47±0.04a	0.25±0.03b	0.32±0.05ab	0.32±0.04ab	0.33±0.03ab	0.36±0.03ab	0.31±0.02ab	0.3±0.01ab
AA	** b/b/a	*** a/b/a	ns	18.4±1.09	21.1±0.86	23.3±0.33	12±0.96	12.2±0.96	14.4±1.37	15.9±1.32	18.6±0.69	23.2±0.41
DPA n6	ns	*** c/a/b	ns	0.37±0.03	0.45±0.05	0.33±0.03	0.95±0.16	0.63±0.09	0.72±0.04	0.56±0.09	0.7±0.11	0.38±0.08

Data are expressed in molar percentage (%) as means ± SEM. Data were analyzed by a two-way ANOVA (anova-2). *P < 0.05; **P < 0.01; ***P < 0.001. Tukey's test was performed for all the groups in which the Surgery:Diet (S:D) interaction was significant or for the main factors (surgery or diet) in which the factors were significant but their interaction was not. Group means with different letters are significantly different (p < 0.05) from other means in the same row. NS, no surgery; S and Sham, simulated surgery, VSG, vertical sleeve gastrectomy; C, control; HF, high-fat ; DC, diet change, HFD in the first 8 weeks before switching to the control diet in the last 4 weeks. AA, Arachidonic acid; ALA, Alpha-linolenic acid; DGLA, dihomo-gamma-linolenic acid; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; FA, Fatty acid; GLA, Gamma-linoleic acid; LA, Linoleic acid; LIG, Lignoceric acid; MA, Myristic acid; MUFA, Monounsaturated fatty acid; NA, Nervonic acid; OA, Oleic acid; PA, Palmitic acid; POA, Palmitoleic acid; PUFA, Polyunsaturated fatty acid; SA, Stearic acid; SFA, Saturated fatty acid; UFA, Unsaturated fatty acid; VAC, Vaccenic acid.

Table S3. Indices, ratios, and enzymes in the liver (all surgical groups)

	2way ANOVA			C			HF			DC		
	Surgery NS/S/VSG	Diet C/HF/DC	S:D	NS	Sham	VSG	NS	Sham	VSG	NS	Sham	VSG
AA/EPA	* b/ab/a	ns	ns	135±23.8	165±30	359±60.3	258±94.6	240±63	277±57.5	169±34	354±113.2	454±77
AA/DHA	ns	***	*	7.8±0.42a	7.7±0.83a	7±0.28ab	5.9±0.5ab	6.3±0.33ab	6.3±0.31ab	5.4±0.2b	6.4±0.4ab	7.8±0.91a
n6/n3	ns	* ab/a/b	ns	11.8±0.5	11.7±0.9	10±0.4	11.7±1	11.4±0.5	12±0.4	9.4±0.2	10.4±0.7	11.2±1.2
SFA/MUFA	***	***	***	2.4±0.26bc	2.8±0.19b	4.5±0.41a	1.1±0.09d	1.1±0.08d	1.7±0.19cd	1.6±0.18cd	2±0.11bcd	4.2±0.36a
SFA/PUFA	*	ns	*	0.79±0.02a	0.77±0.02a	0.92±0.02a	0.91±0.05a	0.92±0.06a	0.84±0.03a	0.83±0.04a	0.89±0.02a	0.95±0.02a
MUFA/PUFA	ns	*** c/a/b	ns	0.34±0.03	0.28±0.01	0.21±0.01	0.84±0.07	0.87±0.09	0.53±0.05	0.56±0.05	0.45±0.02	0.23±0.02
Δ5	***	ns	**	43.6±5.2c	45.6±3c	99.8±11.1a	41.3±6.3c	41±5.8c	45.5±7.4c	46.5±6.5c	60.7±3.4bc	78.8±2.6ab
Δ6	* a/a/a	ns	ns	0.02±0.002	0.022±0.002	0.014±0.001	0.017±0.002	0.017±0.001	0.015±0.001	0.019±0.002	0.018±0.002	0.018±0.001
SCD1	*	***	***	0.2±0.02a	0.16±0.01ab	0.18±0.01ab	0.03±0d	0.04±0d	0.03±0cd	0.08±0.01c	0.05±0.01cd	0.14±0.01b
SCD16	***	***	*	0.09±0.01a	0.06±0abc	0.05±0bc	0.04±0.01bc	0.05±0.01bc	0.02±0c	0.07±0.01ab	0.04±0bc	0.05±0bc
SCD18	***	***	*	0.69±0.1c	0.46±0.06cd	0.26±0.03d	2.09±0.25a	2.15±0.25a	1.37±0.28ab	1.42±0.29ab	0.86±0.07bc	0.3±0.03d
EFASTI	***	***	**	3±0.29cd	3.6±0.17bc	4.8±0.31a	1.2±0.1f	1.2±0.11f	2±0.17ef	1.9±0.15ef	2.2±0.09de	4.4±0.33ab
Elongase	ns	ns	**	2.5±0.32b	3.8±0.24ab	3.3±0.28ab	3.5±0.53ab	3±0.39ab	4.3±0.22a	2.6±0.24b	4±0.3ab	3±0.37ab
DNL	**	ns	***	0.95±0.03ab	0.93±0.04ab	1.2±0.03a	1.1±0.05ab	1.1±0.06ab	0.89±0.03b	1±0.07ab	1.1±0.07ab	1.2±0.01a

Data are expressed as means ± SEM. Data were analyzed by a two-way ANOVA (anova-2). *P < 0.05; **P < 0.01; ***P < 0.001. Tukey's test was performed for all the groups in which the Surgery:Diet (S:D) interaction was significant or for the main factors (surgery or diet) in which the factors were significant but their interaction was not. Group means with different letters are significantly different (p < 0.05) from other means in the same row. NS, no surgery; S and Sham, simulated surgery, VSG, vertical sleeve gastrectomy; C, control; HF, high-fat ; DC, diet change, HFD in the first 8 weeks before switching to the control diet in the last 4 weeks. SCD1, stearoyl-CoA desaturase 1 (16:1n-7/18:1n-9); SCD16, stearoyl-CoA desaturase 16 (16:1 n-7/16:0); SCD-18, stearoyl-CoA desaturase 18 (18:1 n-9/18:0); Δ5, delta-5-desaturase (20:4 n-6/20:3 n-6); Δ6, delta-6-desaturase (18:3 n-6/18:2 n-6); DNL, De Novo Lipogenesis (16:0/18:2 n-6); Elongase (18:1 n-7/16:1 n-7); EFASTI, Essential Fatty-acid Status Index (∑n-3 +∑n-6)/(∑n-7+∑n-9).

Table S4. Intake (calories and nutrients) at week 12

	2way ANOVA			C			HF			CD		
	Surgery NS/S/VSG	Diet C/HF/DC	S:D	NS	Sham	VSG	NS	Sham	VSG	NS	Sham	VSG
Intake w12	**	***	**	21.8±0.52cd	22.8±0.13bc	19.4±0.96d	21.4±0.83cd	23±0.23bc	15.9±0.45e	24.9±0.57b	27.5±0.44a	22.7±0.44bc
Calories	*	***	***	63.2±1.5cd	66±0.38cd	56.4±2.8d	109±4.2a	117±1.2a	80.9±2.3b	72.1±1.6bc	79.7±1.3b	65.8±1.3cd
Protein	*	***	***	3.1±0.07de	3.3±0.02cde	2.8±0.14e	5±0.19a	5.4±0.05a	3.7±0.11bc	3.6±0.08bcd	3.9±0.06b	3.2±0.06cde
Carbohydrate	*** b/a/c	*** b/c/a	ns	10.5±0.250	10.9±0.060	9.3±0.460	5.9±0.230	6.3±0.060	4.3±0.120	11.9±0.270	13.2±0.210	10.9±0.210
Fat	ns	***	***	0.9±0.02c	0.9±0.01c	0.8±0.04c	7.3±0.28a	7.9±0.08a	5.4±0.16b	1±0.02c	1.1±0.02c	0.9±0.02c
Saturated	ns	***	***	0.13±0c	0.14±0c	0.12±0.01c	2.7±0.1a	2.9±0.03a	2±0.06b	0.15±0c	0.16±0c	0.14±0c
Monounsaturated	ns	***	***	0.15±0c	0.16±0c	0.14±0.01c	3.4±0.13a	3.7±0.04a	2.5±0.07b	0.17±0c	0.19±0c	0.16±0c
Polyunsaturated	ns	***	***	0.46±0.01de	0.48±0cde	0.41±0.02e	1.2±0.04a	1.2±0.01a	0.9±0.02b	0.52±0.01cd	0.58±0.01c	0.48±0.01cde
MA (C14:0)	-	-	-	0	0	0	0.1±0a	0.11±0a	0.07±0b	0	0	0
PA (C16:0)	ns	***	***	0.11±0c	0.11±0c	0.1±0c	1.72±0.07a	1.85±0.02a	1.27±0.04b	0.12±0c	0.14±0c	0.11±0c
POA (C16:1)	-	-	-	0	0	0	0.21±0.01a	0.22±0a	0.15±0b	0	0	0
SA (C18:0)	ns	***	***	0.02±0c	0.02±0c	0.02±0c	0.84±0.03a	0.9±0.01a	0.62±0.02b	0.02±0c	0.03±0c	0.02±0c
OA (C18:1)	ns	***	***	0.15±0c	0.16±0c	0.14±0.01c	3.1±0.12a	3.4±0.03a	2.3±0.07b	0.17±0c	0.19±0c	0.16±0c
LA (C18:2)	ns	***	***	0.44±0.01de	0.46±0cde	0.39±0.02e	1.01±0.04a	1.08±0.01a	0.75±0.02b	0.5±0.01cd	0.55±0.01c	0.45±0.01cde
ALA (C18:3 n3)	ns	***	***	0.02±0c	0.02±0c	0.02±0c	0.12±0a	0.13±0a	0.09±0b	0.02±0c	0.03±0c	0.02±0c

Data are expressed in grams/day as the mean ± SEM. Data was analyzed by a two-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Tukey post-test was performed on all groups when the interaction Surgery:Diet (S:D) was significant, or on the main factors (surgery or Diet) when factors were significant and interaction was not significant. Groups with the same letter do not show differences between them. NS, No Surgery; S and Sham, simulated surgery, VSG, Vertical Sleeve Gastrectomy; C, control, DIO, Diet Induced Obesity; D+C, first 8 weeks fed with HFD diet and switched to control diet the last 4 weeks. Mean of daily intake at week 12, in calories and applied to diet composition

4. GLOBAL RESULTS

In this section we will present a global overview of the results obtained in this study, which are published in the two articles, written in the third article, and also further unpublished material. For that, we will start by analyzing the intake evolution for each diet and group during the 12 weeks of the experiment, and how it was modified by the surgical options. We will proceed with how diet and surgery affected the anthropometrical parameters (body weight (BW) and organ weight), followed by the effect on the biochemical parameters. Later, we will analyze the changes in GM. Finally, we will end with the effects of diet and surgery on the FA composition in brain tissue, liver tissue, and visceral WAT.

4.1. FOOD INTAKE

In this study, we fed the C group a standard chow diet , and the HF and DC groups a HFD. The HFD had a calorie composition of 18 % protein, 21% carbohydrates, and 60% fat, with lard (rich in SFA) as the main component (for comparison to chow diet, **table 1, article 3**).

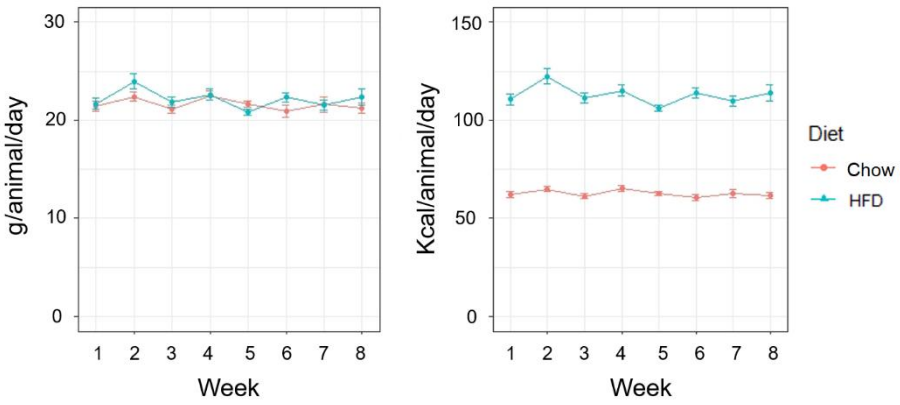

Figure 10. Pre-surgery daily intake. Daily intake per week and animal, in grams and in calories, for each diet during the first 8 weeks. HFD stand for high-fat diet.

During the first 8 weeks of study, all the groups had a similar daily intake (g), but the groups fed a HFD had a much higher calorie intake when compared to the group fed a chow diet ($p<0.01$, Welch's t-test) due to the higher caloric density of the HFD (**figure 10**).

At the end of the 8th week, animals underwent one of the surgical options: no surgery (NS), simulated surgery (sham) or VSG. The DC group, that had been fed a HFD, changed then to the chow diet. The change of diet was intended to represent the change in dietary habits that occur when humans follow a diet with reduced calorie content, combined or not with BS. The NS groups continued the following 4 weeks with a similar intake than in the previous weeks, with HF-NS maintaining the higher calorie consumption, but DC-NS now was eating less amount of calories ingested due to the change of diet (**figure 11**).

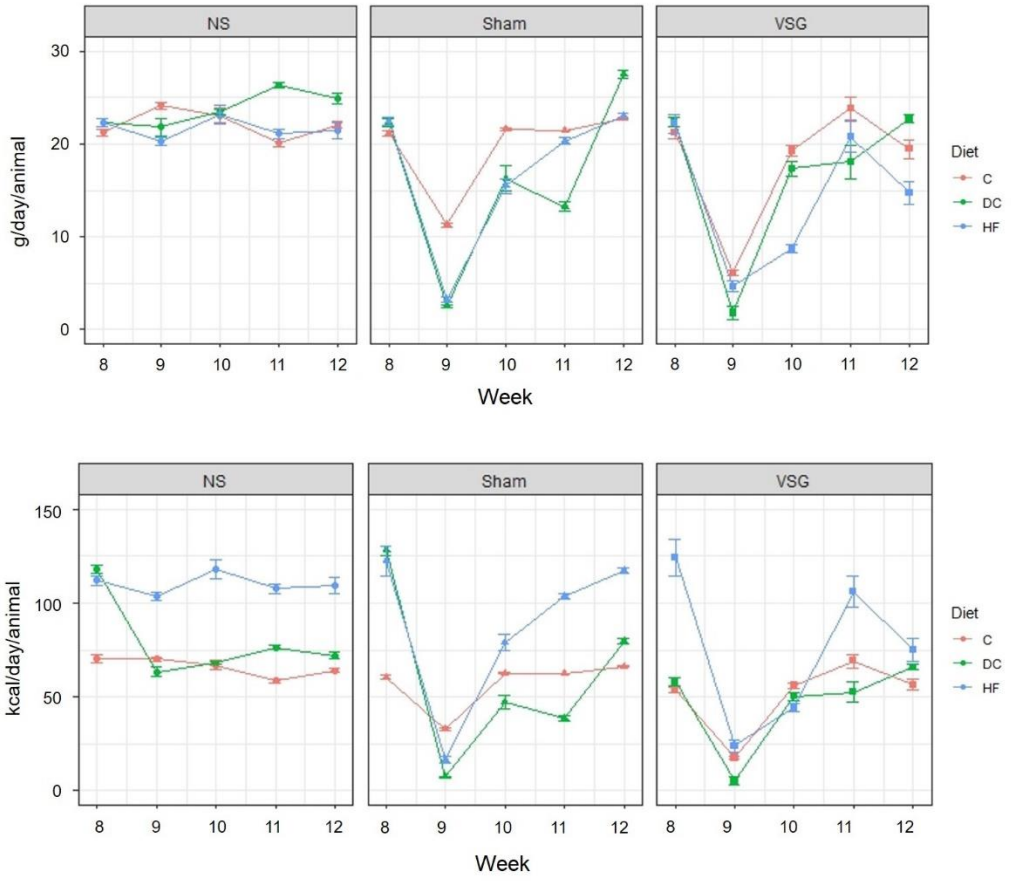

Figure 11. Post-surgery daily intake. Daily intake per week, in grams (upper panel) and in calories (lower panel), for each diet during the four last weeks, with surgery and change of diet taking place at week 8. C stands for control group, and HF for high fat group, DC for diet change group.

There was a reduction in the intake at week 9 for sham groups, probably as a result of the pre-operative fasting. Daily intake was increased at week 10, with C-sham eating as C-NS. At week 11, HF-sham was also eating a similar amount as HF-NS. At the end of the study, sham

groups were eating the same amount as NS groups, except for DC-sham, that after a different feeding evolution, ended up with the highest intake in grams, but still eating fewer calories than the HF group. The intake in all groups was heavily affected by the VSG, probably due to the post-operatory diet which consisted of two days with a semi-fluid diet, followed by a progressive reintroduction of food pellets mixed with liquid. At the 10th and 11th week, food intake progressively increased in all groups until the food intake was similar to the NS groups. At week 12, chow diet-feed groups tended to decrease the amount of food ingested, but only HF-VSG was eating significantly fewer calories than its unoperated match. The amount of calories that DC-VSG ingested at this point was similar to both C-VSG and HF-VSG.

4.2. EFFECT ON ANTHROPOMETRICAL PARAMETERS

4.2.1. *EFFECT ON BODY WEIGHT*

The increased energy intake in the HFD-fed groups caused a higher body weight gain (BWG), visible from the first week of the experiment and maintained during the 8 first weeks (**figure 2-a, article 2**). The higher weight gain translated into a higher body weight in the HFD-fed group (HF and DC groups) compared to the group fed the chow diet - at week 8 (**figure 2, article 1**). There was a tendency to a reduced BWG after the 6th week in C-groups, while HF kept the BWG at a similar pace during the first 8 weeks.

After the 8th week, the groups without surgery (C-NS, HF-NS, and DC-NS) continued increasing their body weight, with C-NS still having a moderate growth compared to HF-NS (**figure 12**). The change from HFD to chow diet did not seem to affect much the weight gain in DC-NS at the beginning, as DC-NS kept a similar BWG, with a higher BW than HF-NS (**figure 2-a, article 2**). After the 10th week, DC-NS slowed down the BW increase, unlike HF-NS, stabilizing its weight until the end of the study at week 12. After sham surgery, all groups lost weight compared to their pre-surgery weight at week 8 (% weight loss, **figure3, article 1**), but all started to regain weight at week 9. All groups caught up their pre-surgery weight at the end of the 12th week but at a different pace depending on the diet, with HF the

first as the weight loss was small, followed by DC-sham, and lastly C-sham, catching up after the 11th week.

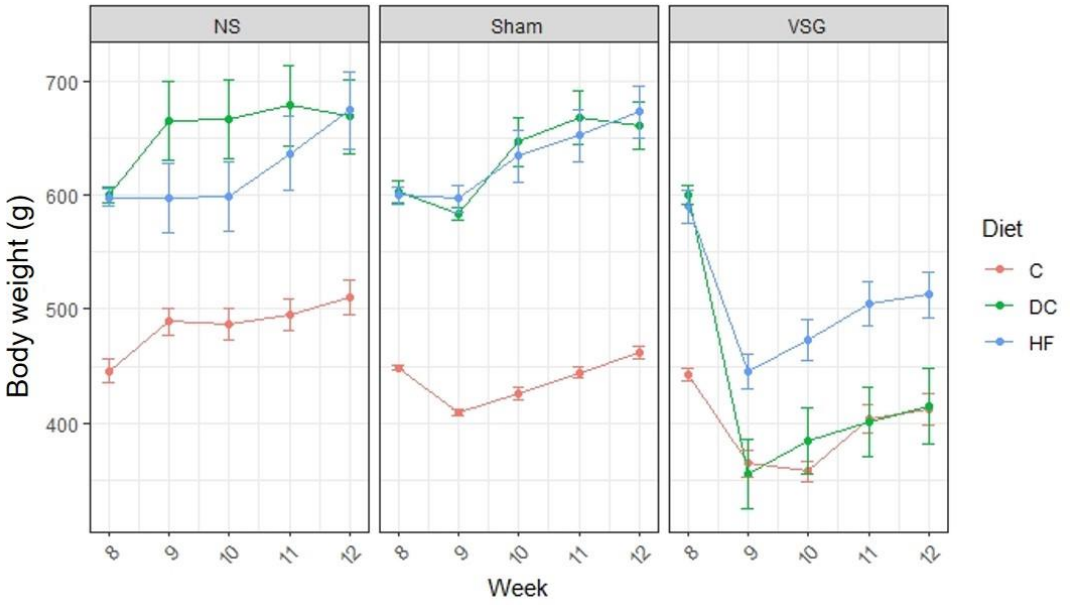

Figure 12. Post-surgery body weight. Evolution of the body weight after surgery, separated by surgical option: No surgery (NS), simulated surgery (sham) or vertical sleeve gastrectomy (VSG). Color lines represent the different diet groups: control (C), diet change to chow diet (DC), and high fat (HF)

After **VSG** is when we have the most pronounced weight decreases in our study. All groups suffered big reductions in weight, being DC-VSG the group that lost the most. After the 9th week, HF-VSG and DC-VSG started to regain weight, although none of the three groups catch up the pre-surgery weight. Besides leading to a higher weight loss, the change of diet also contributed to a lower BWG, compared to HF-VSG.

4.2.2. *EFFECT ON ORGAN WEIGHT*

Besides affecting the whole body weight, the HFD, the diet change, and the surgical intervention also affected the weight of some organs (**figure 13**). As described in the first article, (**table 2, article 1**), the HFD increased the amount of WAT, mostly the lumbar but also the epididymal depot, the liver, the kidney, and also the heart. There was no difference in organ weight between HF-NS and DC-NS. No differences were neither observed between sham and their respective NS groups, except for DC-sham, where kidneys weighted less than in DC-NS, (3.3±0.2 vs 3.3±0.1). After VSG, the size of WAT was heavily reduced, especially

in DC which reduced from 38.4 g in DC-NS to 5.5 g. The liver, the size of which was increased in the groups that were fed a HFD, was also reduced after VSG in HF and DC

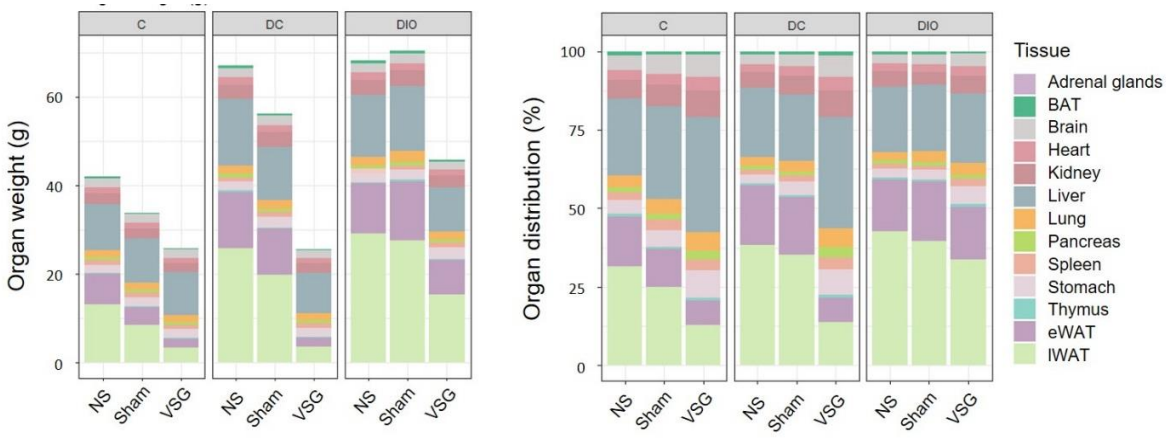

Figure 13. Organ weight expressed as the mean (g), or as relative percentage (%), per group at week 12. AG stands for adrenal glands, BAT for brown adipose tissue, eWAT for epididymal white adipose tissue, lWAT for lumbar white adipose tissue.

groups. We did not record any difference in the stomach weight in the VSG groups compared to either sham or NS.

Besides increasing in weight, the liver of the HF rats had a higher amount of fat (2.8% in HF-NS, compared to 0.94 in DC-NS), an increase reflected in the higher amount of hepatic TG (**table 2, article 3**). Interestingly, hepatic fat was not affected by surgery but only by diet. The change of diet in DC reduced the amount of hepatic fat but remained still higher than in C groups. On the other hand, the amount of TG was reduced to C levels with the change of diet.

4.3. EFFECT ON BIOCHEMICAL PARAMETERS

The use of a HFD in animal models is known to be a useful and effective method to induce obesity and also the associated metabolic alterations such as dyslipidemias and IR.

4.3.1. *LIPID PROFILE*

We analyzed the plasma lipid profile to measure the changes induced by the HFD and how VSG modified them. We did not observe any of these alterations that indicate the apparition of dyslipidemia (**table 2, article 3**), despite the higher body weight and higher WAT weight in HF-NS and DC-NS. There were no significant differences in total cholesterol (**Chol**), HDL, LDL, and NEFA between C-NS, HF-NS, or DC-NS. Regarding TG, levels dropped during the feeding with a HFD, and increased with the change of diet towards the levels of C-NS. The VSG increased levels of C and LDL in rats fed with chow diet (C-VSG and DC-VSG) but did not in HFD-fed animals.

4.3.2. *GLUCOSE HOMEOSTASIS*

We also measured glucose and several parameters related to glucose homeostasis (**figure 2-b, article 2**, and **table 2, article 3**). We did not observe any differences between groups in glucose values, but we found increased values of glucagon in HF groups, as well as a tendency to higher insulin levels in HF groups too. Insulin levels remained stable in HF-VSG, but when surgery (and also sham) was combined with the diet change, it heavily reduced its levels. In fact, insulin levels in DC-sham or DC-VSG were lower than in C groups, even though the differences were not significant. This decrease in insulin, without altering the glucose levels, led to a higher insulin sensitivity index (**ISI**) in DC-sham and DC-VSG, and also tended to be higher in C-sham and C-VSG. A similar pattern for the homeostatic model assessment for IR (**HOMA-IR**), and for β cell (**HOMA-β**).

4.3.3. *HORMONES*

The hormones leptin and ghrelin were also measured in this study (**figure2-b, article 2**). Leptin tended to increase in groups fed a HFD, without being significantly different from C-NS. Neither sham nor the change of diet alone affected leptin levels. On the other hand, VSG reduced leptin levels, but only in the groups that were currently fed with a chow diet. Unacylated ghrelin tended to be lower in the HFD-fed groups, but only as long as the diet continued. The change to a chow diet increased ghrelin values to control levels.

Leptin positively correlated with the amount of fat (sum of the epidydimal and lumbar WAT)

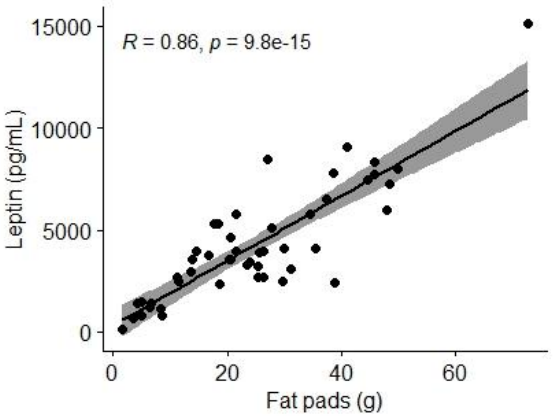

Figure 14. Pearson correlation between leptin and amount of adipose tissue

4.4. EFFECT ON THE GUT MICROBIOTA

The GM community was heavily affected by the administration of the HFD and by the different surgical variables.

4.4.1. *DIVERSITY*

The HF groups had reduced alpha-diversity (**figure 3-a, article 2**), which translates in a reduction of the number of species found in the gut. The change of diet did not improve the alpha-diversity and remained low in DC-NS. The VSG, but also sham, contributed to further lower the alpha-diversity in HF groups, but also in C. Interestingly, when surgery (either VSG or sham) was combined with the change of diet it increased the richness of species living in the gut, restoring the richness to C-NS levels. Regarding beta-diversity (**figure 3-b, article 2**), the diversity of species compared to other samples, or how similar samples are between them, the HF groups were clustered together despite undergoing surgery. The DC groups behaved similarly, with a more widespread pattern, but clustered together, overlapping some of the area corresponding to the C groups.

4.4.2. *TAXONOMY*

We have also observed changes in the taxonomy in our study, with the HF groups having a higher portion of the *Bacteroidetes* phylum, dominated by the *Bacteroidaceae* family, while the *Bacteroidales S24-7* was heavily reduced (**figure 4, article 2**). We can see that several families do not longer appear in the HF-sham and HF-VSG, in accordance with what was shown in the diversity plots. On the other hand, the group DC-NS, which continued having a low richness diversity, seems to have restored some of the taxonomical variances at the family level. Not only that, but the phylum *Verrucomicrobia,* scarcely present in the C groups, appears here with approximately 20% of the relative abundance. This detail is interesting and shows how complex the interpretation of GM data can be, as an increased number of families can still translate into a reduced number of species.

4.4.3. *SHORT CHAIN FATTY ACID ANALYSIS*

Circulating SCFAs were measured in plasma samples by gas chromatography combined with mass spectrometry (**GC-MS**), following the same methodology as for the analysis of FA as in article 3. Circulating SCFAs (acetic, butyric, and propionic) were affected by the combination of diet and surgery (**figure 14**).

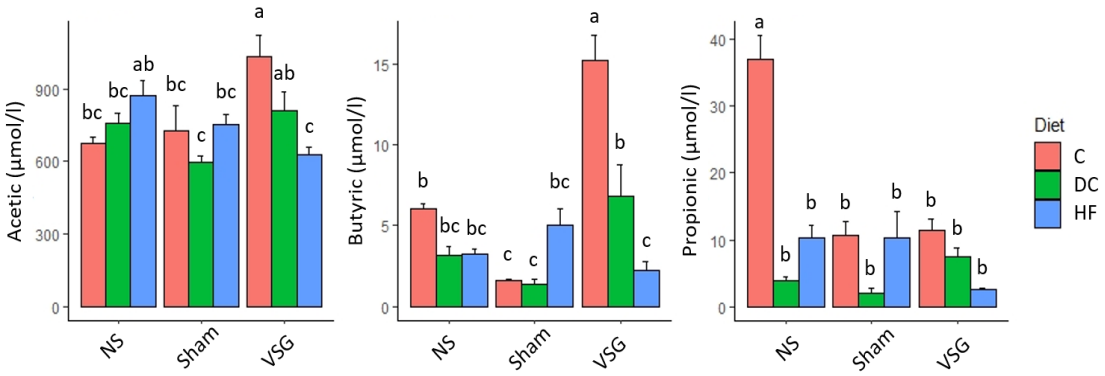

Figure 15. Circulating levels of acetic, butyric and propionic. Surgical groups: No surgery (NS), simulated surgery (sham) or vertical sleeve gastrectomy (VSG). Color groups bars represent the different diet groups: control (C), diet change to chow diet (DC), and high fat (HF). Data are expressed as means ± SEM. Data were analyzed by a two-way ANOVA and Tukey's test was performed for all the groups in which the Surgery:Diet (S:D) interaction was significant. Different letters indicate significantly different means ($p < 0.05$) from other means in the plot, while common letters imply no differences between means.

Acetic content was the highest of the three measured SCFA. No differences were seen between sham and NS. In general, values tended to increase with the HF, but decreased when combined with VSG in HF-VSG. On the other hand, combining VSG with standard chow tended to increase acetic concentration. Butyric tended to reduce with the sham surgery but increased in C-VSG. Propionic was increased after surgery in C-VSG, and DC-VSG also tended to increase. Propionic was reduced in all groups, compared to C-NS.

The values of SCFA were correlated with the biochemical parameters and also with the liver estimated enzymatic activities and the different FA (**figure 15**). Acetic and butyric positively correlated with plasmatic lipid profile, except for TAG, while propionic correlated negatively with glucagon. Regarding liver results, acetic and butyric correlated positively with DNL

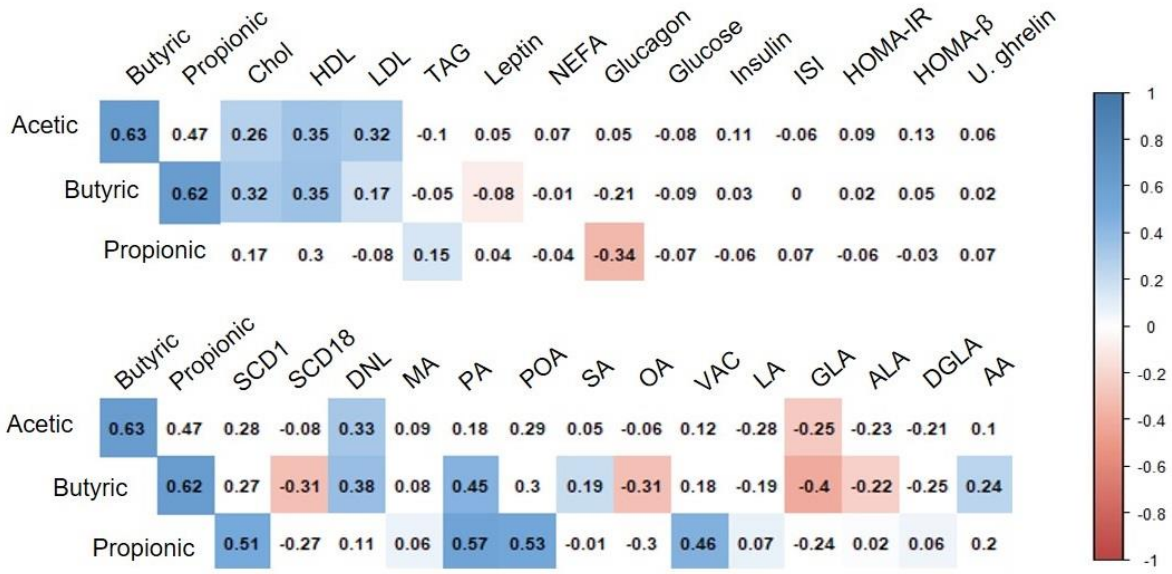

Figure 16. Correlogram showing Spearman correlation coefficient values between SCFA and biochemical parameters (upper) and estimated liver enzyme activity and FA (lower). Non-significant results ($p<0.05$) are shown in white.

4.5. EFFECT ON FATTY ACID COMPOSITION

Due to the use of a HFD to induce obesity, and the accumulation of fat in the liver of obese subjects and animals, we analyzed the FA composition of three organs: the liver, for its role as a metabolic organ, the WAT, for its role as energy storage and a place where fat is

ectopically accumulated during obesity, and the brain, for being an organ with a lipidic composition. An added interest in analyzing the FA composition is the few studies published about it and the difficulty to do such analyses in humans. Regarding the FA composition data, the article with the liver data is under review, and the data of WAT and brain are in the article writing process.

4.5.1. *FATTY ACID PROFILE IN LIVER TISSUE*

Besides increasing in fat content, there were several changes in the FA composition of the liver (**table 3, article 3**). While the SFA decreased when animals were fed a HFD, and increased when animals underwent VSG, the MUFA portion showed an inverse pattern. When looked in detail, the MUFA oleic acid (**OA**) increased in the HF groups, decreasing in HF-VSG. The change to the chow diet also contributed to a further decrease, so that DC-VSG had no differences in OA when compared to C groups. As for PUFA, the proportions decreased with the HFD, and had a partial recovery with the change to chow diet. The HFD also decreased the activity of stearoyl-CoA desaturase 1 (**SCD1**), estimated as a product-to-precursor ratio.

The hepatic OA content positively correlated with the amount of hepatic fat.

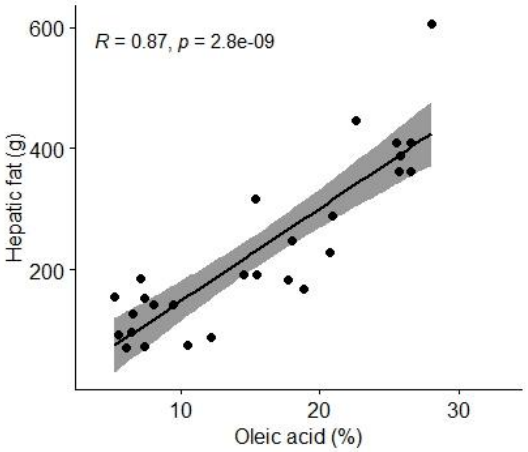

Figure 17 Pearson correlation between hepatic fat and oleic acid liver content

4.5.2. *FATTY ACID PROFILE IN WHITE ADIPOSE TISSUE*

The analysis of visceral WAT FA profile was performed with the same methodology as the analysis of the FA profile in the liver, specified in article 3.

The different surgical options, the ingestion of a HFD, and the change back to a standard chow modified several of the FAs forming the WAT (**table 2**). Sham did affect the profile of several FAs, but not as much as VSG. The proportion of SFA increased in the HF groups, especially due to higher palmitic acid (**PA**) and stearic acid (**SA**) proportions, but decreased with the change of diet in DC-NS. The proportion of the saturated SA remained higher with the change to control diet, surgery, or the combination of both. The MUFA proportion was mainly affected by the changes in the OA profile, which heavily increased by the HFD. The change to control diet in DC reduced its levels, especially when combined with surgery in DC-VSG, without reaching the values for C groups. Palmitoleic acid (**POA**) showed an inverse pattern, decreasing with the ingestion of a HFD and increasing after VSG in the C and DC groups. Concerning PUFA, we observed an inverse pattern again. Proportions of PUFA, both n3, and n6, decreased with the HFD, and increased with the change of diet, remaining lower than C-NS. When combined with surgery, DC-VSG tended to increase, while C-VSG decreased. Looking into detail, the levels of linoleic acid (**LA**) and gamma-linoleic acid (**GLA**) decreased with the HFD. The LA increased with the change of diet, but without reaching the C levels, although no effect was seen in GLA. Surgery increased the LA and GLA values in both HF and DC, but had the opposite effect on C, decreasing the content.

4.5.3. *FATTY ACID PROFILE IN BRAIN TISSUE*

The analysis of visceral brain FA was performed with the same methodology as the analysis of the FA in the liver, specified in article 3.

The FA composition in brain samples was scarcely affected by either diet or surgery, and most of the profiles remained stable (**table 3**), no differences were seen between NS and respective sham groups. The SFA proportion, despite being affected by the different diets, showed no differences between groups. In detail, the saturated myristic acid (**MA**) increased

with the HFD, but was normalized with either with surgery, change of diet, or both combined. Regarding MUFA, only surgery had some effect, like in POA, where VSG tended to increase its values (except in DC-VSG). A similar pattern was observed for PUFA, with a tendency of lower proportions in VSG groups. On the other hand, the essential LA increased with VSG, as well as n6 docosapentaenoic acid (**DPA n6**) who showed a similar tendency.

Table 2. Visceral WAT fatty acid profiles and ratios

WAT	2way ANOVA			C			HF			DC		
	Surgery NS/S/VSG	Diet C/HF/DC	S:D	NS	Sham	VSG	NS	Sham	VSG	NS	Sham	VSG
SFA	***	***	***	25.9±0.3d	24.09±0.2e	29.31±0.2bc	31.36±0.3a	31.35±0.4a	32.4±0.2a	29.42±0.1bc	29.07±0.3c	31.19±0.9ab
MA	***	*	***	1.5±0.07b	1.3±0.07bc	2±0.05a	1.4±0.04bc	1.4±0.01bc	1.2±0.03c	1.3±0.05bc	1.3±0.02c	1.6±0.01b
PA	***	***	***	20.98±0.31bc	19.7±0.18c	22.27±0.26ab	22.93±0.39a	23.17±0.24a	22.23±0.14ab	20.83±0.34bc	20.22±0.12c	23.53±1a
SA	**	***	***	3.4±0.14de	3.1±0.36e	5±0.42cd	7±0.35bc	6.8±0.47bc	9±0.19a	7.3±0.35ab	7.6±0.42ab	6.1±0.11bc
MUFA	**	***	***	33.8±0.7ef	32.1±0.4f	35.4±0.4e	49.3±0.3a	49.5±0.4a	43.3±0.1c	45.3±1.2bc	46.5±0.5b	40.2±0.1d
POA	***	***	***	4.4±0.37b	3.1±0.13c	6.1±0.14a	2.1±0.12d	2.1±0.15d	1.5±0.1d	2.3±0.26cd	1.9±0.11d	4.5±0.53b
OA	ns	***	***	25.15±0.22e	25.02±0.4e	25.27±0.12e	43.04±0.18a	42.78±0.26a	37.88±0.17c	39.24±0.84bc	40.5±0.43b	32.02±0.78d
VAC	ns	ns	ns	4.3±0.2	4±0.12	4±0.27	4.2±0.09	4.7±0.22	3.9±0.1	3.8±0.18	4.1±0.12	3.6±0.13
PUFA	***	***	***	38.5±0.9b	41.5±0.2a	32.9±0.1c	18.3±0.3f	18.1±0.3f	23.3±0.3e	24±1.1de	23.3±0.5e	27.5±1d
n3	***	***	***	2.17±0.1a	2.34±0.05a	1.71±0.05b	0.42±0.02de	0.39±0.02e	0.47±0.03de	0.71±0.07cd	0.65±0.03cd	0.86±0.21c
ALA	***	***	***	2.1±0.09a	2.2±0.04a	1.6±0.05b	0.4±0.02ef	0.4±0.02f	0.5±0.03def	0.7±0.07cd	0.6±0.03cde	0.9±0.21c
DHA	ns	ns	ns	0±0	0.1±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
DPAn3	ns	ns	ns	0.04±0.01	0.06±0.01	0.03±0.01	0±0	0±0	0±0	0±0	0±0	0±0
n6	***	***	***	36.3±0.8b	39.1±0.2a	31.2±0c	17.9±0.3f	17.7±0.2e	22.8±0.3f	23.3±1.1e	22.6±0.4e	26.7±0.8e
LA	***	***	***	35.7±0.74b	38.3±0.27a	30.5±0.09c	17.7±0.29f	17.4±0.26f	22.6±0.24e	23.1±1.04e	22.4±0.43e	26.4±0.8d
GLA	*	*	*	0.08±0.01ab	0.12±0.01a	0.14±0.05a	0.06±0.01bc	0.05±0.01bc	0.05±0bc	0.05±0bc	0.05±0c	0.07±0abc
DGLA	** a/a/a	*** a/b/c	ns	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0±0.01	0±0	0±0	0±0
AA	***	***	*	0.4±0.05b	0.6±0.03a	0.5±0.02ab	0.1±0.01c	0.1±0.03c	0.1±0.02c	0.1±0.02c	0.1±0.03c	0.2±0.02c
DPAn6	***	***	*	0.04±0.01b	0.06±0a	0.04±0b	0±0-	0±0-	0±0-	0±0-	0±0-	0±0-
n6/n3	ns	*** c/a/b	ns	17±0.48	17±0.39	18.9±0.62	43.1±1.42	47.3±2.5	49.8±3.5	33.4±1.78	35.3±1.16	33±7.18
SFA/MUFA	***	***	*	0.7±0.01ab	0.7±0.01bc	0.8±0.02a	0.6±0.01c	0.6±0.01c	0.7±0.01b	0.6±0.02c	0.6±0.01c	0.8±0.02ab
SFA/PUFA	***	***	***	0.68±0.02e	0.58±0e	0.88±0.01d	1.72±0.04a	1.74±0.04a	1.39±0.02b	1.24±0.06bc	1.26±0.03bc	1.14±0.07c
PUFA/MUFA	***	***	***	1.11±0.04b	1.24±0.02a	0.92±0.02c	0.37±0.01f	0.36±0.01f	0.53±0.01e	0.52±0.04e	0.49±0.01e	0.67±0.02d

Data are expressed in molar percentage (%) as means ± SEM. Data were analyzed by a two-way ANOVA (anova-2). *P < 0.05; **P < 0.01; ***P < 0.001. Tukey's test was performed for all the groups in which the Surgery:Diet (S:D) interaction was significant or for the main factors (surgery or diet) in which the factors were significant but their interaction was not. Group means with different letters are significantly different (p < 0.05) from other means in the same row, while common letters imply no differences between means. NS, no surgery; S, simulated surgery; VSG, vertical sleeve gastrectomy; C, control; HF, high-fat; DC, diet change, HFD in the first 8 weeks before switching to the control diet in the last 4 weeks. AA, arachidonic acid; ALA, alpha-linolenic acid; DGLA, dihomo-gamma-linolenic acid; DHA, Docosahexaenoic acid; DPA, docosapentaenoic acid; GLA, gamma-linoleic acid; LA, linoleic acid; MA, myristic acid; MUFA, monounsaturated fatty acid; OA, oleic acid; PA, palmitic acid; POA, palmitoleic acid; PUFA, polyunsaturated fatty acid; SA, stearic acid; SFA, saturated fatty acid; UFA, unsaturated fatty acid; VAC, vaccenic acid

Table 3. Brain fatty acid profiles and ratios

Brain	2way ANOVA			C			HF			DC		
	Surgery NS/S/VSG	Diet C/HF/DC	S:D	NS	Sham	VSG	NS	Sham	VSG	NS	Sham	VSG
SFA	ns	* a/a/a	ns	49.1±0.5	48.8±0.2	47.6±0.5	47.6±0.5	48.7±0.2	48±0.4	47.7±0.4	48±0.6	48.6±0.2
MA	*	**	***	0.25±0.02cd	0.23±0.01d	0.27±0abcd	0.3±0.01ab	0.31±0.01a	0.27±0.01abcd	0.28±0.01abcd	0.28±0.01abc	0.26±0abcd
PA	*	*	*	23.2±0.44a	22.9±0.27a	22.1±0.3a	22.2±0.27a	22.9±0.14a	22.5±0.29a	22.3±0.21a	22.4±0.29a	22.8±0.13a
SA	ns	ns	ns	25.6±0.12	25.6±0.09	25.2±0.21	25.1±0.25	25.4±0.18	25.2±0.13	25.1±0.2	25.3±0.33	25.5±0.11
MUFA	** a/a/a	ns	ns	22.1±0.3	21.3±0.3	23.2±0.4	22.7±0.4	21.7±0.1	22.4±0.4	23±0.4	22.8±0.5	22.6±0.2
POA	*	*	*	0.48±0.01ab	0.43±0.01ab	0.5±0.02a	0.43±0.01ab	0.45±0.04ab	0.43±0.01b	0.49±0.01ab	0.43±0.01ab	0.47±0.01ab
OA	** a/a/a	ns	ns	17.8±0.28	17.2±0.19	18.8±0.34	18.6±0.33	17.8±0.16	18.4±0.37	18.7±0.33	18.7±0.37	18.4±0.17
VAC	ns	ns	ns	3.9±0.03	3.6±0.09	3.9±0.07	3.7±0.04	3.5±0.04	3.6±0.05	3.8±0.08	3.7±0.12	3.7±0.06
PUFA	** ab/a/b	ns	ns	23.1±0.3	24.1±0.2	22.8±0.2	23.5±0.2	23.9±0.3	23.5±0.3	23.2±0.3	23.1±0.3	22.8±0.1
n3	ns	ns	ns	11.5±0.3	11.9±0.2	11.1±0.2	12±0.2	12±0.4	11.9±0.2	12.1±0.2	11.6±0.3	10.9±0.1
DHA	ns	ns	ns	11.4±0.33	11.8±0.21	11±0.24	11.9±0.16	11.9±0.37	11.8±0.19	12±0.18	11.6±0.27	10.8±0.11
DPAn3	ns	ns	ns	0.08±0.01	0.07±0.01	0.08±0.01	0.07±0	0.08±0	0.08±0.01	0.08±0.01	0.07±0	0.07±0
n6	ns	ns	ns	11.6±0.2	12.3±0.3	11.8±0.2	11.5±0.1	11.9±0.1	11.6±0.3	11.1±0.2	11.5±0.2	11.9±0.1
LA	ns	ns	*	0.71±0.02b	0.7±0.03b	0.74±0.04ab	0.68±0.01b	0.68±0.04b	0.9±0.07a	0.72±0.02b	0.69±0.03b	0.71±0.04b
DGLA	ns	ns	ns	0.18±0.01	0.16±0.01	0.18±0.01	0.17±0.01	0.18±0.01	0.18±0.01	0.2±0.02	0.17±0.01	0.18±0.01
AA	ns	ns	ns	10±0.17	10.4±0.35	10±0.17	9.9±0.08	10.3±0.15	9.6±0.19	9.7±0.15	9.9±0.19	10.1±0.09
DPAn6	ns	**	**	0.8±0.05a	0.94±0.05a	0.81±0.06a	0.82±0.08a	0.75±0.04ab	0.87±0.08a	0.52±0.04b	0.71±0.04ab	0.94±0.08a
n6/n3	ns	ns	ns	1.2±0.04	1.3±0.05	1.3±0.04	1.2±0.02	1.2±0.05	1.2±0.04	1.1±0.03	1.2±0.04	1.3±0.01
SFA/MUFA	* a/a/a	ns	ns	2±0.05	2.1±0.04	1.9±0.06	1.9±0.06	2.1±0.02	2±0.05	1.9±0.05	1.9±0.07	2±0.03
SFA/PUFA	ns	* a/a/a	ns	1.96±0.05	1.86±0.02	1.91±0.01	1.86±0.01	1.87±0.02	1.89±0.02	1.9±0.02	1.91±0.03	1.96±0.02
PUFA/MUFA	* ab/a/b	ns	ns	1.04±0.03	1.13±0.03	0.98±0.03	1.02±0.03	1.1±0.01	1.03±0.03	0.99±0.03	1.01±0.04	1±0.01
AA/DHA	ns	ns	ns	0.88±0.03	0.89±0.04	0.92±0.03	0.83±0.02	0.87±0.04	0.82±0.03	0.81±0.02	0.86±0.03	0.93±0.01

Data are expressed in molar percentage (%) as means ± SEM. Data were analyzed by a two-way ANOVA (anova-2). *P < 0.05; **P < 0.01; ***P < 0.001. Tukey's test was performed for all the groups in which the Surgery:Diet (S:D) interaction was significant or for the main factors (surgery or diet) in which the factors were significant but their interaction was not. Group means with different letters are significantly different (p < 0.05) from other means in the same row, while common letters imply no differences between means. NS, no surgery; S, simulated surgery; VSG, vertical sleeve gastrectomy; C, control; HF, high-fat; DC, diet change, HFD in the first 8 weeks before switching to the control diet in the last 4 weeks. AA, arachidonic acid; ALA, alpha-linolenic acid; DGLA, dihomo-gamma-linolenic acid; DHA, Docosahexaenoic acid; DPA, docosapentaenoic acid; GLA, gamma-linoleic acid; LA, linoleic acid; MA, myristic acid; MUFA, monounsaturated fatty acid; OA, oleic acid; PA, palmitic acid; POA, palmitoleic acid; PUFA, polyunsaturated fatty acid; SA, stearic acid; SFA, saturated fatty acid; UFA, unsaturated fatty acid; VAC, vaccenic acid

5. Discussion

Obesity, as well as other diseases, is a complex situation that can be influenced by multiple variables, which difficult the study in humans, and makes the use of animal models a valuable complementary tool. As we mentioned in the introduction (1.3. Rodents as an animal model for obesity), the study of obesity has benefited from the use of animal models to whom obesity is induced, either genetically or dietary, in a controlled environment. The induction of obesity through diet, in the DIO models, has allowed the scientists to observe not only the final stages where obesity is established, but the progression from a normoweight to the different stages of overweight and obesity, investigating the physiological evolution of the process.

Inducing obesity

In this study, we successfully induced obesity after the administration of a HFD. The group fed a HFD had a higher calorie intake that was maintained during the 8 weeks pre-surgery, but also during the 4 following weeks in the NS groups. The recorded intake was stable, in both chow and HFD groups. We did not observe an initial higher intake in the first weeks of a HFD, described in other studies(35,142), which normalizes after some time. Apparently, rats find an improved palatability in the HFD, and thus increase the intake until they become used to the new taste (92). As a consequence of the elevated calorie content, the BW of the HFD-fed group was 32% higher than the chow-fed group (591 ± 16.1 g, compared to 447 ± 11.3 g) at week 8. The observed weight difference between groups is similar or higher than other studies also inducing obesity with a HFD (94,98,143) The bodyweight of C-NS and HF-NS groups (no surgery, nor change of diet) continued unaltered until the end of the study and were used as controls. As expected, the adipose tissue of the HF-NS, i.e. estimated as the sum of the eWAT and lWAT, was increased and represented almost 50% of the total organ weight. The liver, which also increased weight, had a greater content of fat (almost 3 times more than the C-NS) indicating the possible apparition of hepatic steatosis, a common comorbidity in obesity (59,144,145). The higher fat content also affected the physical

appearance of the liver, with the HF-NS liver having a paler coloration compared to the one from C-NS (**figure 18**).

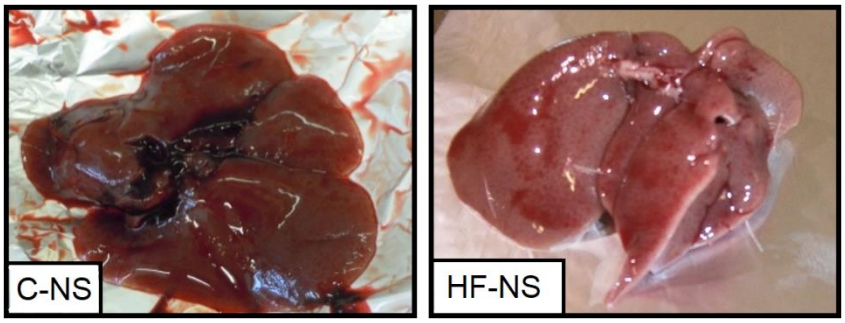

Figure 18. Appearance of a C-NS liver and a HF-NS liver.

After the 12 weeks of diet, the HF-NS group did not show alterations on the circulating lipid pool that could indicate the apparition of dyslipidemia, as other groups described (146). Not only that, but the continued HFD led to a decrease in TG in HF-NS, which we found surprising and contradicts the studies that associate a HFD with hypertriglyceridemia (143,144). Nevertheless, a decrease in TG after a HFD was already described (145,147,148), and it was associated with a reduction in hepatic TG secretion (147). This reduction seems to be a side effect of the stress that a HFD exerts on the endoplasmic reticulum, which in turn limits the secretion of apolipoprotein B100, necessary for the formation of VLDL. Others have observed the apparition of dyslipidemia only when the HFD was supplemented with Chol (148).

The apparition of alterations in the glucose metabolism is also associated with both obesity and the use of a HFD in animal studies. In our case, we did not observe any differences in glucose levels between C-NS and HF-NS, but we observed a tendency to increased levels of insulin, and a worsened HOMA-IR and HOMA-β in the HFD-fed group. Taking those parameters together, we can speculate that there was a tendency toward developing IR, a previous stage to become diabetic, in the HFD-fed animals, showing a worsened glucose homeostasis. Previous studies have found similar results, with no alterations in glucose values, but altered insulin and HOMA-IR (142). Other authors have found glucose alterations with the use of a HFD, although the altered glucose parameters are more related to diets higher in sugar, CAF diets, or HFHS diets (76).

The use of a HFD is not exempt from debate. The HFD is one of the most used diets in research studies about obesity and related comorbidities as dyslipidemias (146,149–151), NAFLD (59,144,152), as well as for GM modifications (127,138,153,154), but it has several

drawbacks. The HFD lacks the variety and heterogeneity present in the human western diets, and the elevated high-fat content is not representative enough of the human dietary habits, with a maximum of 30% of the total ingested calories coming from fat, together with the lack of refined sugars (35). Other diets have been developed and are a feasible alternative, such as the CAF, another DIO diet which consists of supplementing a standard food with human snacks, high in sugar and fat, and low in fiber and micronutrients, making it more representative to the human dietary habits (91). But despite the benefits of resembling the WD diet, the snacks used in different CAF diets may vary, altering the diet composition between experiments; and rodents may show preferences over a type of snack than another, creating intake differences in the same experiment. The lack of standardization makes it difficult to reproduce (35). Also, snack supplementation may lead to nutritional deficiencies, due to the lower nutritional concentration, while adding additives such as preservatives that may interfere with the experimental outcome (92). There are currently new diets that are more representative of the human WD, that are standardized, and that may offer a more appropriate diet than the HFD used in this study (92,155). Besides the different views on the HFD, another factor we did not take into account was the choice of chow diet, as some advice the use of refined low-fat diets as CD, instead of standard controls chows that are less standardized and may add more variability than the lower fat content ones (156).

Tackling obesity

Two actions were taken in this study in order to tackle obesity: the performance of a dietary intervention (DC-groups), i.e. changing from a HFD to a chow diet; and a VSG (VSG groups). These two actions were combined in the group DC-VSG, which aimed to simulate a behavioral change, after VSG.

Changing diet had little impact on the amount of ingested food, and we only observed a modest increase in the 11th and 12th weeks in all DC groups. Both VSG and sham groups lowered the intake in the first-week post-surgery, much of it due to the food-restriction prior to surgery (and after surgery in the VSG groups). Surprisingly, VSG and sham groups showed a rapid increase in their intake which contributed to the recovery of the pre-surgery intake already after two weeks, similarly to what is described (157). Other groups have registered a maintained lower intake for several weeks (98,158). It is described that rats try to compensate fasting situations, and that VSG rats have the capacity to increase meal frequency due to the

physical restriction of the reduced stomach (95,99). In our study, we measured the food consumption every two days, and thus we did not take into account the feeding pattern. It could be plausible that rats were compensating the previous fasting situation by increasing their feeding frequency, as we did not observe any dilatation in the stomachs of VSG-rats as others have (94,157). Despite the recovered intake, all VSG groups tended to eat less calories compared to the pre-surgery intake at week 8.

The change to chow diet did not reduce BW, although it did reduce the growth rate, leading to a trend towards BW stabilization for DC-NS after the 9th week. On the other hand, all VSG groups had a clear reduction in BW, as was expected. We observed a generalized reduction in the first week, with DC-VSG being the one losing the most, with a reduction of more than 20% of the pre-surgery weight. Other authors recorded the maximal weight loss in the second week which is probably due to the maintained lower intake (98). In our study, rats started to regain weight from the second week on, but at the end of the study the BW continued below the pre-surgery weight. In other studies, rats started regaining weight after the first week (159) and even recovered the pre-surgery weight around the third week (96,160). It is important to mention that rats keep gaining weight during their whole adult life. A rat that is not gaining weight, or gaining less than the habitual weight is also regarded as a weight loss, and thus the desired effect of the VSG (95).

The observed weight reduction in VSG groups was mainly distributed in the adipose tissue, as earlier described (99). Some studies observe an adipose redistribution after VSG, with more subcutaneous adiposity and reduced visceral and lumbar fat (159). Even though we also took samples of visceral and subcutaneous WATs, we only weighted the epidydimal and lumbar fat depots, making it impossible to assess if there also was a redistribution in our study. The liver, which is an organ typically affected during obesity, and in which VSG is known to improve its condition (152), normalized its weight after VSG in both HF and DC groups. On the other side, the hepatic fat content was only affected by diet. The VSG intervention had no effect on hepatic fat content when the HFD continued. Although not significant, there was a tendency to a higher decrease when the change of diet was combined with VSG. Taking into consideration that we performed an almost 80% resection of the stomach, we expected that the remaining pouch of VSG animals would weigh less than their NS counterparts, but we could not find any significant differences. After VSG, the stomach might have been dilated (94,157) but it could also be due to the formation of scarring tissue and adherences, visible upon post-mortem visual examination of the stomachs (data not

shown). On the other hand, in 2010, Frühbeck and collaborators already described that both sham and VSG operated obese Zucker rats showed cardiac hypertrophy, as well as an improvement in blood pressure, although it was not clear if both data were related (161). We observed the inverse result, with lesser weight after VSG in HF and DC.

The diet change did not have much effect on the altered glucose metabolism, nor did VSG in the groups that continued with the HFD, as described in previous studies (158,162). In a study investigating the diabetes recurrence after BS it was observed that continuing a HFD reversed the improvements after VSG, due to re-impaired insulin sensitivity (163). On the other hand, we observed a drastic improvement in the group that combined VSG together with a change to chow diet. Surprisingly, the improvement was also seen in the sham group, which indicated that the stomach resection was not responsible for this improvement. The improvement of glucose metabolism after VSG is thought to be due to several factors such as calorie restriction and hormone regulation, with ghrelin as one of the involved hormones (62). We were surprised to not observe any difference in ghrelin levels between VSG and NS groups, as ghrelin is described to be decreased after VSG (25,29,158). Ghrelin production is often attributed mostly to the stomach, but it has also been described to take place in the intestine and the WAT, which may compensate for the decreased stomach production after VSG (33). Leptin, increased in the groups fed with a HFD even after the diet change, strongly correlated with the adipose tissue mass (**figure 14**), as already described (164).

Modulating the gut microbiota

As already described, the prolonged feeding with a HFD led to substantial changes, in diversity and composition, in the GM, leading to the characteristic dysbiosis (127,137,138,153,165). It is now clear that the "obese phenotype" seen in animal and human studies rely more on factors like diet rather than on obesity itself (134,137,138). In our case, we could observe some minor positive effects in the DC-NS group, such as improved beta diversity and taxonomical shifts, even though the BW was similar to HF-NS. On the other hand, no improvements were seen in HF-VSG, despite having a lower BW than HF-NS. Our data support the fact that GM alterations are mostly related to diet, and not to body weight itself. Interestingly, we did observe that in combination with VSG (or with sham) the diet change increased the richness of species living in the gut, restoring the alpha diversity to C-NS levels. This might be due to the use of a non-refined chow diet that may add a higher

content of fiber which in turn benefits the microbiota wellbeing (156) especially when it is combined with VSG or sham. Both surgery types share in common the administration of enrofloxacin as prophylactic antibiotic treatment during the pre and the post-operative care. The effect that antibiotic administration on the GM community was not taken enough into account at the beginning of the study. We would have benefited from including a NS group exposed to the same conditions as Sham and VSG groups (i.e. antibiotic, analgesic, and fasting), even though some authors describe a GM normalization after 7 days of antibiotic administration (Vaughn 2017). This extra group would have provided useful information regarding the effect of the antibiotic *per se*, without the added surgical stress, as seen in other studies (134). Besides the deleterious effect, the antibiotic administration had a positive outcome when combined with the change of diet, as mentioned above. It might be that the antibiotic treatment prior to surgery, and the consequent wipe-out of species, was necessary in order to facilitate bacterial recolonization and recover some GM community lost after the HFD (166–168).

One of the main products of GM are the SCFAs, whose role in obesity is still discussed. The SCFAs are described to be increased during obesity (33,41,169), but are also linked to beneficial health effects such as improved glucose homeostasis and appetite regulation (130,170). Many studies focus on fecal SCFA concentration, but we choose to analyze the plasmatic levels of SCFAs instead of fecal levels, as circulating SCFAs are more linked to metabolic markers (130). As expected, acetic was the main circulating SCFA, as butyrate is mainly used by colonocytes as an energy source (109). Our obese groups did not show increased levels of SCFAs, and only acetic had a modest tendency to be increased with the HFD in HF-NS group. On the contrary, we found higher levels in the C groups, with higher concentrations of acetic and butyric in the C-VSG groups, and higher propionic in the C-NS group. As SCFAs are described to influence several hormones and lipogenesis (33,41,102,130,132), we correlated the SCFAs results with our biochemical parameters (**figure 16, upper chart**), and with liver FA and enzymes (**figure 16, lower chart**). Concentrations of acetic and butyric acid positively correlated with plasmatic lipid profile, except TAG and NEFA, which we found interesting as we could not see signs of dyslipidemia. On the other hand, propionic positively correlated with TAG, and negatively with glucagon. We did not find any correlation between the analyzed SCFAs and the hormones leptin, ghrelin, or insulin, as others have found (33,130), but the exact relationship between these parameters is still uncertain (33).

Regarding FA and lipid metabolism, circulating levels of acetic acid are described to correlate with some lipid profile values, DNL, and Chol formation in the liver, while propionic inhibit the lastly mentioned (171,172). Also, several studies point out that SCFAs are used as a substrate for DNL (109,173). We do observe a positive correlation between acetic or propionic and DNL or Chol, even though DNL was unaltered in our study. Regarding propionic, we could not observe any negative correlation with DNL and, on the contrary, we found that it was positively correlated with SCD1, as well as the FAs PA, POA, and VAC. The enzyme SCD1 is closely related to DNL, and PA is one of the major DNL products (174,175).

Although it is a common feature seen in many scientific articles, focusing on specific microbes and attribute determinate roles to them is controversial, as GM is flexible and adapts depending on the external factors (diet, housing, animal strain) (35), as well as many species being functionally redundant with others (41,100,176). Still, two families captured our attention in our study. First, the family *Verrucomicrobiaceae*, with *Akkermansia Mucinphylla* as the only member of this family, that increased with the change of diet in the DC-NS group, representing almost a 20% of the total abundance (**article 2, figure 4**). This increase was not observed when the change of diet was combined with surgery, suggesting that antibiotic administration hindered the rise of this family. *A. Mucinphylla* is considered a beneficial organism, associated with improved glucose metabolism and lower adiposity (177). In our study *A. Mucinphylla* positively correlated with HOMA-IR, but also with adiposity (article 2, figure 5), which may suggest that changing diet alone positively affected the GM composition, without reducing body weight. Second, the family *Bacteroidales S24-7*, which was almost depleted in the HF groups (**article 2, figure 4**). In agreement with our results, reductions of this family are associated with loss of diversity, observed during feeding with a HFD, and even with harmful situations such as dextran sodium sulfate induced colitis (178–180). An increase in *Bacteroidales S24-7* is seen when those situations are either treated, or improved, and is even associated with improvement in NAFLD (178–181). In accordance to what is described, we observed that restored abundances in the DC-VSG, after the combination of change of diet with surgery, but not with the diet change alone, or with the VSG if the HFD were continued.

Fatty acid composition and metabolism

The feeding with a HFD led to several modifications in the FA composition of the liver and visceral WAT (**article 3, table 2; table 3;** and resumed in **figure 19**). One of the biggest modifications was the increase in MUFA, mainly OA, in the HF groups which surprised us at first, as we expected a higher portion of SFA due to the elevated amount of fat in the diet. Nonetheless, the HFD, composed mainly of lard, had 20 times more SFA than the chow diet, but also 20 times more MUFA. The higher MUFA in a lard-based diet might be unexpected at first, but mammalian fats have PA and OA as the main FA components (182,183). The calorie excess derived from diet, either from fat as in our study, or from carbohydrates, is cleared through the formation of TG (31,45,46).

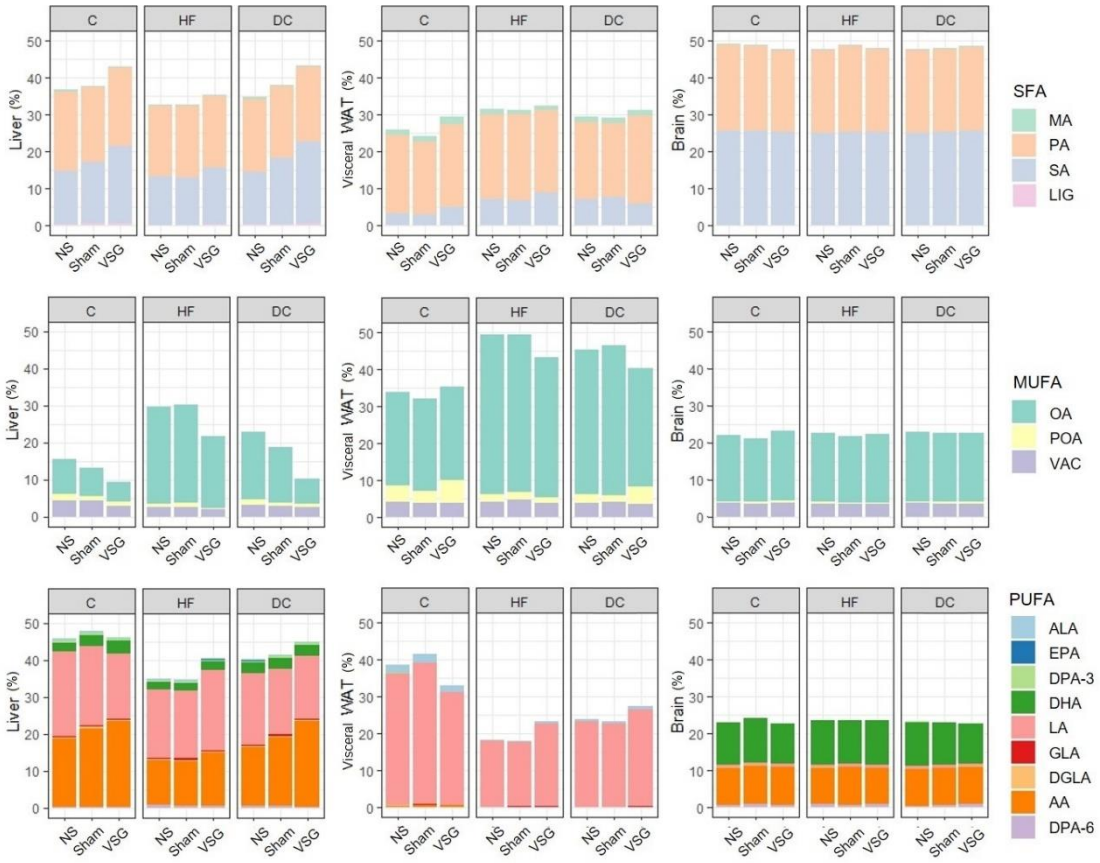

Figure 19 Levels of the FA at the 12th week in Liver, visceral WAT and brain tissue, expressed in molar percentage (%). In saturated fatty acids (SFA): Myristic acid (MA), palmitic acid (PA), and stearic acid (SA). In monounsaturated fatty acids (MUFA): Oleic acid (OA), palmitoleic acid (POA), and vaccinic acid (VAC). In polyunsaturated fatty acids (PUFA), the n3 PUFA α-Linolenic acid (ALA), and the n6 PUFA Linoleic acid (LA), Arachidonic acid (AA, Dihomo-gamma-linolenic acid (DGLA) and Gamma-linolenic acid (GLA)

The enzymes of the stearoyl-CoA desaturases (SCDs) family incorporate a desaturation in the SFA transforming them into lesser deleterious MUFA, such as from SA to OA, which are incorporated into phospholipids, Chol esters, and TG, and finally stored in the AT (21,31). The SCDs are enzymes described to be increased during obesity (184,185), as was SCD18 in our study (**table 4, article 3**). Due to both the increased dietary content, but also the elevated estimated SCD activity, the OA was highly elevated in the HF-NS compared to C-NS, both in the liver or in the visceral WAT tissue. As expected, the amount of hepatic fat positively correlated with the OA content (**figure 17**).

The change of diet and the VSG had a similar effect on the FA composition of both liver and visceral WAT. The content of OA was reduced in both cases, probably as a consequence of the weight lost after VSG in HF-VSG, or due to the reduction of dietary OA intake in the DC-NS. The other FA had modest modifications, several of them were too small to be significant. Changes after RYGB or after diet intervention gave similar modifications in human serum samples, except for n3 PUFAs which decreased with surgery but increased with the dietary intervention (186). Our visceral WAT results agree with those findings as we observed a similar variation in HF-VSG and DC-NS when compared to HF-NS, with decreased MUFA and increased PUFA. In our study, n6 PUFA differed instead of n3, increasing in DC-NS but not in HF-VSG, probably due to the maintenance of the HFD after VSG. Interestingly, we observed a better improvement in the composition of both tissues when the change of diet and VSG were combined. As Walle et al mention in their work with human patients (186), dietary intake of MUFAs and PUFAs were determinant for their respective FA profile, while the SFA profile was more weight loss-related. We benefit from an animal model with a controlled environment and diet, knowing that the modifications observed are due to controlled interventions.

On the other hand, the FA composition of the brain tissue remained mostly stable (**table 3**), as seen in other studies (187). The different FAs have mainly structural roles in the brain. One of the main components of myelin is OA (188), which accounted for approximately 15% of the FA composition in our results. The PUFAs are an essential neuronal cell component, with AA being present in all neuronal cells, and DHA present in neuronal membranes (189) as well as contributor to brain signaling (190). Our results are consistent with the former described, as we quantified OA as the main MUFA, and AA and DHA as the two main PUFA. In general, FAs in the brain remain stable, non-affected by neither diet nor surgery modifications. The composition of membrane phospholipids in the brain is

influenced by the n6/n3 PUFA ratio (189), which also remained stable in our study. The brain fatty acid composition is affected by aging more than by diet, as seen in a study by Gimenez da Silva et al. They fed mice with a HFD or a high-carbohydrate diet and observed initial differences that fade towards the end of the experiment. The group fed a HFD had a faster lipid accumulation in the brain, but after 56 days there were almost no differences between diets, resulting in a similar amount of FA (191). As our study was 12 weeks long, the initial differences that might have appeared with the HFD were probably faded when samples were analyzed. Brain FA composition is mostly stable, but disturbances on it are related to several neurological and neurodegenerative disorders in humans (192).

The FAs, besides their role as molecules for energy storage, also have an important role in the composition of cell membranes, as well as a signaling molecules. The balance between FA ratios determined by the ratios between SFAs, MUFAs, and PUFAs, rather than specific FA, tend to be stable, as modifications in the saturation of the FA alters cell membranes properties, such as fluidity, stability, but also functionality (182,193). For example, modifications on the lipidic composition of the mitochondrial membrane are described to alter mitochondrial respiration (194) or associate with metabolic syndrome (195). In our study, the ratio SFA/MUFA tissue decreased in the HF-groups, as well as in the DC-NS, in liver and visceral WAT. Despite a significant result, it is difficult to interpret how membrane fluidity is affected by our results as FA composition was analyzed for the total lipids of the sample. Other studies analyze the FA compositions of different lipid fractions, i.e. TG, Chol esters and phospholipid fractions, allowing the identification of separate structures (186,196,197). It is plausible that our results were mainly affected by the increased fat content in both liver and visceral WAT tissue and thus, not marking membrane alterations. This adds some difficulties when comparing our results with other studies, as the lipid profile varies between TG, Chol esters and phospholipid fractions, while we have a total amount value.

Besides the structural roles, the PUFA have been studied for their signaling properties, especially during injury and inflammation. The n3 and n6 PUFA serve as precursors of SPM, modulators of the resolution, and clearance of the inflammation occurring in the body (50,51) (**figure 20**). Also, the AA present in cell membranes is released when cell injury occurs, in order to form eicosanoids and modulate inflammation, among other functions (182). Having in mind that rats fed a HFD ingested higher proportions of both LA and ALA (**supplementary table 4, article 3**) it is interesting to see that both LA and ALA, as well as general PUFA levels, were decreased in both liver and visceral WAT. The lower PUFA

values, despite the higher intake, may indicate that PUFAs are more used. Knowing that obesity is characterized by a low inflammatory state, we may hypothesize that there is a bigger usage of the SPM molecules, in order to contain inflammation.

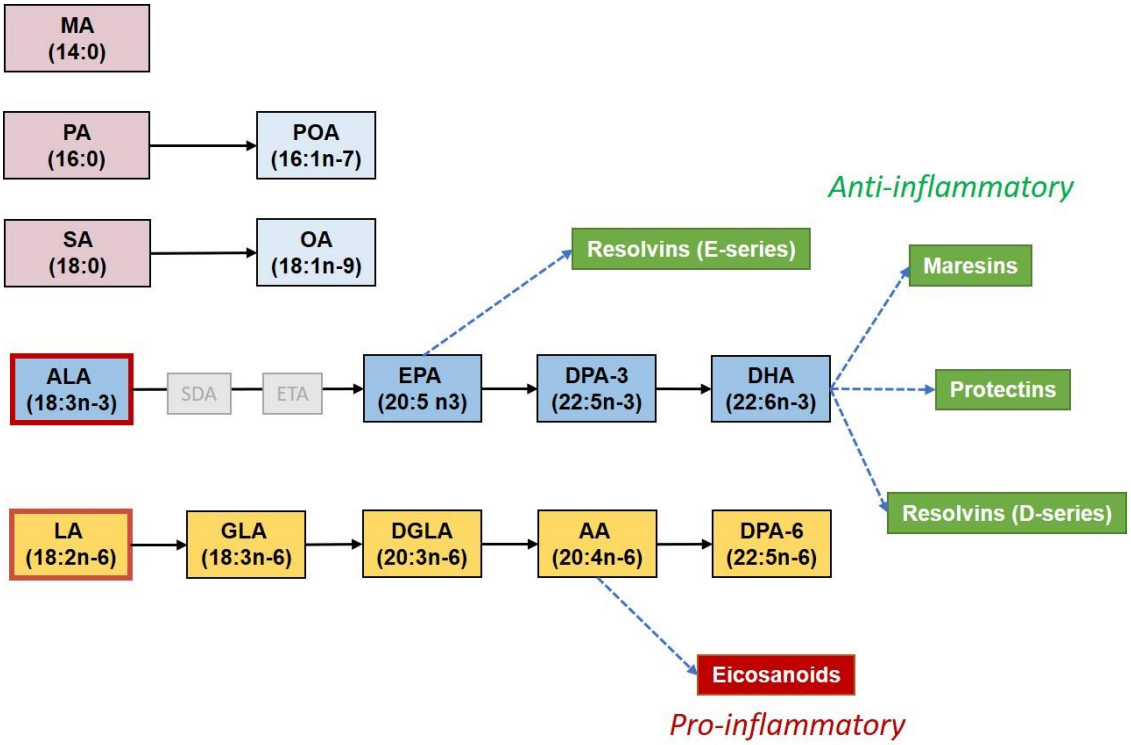

Figure 20. Biosynthesis of fatty acids and the derived anti-inflammatory and pro-inflammatory products. Adapted from Hussain et al (188), and Spike et al. (50). In pink, the saturated fatty acids. In light blue, the monounsaturated fatty acids. In dark blue, the n3 polysaturated fatty acids. In yellow, the n6 polysaturated fatty acids. Highlighted with the red contour, the essential PUFA.

We feel that it is important to mention that the analyzed desaturases activities in our study were estimated as a product-to-precursor ratio, a methodology criticized by some authors. Estimated activities are described to correlate with the mRNA (198) and are used in many studies (184,185,198–200). Even though, it would have been preferrable to have data on desaturase mRNA expression, we lacked the means to analyze it for this study and thus, we decided to continue presenting the data but mentioning the possible drawback.

The complex interplay: connecting the dots

As we have seen, obesity is a multifactorial disease that begins with an imbalance between the calories ingested versus the expended. Besides the increased body weight and adipose tissue mass, it is associated with altered parameters that lead to comorbidities. Glucose

homeostasis, several hormones, FA metabolism, and GM composition are the ones studied in this work. It has become lately clear that the apparition of comorbidities is the result of a complex interplay between multiple factors and alterations. The studied parameters in this work (the HFD, the adipose tissue, the GM, and the FA composition), have been analyzed individually and treated as independent factors, but have a role in this interplay, contributing to the disease progression (**figure 21**). Here, as a final thought, we try to put these parameters together, to see a global result.

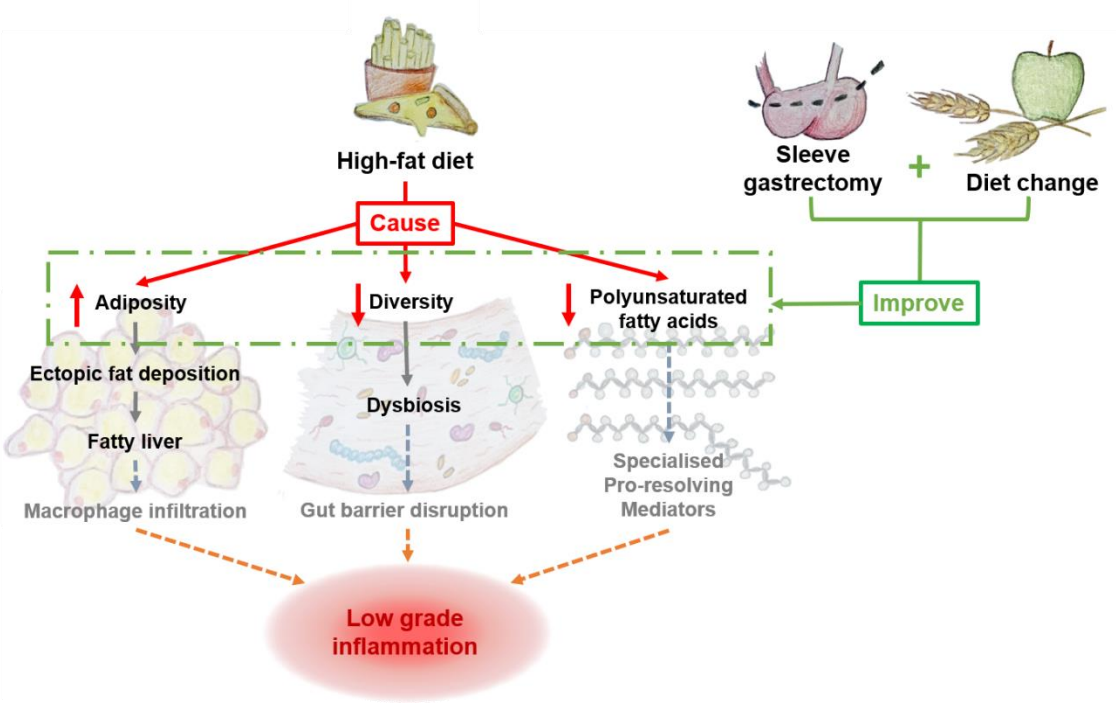

Figure 21. The interplay between the studied parameters. The high fat diet cause modifications in adiposity, gut microbiota and fatty acid composition that lead to further modifications contributing to low grade inflammation. The combination of diet change and sleeve gastrectomy improved the initial modifications caused by the high fat diet. The parameters studied in this thesis appear in black color, connected with solid lines. Parameters not studied appear in grey color, connected with dashed lines.

The characteristic low-grade inflammatory state in obesity has been mainly associated with adipose tissue. The inadequate diet, in this case a HFD, with the subsequent increased calorie intake, that increases fat mass to a point where adipocytes cannot cope with the TAG influx, would lead to ectopic fat deposition in the liver and other organs, as we have seen in our results (**table 2, article 3**). The infiltration of macrophages in WAT and liver has been studied for long as one of the main sources of the low-grade inflammation (47–49). On the other hand, due to a reduced intake of fibers and increased consumption of fats (and sugars in the WD), the GM community starts losing diversity, which translates into the loss of beneficial commensal species and taxonomical shifts. The consequent dysbiosis, observed in

this work, weakens the gut-barrier allowing infiltration of bacterial products, such as LPS or flagellin (201). Once these bacterial products reach the bloodstream, are recognized by the TLR4 and TLR 5, starting a signaling cascade that contributes to the low-grade inflammation, in what is called metabolic endotoxemia (21,201).

As the third player seen in this work, the intake of a HFD lead to substantial changes in the FA composition of the liver and visceral WAT. The long chain FA (LCFA) belonging to the PUFA family have important roles as precursors for the SPM (50). Studies have shown that a HFD, rich in SFA, is linked to increased pain response, as well as prolonged inflammation after surgery in animal models. Some authors attributed this response to altered regulation of the immune system, increased endoplasmic reticulum stress (which is involved in pain perception), and the alteration of pro-inflammatory markers and pain modulators (145,202). However, they did not analyze inflammation resolution, and thus we cannot know if it may be related with the observed FA alterations. Both studies observed a normalization after some weeks of switching to the chow diet.

Future perspectives: Could we take more advantage of bariatric surgery?

Obesity and related comorbidities are one of the main burdens of health care systems around the globe. To stop this increasing problem, governments should work towards policies against sedentarism, as well as implementing interventions and recommendations encouraging a healthier diet (2,16,17,20). As the formerly mentioned approach are long-term interventions, bariatric surgery is currently the best option to treat obesity, and despite being a common procedure, it is widely studied to elucidate the mechanism of action, as well as how to improve its benefits. Also, there is much being studied regarding diet and the different effects that some foods have on the organism.

Having in mind that BS is a major surgery and that there is an antibiotic administration as well as a need to change dietary habits, the post-surgery scenario might be suitable to add dietary modifications that could increase the benefits from this intervention. The antibiotic administration leads to the loss of many species, offering an opportunity to beneficial species to colonize when given the right situation. Studies with prebiotics have proved beneficial for hepatic lipid metabolism, and even helped counteract the effects of the HFD (172,176,203).

Other types of dietary interventions that can accompany VSG may be supplementation of SCFA or n3 PUFAs. Supplementation with SCFAs has proven beneficial in decreasing TAG and improving IR without increasing BW in HFD mice (172). On the other hand, supplementation with PUFA has also proven beneficial for lipid metabolism and hepatic steatosis (197,204,205)by suppressing hepatic lipogenesis (195), and could contribute to a better resolution of the inflammation. Fecal microbiota transplant, i. e. transplanting microbiota from a healthy donor into patients with altered microbiota, is also a potential method to treat obesity and the associated diseases that are currently studied (169).

What is clear in our study is that animals benefitting from a change of diet after VSG had an improved outcome compared to the ones who continued with the HFD after surgery. Continuing with the HFD counteracted many of the beneficial effects attributed to VSG, as we could see in several biochemical parameters that did not improve, or in the maintained dysbiosis. At the same time, a change of diet alone was not enough to revert the effects caused by a prolonged HFD, as we observed in maintained body weight and hepatic fat content. Combining VSG with a change of diet led to the highest weight reduction, the highest reduction of adipose tissue, the most improved glucose homeostasis, improved GM, and improved FA profile. All the former only were restored to C levels when both studied variables, surgery, and change of diet, were combined.

6. CONCLUSIONS

The results of our research presented in this thesis provide complementing evidence that the combination of VSG and a change of diet is able to improve, and even revert, most of the modifications caused by the high-fat diet. The main conclusions are:

- The HFD increased BW and adiposity in the body and liver, reduced GM diversity, altered GM composition, and modified FA composition in WAT and liver. All the aforementioned effects are linked to the comorbidities associated with obesity.
- Animals continuing on a HFD and subjected to VSG reduced BW but did not reduce hepatic fat, nor could improve GM diversity and composition. Thus, surgery alone was not sufficient to revert the effects of the HFD.
- Switching from a HFD to a chow diet did not reduce BW but reduced hepatic fat, and did not improve GM diversity, but restored some of its taxonomy. Despite some improvements, diet change alone was not enough to revert the effects of the HFD.
- The combination of diet change and VSG had a higher BW reduction, reduced hepatic fat, improved glucose homeostasis, increased GM diversity and partially restored GM taxonomy, and also partially reverted the modifications in FA composition in liver and WAT. Thus, the combination of both treatments is the most effective intervention to ameliorate obesity /the effects of diet-induced obesity.
- Taking into consideration the effect of antibiotic administration prior to surgery on GM, the benefits of the VSG and the change of diet might be enhanced with prebiotics or SCFA supplementation with prebiotics or SCFA, which improve GM dysbiosis and contribute to GM restoration.

7. BIBLIOGRAPHY

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