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“Obesity and post-bariatric surgery: searching for predictive biomarkers”

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Abbreviations: AT, Adipose Tissue; ASV, Amplicon Sequence Variants; BAT, Brown Adipose Tissue; BMI, Body Mass Index; BS, Bariatric Surgery; CVD, Cardiovascular Disease; DADA2, Divisive Amplicon Denoising Algorithm 2; ddPCR, Droplet Digital PCR; Drp1, dynamin-related protein 1; FA, Fatty Acid; FMT, Fecal Microbiota Transplantation; GF, Germ-free; HbA1c, Glycated Hemoglobin; HDL, High-Density Lipoprotein; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; HOMD, Human Oral Microbiome Database; IBD, Inflammatory Bowel Disease; IL-6, Interleukin-6; IL-8, Interleukin-8; IR, Insulin Resistant; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, KEGG Orthology; MaAsLin2, Microbiome Multivariable Associations With Linear Models 2; MCP-1, Monocyte Chemoattractant Protein-1; MS, Metabolic Syndrome; mtDNA-CN, Mitochondrial DNA Copy Number; NCD, Noncommunicable Disease; PBMC, Peripheral Blood Mononuclear Cell; PCoA, Principal Coordinate Analysis; PD, Phylogenetic Diversity; PERMANOVA, Pairwise Permutational Multivariate Analysis of Variance; PGC1-α, Peroxisome Proliferator-activated Receptor-γ Coactivator 1-α; PICRUSt2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2; PRRs, Pattern Recognition Receptors; QIIME2, Quantitative Insights Into Microbial Ecology Version 2; ROS, Reactive Oxygen Species; RT-PCR, Real-Time PCR; SAT, Subcutaneous Adipose Tissue; SIRT3, Sirtuin 3; TCA, Tricarboxylic acid; TG, Triglycerides; TNF-α, Tumor Necrosis Factor-α; T2DM, Type 2 Diabetes Mellitus; VAT, Visceral Adipose Tissue; WAT, White Adipose Tissue.

Abstract

Background: Obesity and metabolic disorders are multifactorial diseases resulting from the interaction between genetic and environmental factors. A systemic low grade of chronic inflammation, oxidative stress and mitochondrial dysfunction are the main common traits in obese people. In the last years, the relationship between gut microbiota and obesity has been extensively documented. Both animal and human studies have highlighted the role of the gut microbiota and its functional products as environmental factors able to modulate the energy harvest from the diet and fat storage affecting the host metabolism. While lifestyle modifications, a combination of diet, physical activity and behavior therapy are the cornerstones of weight management and prevention of cardiovascular risk factors, these are usually not enough to treat severe obesity, especially to prevent the obesity-related comorbidities onset. In this case, bariatric surgery seems to be the most effective tool in weight loss and reduction of risk of metabolic syndrome development. Several studies, carried out both in animal models and in humans, have shown that bariatric surgery can modify the gut microbiota leading to a "healthier" community. The oral microbiome is the second most complex microbial community in the human body and plays an important role in maintaining a normal oral physiological environment, thus affecting the host health. Nevertheless, the molecular mechanisms that underlie this positive impact of bariatric surgery on the clinical and metabolic profile of morbid obese subjects are still unknown.

Materials and methods: In our longitudinal study, 36 severe obese subjects (mean body mass index, BMI= 44.3) who underwent bariatric surgery and subsequent weight loss (mean BMI= 29.8), were enrolled. Stool, saliva and blood samples were collected pre-surgery and up to 1-year post-surgery. A complete panel of clinical and anthropometric parameters has been collected during the follow up. The gut and oral microbiota have been analyzed by 16S rRNA metagenomics sequencing in stool and saliva samples, respectively. The gene expression levels of Sirtuin 3 (SIRT3), the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1-α) and the dynamin-related protein 1 (Drp1), as mitochondrial molecular players, have been analyzed by real time (RT-PCR) in peripheral blood samples and the mitochondrial DNA copy number (mtDNA-CN) in peripheral blood mononuclear cell (PBMC) has also been measured by droplet digital PCR (ddPCR). All these analyses have been carried out at the different time of the follow up.

Results: The beneficial effect of the bariatric surgery is testified by a significant recovery of a normal clinical profile also associated to the decrease of the anthropometric parameters. A reduction of plasmatic levels of interleukin-6 (IL6), C-reactive protein, tumor necrosis factor-alpha (TNF-α), C-peptide and leptin has been also observed confirming that the intervention is able to reduce the inflammatory status of treated

subjects. These changes are associated to a reduction of several obesity-related comorbidities. The 16s metagenomics studies show a significant increase of alpha and beta diversity and specific changes of microbial composition leading to the restoration of eubiosis. The molecular analysis of mitochondrial players shows a higher expression level of SIRT3, PGC1-α and Drp1 both after six (T1) months and one year (T2) after the intervention. Lastly, a significant reduction of mitochondrial DNA copy number (mtDNA-CN) at the end of the follow up is also observed.

Discussion and conclusion: The clinical-anthropometric healthier profile associated to the gut eubiosis and the restoration of the mitochondrial homeostasis, observed in our cohort after bariatric surgery, strongly suggest a potential microbiota-mitochondria interplay. Interestingly, a bidirectional crosstalk between gut microbiota and mitochondria have been recently proposed. Gut microbiota, by its metabolites, modulates the gene expression levels of proteins implicated in the mitochondrial biogenesis and metabolism as well as the mitochondria concur to guarantee redox balance and gut barrier integrity. This study, focused on the association between gut/oral microbiota and mitochondrial function, explores the potential use of mitochondrial players as key biomarkers for the development of therapeutic strategies to prevent obesity-related comorbidities.

Aims

This experimental thesis is part of a multidisciplinary research aimed to the characterization of molecular mechanisms that underlying the metabolic syndrome development. A longitudinal study based on the enrollment of 36 severe obese subjects who were undergoing bariatric surgery has been performed and several biological matrices were collected both at the baseline and after six and twelve months of the intervention.

- The first aim of this work is to characterize the gut and oral microbiome profiles and their potential crosstalk in bariatric patients.
- The second aim of this work is to perform functional predictions analysis through PICRUST2 algorithm of the oral and gut microbiome before and after intervention.
- Finally, this study aims to investigate the role of mitochondria and their crosstalk with microbiota in restoration of metabolic homeostasis after BS.

Background

Obesity and metabolic disorders are multifactorial diseases resulting from the interaction between genetic and environmental factors. Obesity is defined as an abnormal or excessive fat accumulation of body that results from energy imbalance, i.e., energy intake exceeds energy expenditure, and presents a risk to health. The obese phenotype increases the risk of metabolic syndrome (MS) development, which is defined as the presence of at least three of the following conditions: excess body fat around the waist, high blood pressure, abnormal HDL-cholesterol, high triglycerides levels, hyperglycemia, insulin resistance and liver steatosis (Alberti et al., 2005). Raised body mass index (BMI) and MS can lead to the onset of noncommunicable disease (NCDs) such as, type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), stroke, and some types of cancer, among others. (Bray et al., 2018) (Fig. 1).

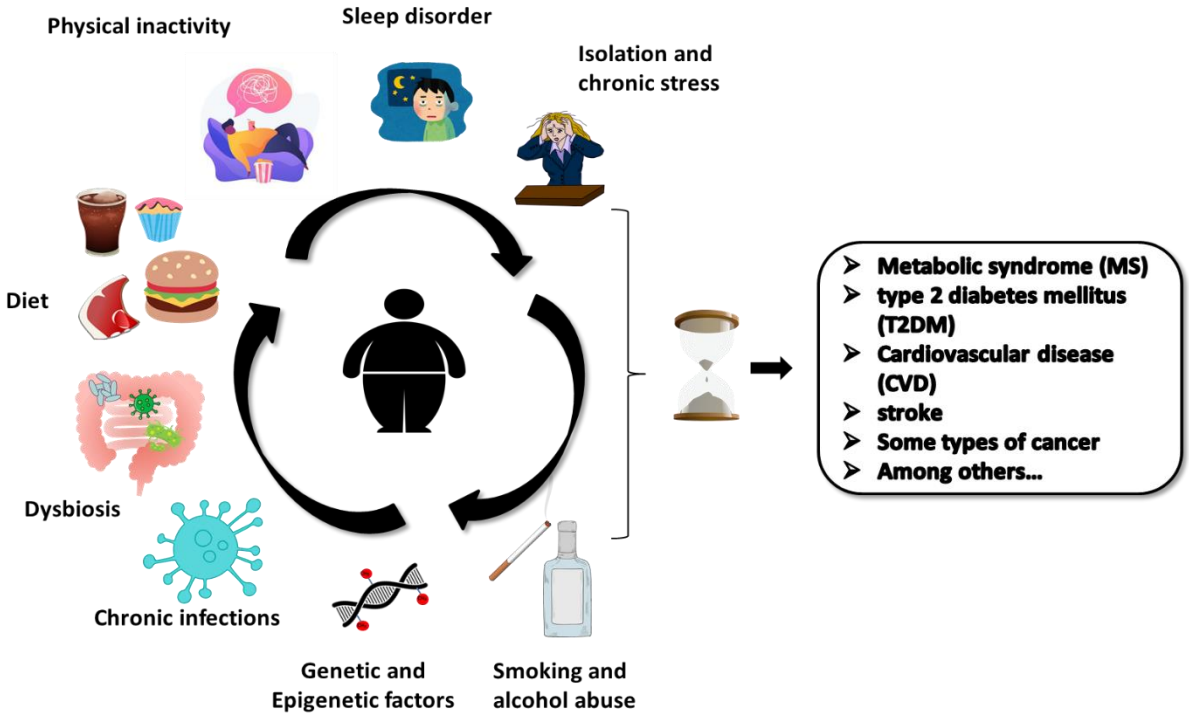

Fig. 1 The complex obesity etiology and development of noncommunicable disease (NCDs).

The incidence of obesity has surged rapidly since 1980 and now affects a third of the world's population (Chooi et al., 2019). One of the most used indices to classify overweight and obesity in humans is BMI, which is obtained by dividing the body weight in kilograms by the square of the height in meters (kg/m^2). Generally, people having BMI ≥ 25 kg⁄m^2 are overweight and people with BMI ≥ 30 kg⁄m^2 are defined as obese. Furthermore, obesity is frequently subdivided into three categories; Class 1: BMI of 30 to < 35, Class 2: BMI of 35 to < 40 and Class 3: BMI of 40 or higher (severe obesity). However, this classification provides only a partial information, that does not consider the entire body composition (lean and fat mass). Previous studies have shown that waist circumference as a measure of fat distribution may improve disease prediction (Zhou et al., 2021). More elaborate techniques, such as bioimpedance-based precision balance and magnetic resonance imaging, are increasingly available to assess body fat distribution, but these measures are not readily available in routine clinical practice. Measurement of biomarkers reflecting the underlying molecular mechanisms for increasing the risk of developing a disease may be an alternative approach to characterize the correct phenotype of obesity.

1 Introduction

1.1 Obesity as a multifactorial disease: the role of adipose tissue

The adipose tissue (AT) is a loose connective tissue composed mostly of adipocytes. Furthermore, the AT contains the stromal vascular fraction of cells, which includes preadipocytes, fibroblasts, vascular endothelial cells, and a variety of immune cells such as adipose tissue macrophages (Birbrair et al., 2013). In humans there are two major types of AT, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is derived from preadipocyte and comprises two major depots, subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) around internal organs. VAT, which is concentrated in the abdominal cavity, is further subdivided into mesenteric, omental, perirenal, and peritoneal depots (Gesta et al., 2007). The main role attributed to WAT is the store of energy in form of triglycerides (TGs), which are stored in a large, unilocular droplet that occupies most of the cell. In addition, WAT also provides mechanical cushion in anatomical regions experiencing high mechanical stress such as the palm, buttocks, and heel. Internally, AT provides cushioning to organs such as the ovaries, adrenal glands, kidney, heart and insulates the body. Furthermore, studies have highlighted the profound influence of adipocytes on other aspects of systemic metabolic homeostasis. In fact, the internalization of lipids in adipocyte cells prevents toxic lipid accumulation (lipotoxicity) in other tissues, such as liver, muscles, and heart.

BAT is a second class of specialized adipocytes that participates in non-shivering thermogenesis through lipid oxidation. Brown adipocytes contain a large number of mitochondria which express the uncoupling protein 1, which stimulates the process of non-shivering thermogenesis (Richard, 2011). Although BAT is readily observed in both infant and adult rodents, it has been proposed that BAT in humans is limited to neonates and is gradually replaced by WAT with aging. However, recent positron emission tomography/computed tomography studies have shown that BAT is viable and functional in human adults (Virtanen et al., 2009). Therefore, different types of AT act as central regulators of energy homeostasis i.e., storing energy and controlling thermogenesis.

1.2 How does adipose tissue regulate energy homeostasis?

Previously it was thought that AT was only responsible for the energy storage depot in the form of TGs and where that energy was needed elsewhere in the body, was released in form of fatty acid (FA). This concept was revised by Friedman's and colleagues in 1990, following the discovery of leptin, the first adipocyte-derived cytokine (Friedman et al., 1991). The release of leptin in response to the change in energy state indicates that adipose tissue acts as an endocrine organ capable of modulating energy homeostasis. Additional cytokines, hormones and peptides secreted by adipocytes, collectively called "adipokines" have been identified. The adiponectin, a 30-kDa protein, can regulate glucose levels, lipid metabolism, and insulin

sensitivity suppressing fibrosis and inflammation. Furthermore, leptin, a 16-kDa hormone protein, regulates the satiety centers in the brain and is also involved in the regulation of the body mass. This adipokine has also reproductive functions, plays a role in fetal growth, proinflammatory immune responses and angiogenesis and increases sympathetic output to promote lipolysis (Farr et al., 2015). In both cases, a deregulation of the expression levels of these cytokines in obesity was observed, leptin levels are directly associated with AT mass and have been shown to be elevated in obese, while adiponectin levels are reduced in obese, insulin resistant (IR), diabetic or dyslipidemic subjects as compared to normal healthy individuals (Considine et al., 1996; Turer & Scherer, 2012).

1.3 Adipose tissue expansion

It is well known that adipose tissue can increase in size both by hyperplasia (cell number increase) and by hypertrophy (cell size increase). The increase in number occurs via replication and differentiation of preadipocytes. It has been shown that the number of adipocytes in each depot is primarily determined early in life and is mostly stable through adulthood. This strongly suggests that difference in adipocyte number between obese and lean subjects could be established earlier in childhood (Spalding et al., 2008). Furthermore, differentiated adipocytes present significant hypertrophic potential (> 100 μm), an adaptive response to nutrient excess that maintains adipose tissue nutrient buffering capacity and protects other tissues from lipotoxicity. It is well established that balance of hypertrophic expansion and adipogenesis within an individual has a profound impact on metabolic health. In fact, the "overflow hypothesis" suggests that the capacity to increase the size and number of adipocytes is limited and that when this limit is exceeded, fat accumulates in ectopic sites and leads to metabolic disease. Moreover, a significant correlation was observed between adipocyte size and increased systemic insulin resistance, while other studies have pointed out that small adipocytes can counteract obesity associated metabolic abnormalities (Lundgren et al., 2007; McLaughlin et al., 2007). Furthermore, during obesity there is remodeling of extracellular matrix components, increase of necrotic areas and hypoxia. This further contributes to the increase secretion of adipokines, pro-inflammatory cytokines and chemokines, including interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP-1). This secretory pattern promotes AT inflammation by recruiting various immune cells, including macrophages (M1) and T and B lymphocytes (Cinti et al., 2005; Jernås et al., 2006). In addition, saturated fatty acids, such as palmitic or stearic acids, activate the Toll-like receptor 4 signaling cascade which plays an essential role in innate immunity, leading to chronic low-grade systemic inflammation, as well as IR (Lee et al., 2003; Frasca et al., 2018) (Fig. 2).

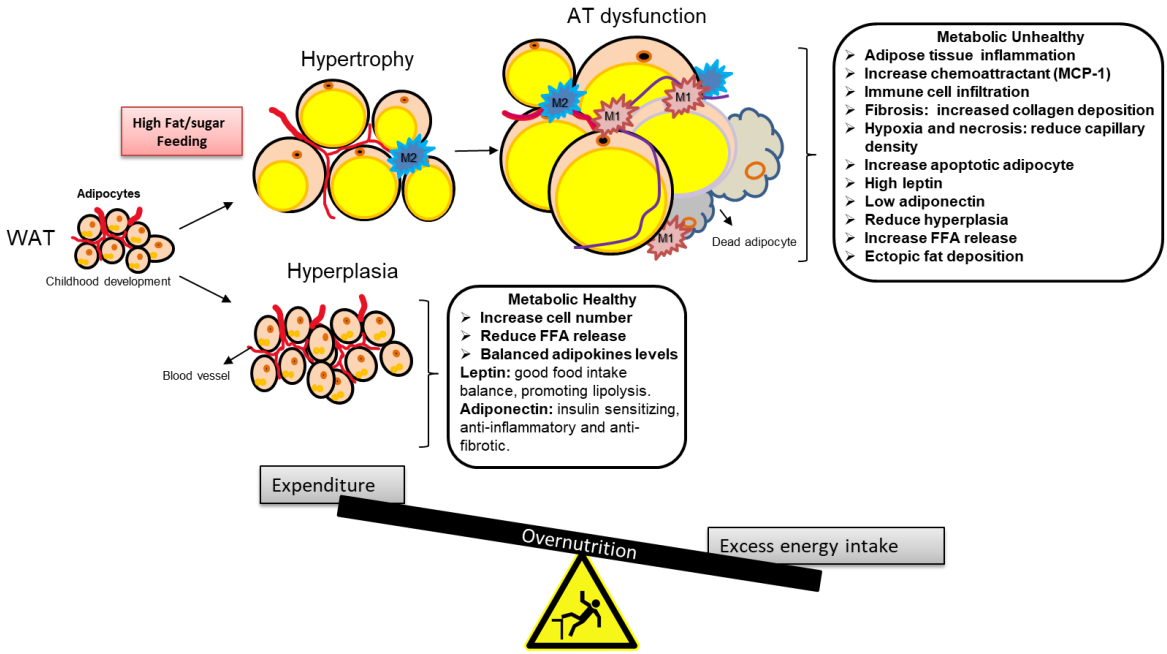

Fig. 2 Hypertrophic *versus* hyperplasic adipocytes. During energy imbalance, adipose tissue expansion occurs by two different mechanisms. **Hypertrophic adipose expansion** is associated with metabolic unhealthy condition, such as adipose tissue (AT) inflammation, proinflammatory cytokines release, immune cell recruitment and polarization of proinflammatory macrophages M1, increased free fatty acids release, hypoxia, fibrosis, increased apoptosis, decreased adiponectin, high leptin and impaired insulin sensitivity. Conversely, **hyperplasic adipose expansion** is linked to metabolic healthy condition, such as balanced adipokines levels, increased adiponectin, reduced free fatty acids release, reduced pro-inflammatory cytokines release, reduced immune cell recruitment, hypoxia, fibrosis and improved insulin sensitivity.

1.4 The loss of mitochondrial function as a primary hallmark of low-grade chronic inflammation in adipose tissue

Mitochondria are specialized organelles that generate most of the chemical energy needed to power the cell's biochemical reactions. The chemical energy produced by mitochondria is stored in the form of adenosine triphosphate (ATP) and plays a pivotal role in maintaining cell survival. Mitochondrial activity and cellular metabolism are in close cooperation. Different source of nutrients such as sugars, amino acids and fatty acids are metabolized and directed towards the tricarboxylic acid (TCA) cycle and, through repetitive oxidation reactions, electrons are stored in the reducing equivalents NADH and FADH2 (Walsh et al., 2018). These carriers deposit electrons into the electron transport chain (ETC) in the inner mitochondrial membrane and use electron flow to pump protons into the intermembrane space. Protons flow down their electrochemical gradient through ATP synthases to generate ATP (Watt et al., 2010). The flow of electrons through the ETC is an imperfect process in which 0.4 to 4% of oxygen consumed by mitochondria is incompletely reduced leading to the production of reactive oxygen species (ROS). Superoxide anions ($O2^{\cdot-}$), are the most abundant

ROS in the mitochondria (Murphy et al., 2009). Overproduction of superoxide anion interacts with many other compounds and generates "secondary" ROS such as, hydroxyl radical and hydrogen peroxide. Since the intracellular ROS generation is a constant process, the cells have developed numerous systems to buffer their excess. A concerted action of antioxidant enzymes such as, superoxide dismutase, catalase, glutathione peroxidases and non-enzymatic antioxidants (Vitamin A, vitamin C, vitamin E, Vitamin Q, and minerals) concurs to maintain the cellular redox homeostasis (He et al., 2017). In addition, mitochondria play a pivotal role in the regulation of ion homeostasis, cell growth, cell signaling and apoptosis (Červinka et al., 1999; Javadov et al., 2020). To ensure mitochondrial functionality, a balance between mitochondrial biogenesis and degradation (mitophagy) must be guaranteed. Mitochondrial dysfunction, generally characterized as a loss of efficiency in oxidative phosphorylation, is often associated to inflammation, oxidative stress, cell death and metabolic alterations (Picard et al., 2016). Moreover, mitochondrial dysfunction and oxidative stress are involved in the onset of the metabolic disorders frequently observed in obese people. Consistently, significant decrease in mitochondrial activity has observed in AT biopsies isolated from obese individuals (Yin et al., 2014). The mitochondrial dysfunction leads to inflammation through modulating inflammatory mechanisms such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or direct inflammasome activation. The activation of these pathways induces an upregulation of inflammatory cytokines and adhesion molecules secretion, resulting in a substantial inflammatory response. Furthermore, it has been reported that the mitochondrial dysfunction also contributes to ectopic fat accumulation altering insulin signaling (López-Armada et al., 2013). In contrast to the widespread "hypoxia theory", Woo and collaborators have recently proposed that mitochondrial dysfunction might be the primary cause of obesity-related AT inflammation and systemic IR (Woo et al., 2019) leading to a new question: is mitochondrial dysfunction the cause or a consequence of AT inflammation?

Recently, mitochondrial DNA copy number (mtDNA-CN) was introduced as a biomarker of mitochondrial dysfunction. MtDNA-CN ranges from 10^3 to 10^4 copies per cell in a tissue-specific manner (Shay et al., 1990). Although cellular regulation of mtDNA-CN is known to be a dynamic and tightly regulated process, the molecular mechanism by which mtDNA-CN is regulated and controlled needs to be fully elucidated (Klingbeil & Shapiro, 2009). However, elevated levels of mtDNA-CN have been associated with altered oxidative stress, age-related disease, immune response activation, response to environmental exposure, and cancer (Ricchetti, 2018; Ashar et al., 2015; Al-Kafaji & Golbahar, 2013; Taylor & Turnbull, 2005). In addition, alterations in mtDNA levels often accompany key pathophysiological changes during the transition from health to disease (Butow & Avadhani, 2004). In a recent study, a significant increase in mtDNA-CN was observed in fat deposits of obese patients, affected by T2DM, compared to either obese patients without

diabetes or the normal weight counterpart. Interestingly, a significant correlation between this mitochondrial marker and the leptin and proinflammatory cytokines levels was also observed (Litvinova et al., 2019).

1.5 The crosstalk between the gut microbiota and the mitochondria

As previously mentioned, adequate nutrient levels are essential for mitochondrial function. On the other hand, over-nutrition, promotes mitochondrial dysfunction leading to effects on metabolism. Gut microbiota is a key factor able to affect energy balance given its widely reported role in the development of metabolic dysfunctions in obese people. In agreement, recent studies have highlighted a bidirectional interaction between mitochondria and microbiota (Vezza et al., 2020; Saint-Georges-Chaumet & Edeas, 2016; Mottawea et al., 2016).

1.6 Gut microbiota and obese phenotype

The human microbiome is a complex and dynamic ecosystem, which presents approximately 3.8×10^{13} of bacterial cells in the colon which account for approximately 0.2 kg of weight in a healthy adult man of 70 kg (Sender et al., 2016). The gut microbial ecosystem starts its development from the birth and can be affected by birth mode, infant feeding, lifestyle, host genetics and drug consumption (Jakobsson et al., 2014; Rodríguez et al., 2015; Fan & Pedersen, 2021).

The first evidence of a link between obesity and gut microbiota derives from studies in germ-free (GF) mice that, under a high fat diet regimen, are protected against obesity versus their counterpart with microbiota. As a proof of evidence, GF mice undergoing fecal microbiota transplantation (FMT) from obese animals showed significant weight gain compared to FMT from lean animals (Turnbaugh et al., 2006). Further studies, at the molecular level, have shown that an increased phosphorylation of AMP-activated protein kinase and a lipoprotein lipase activity characterized the persistent lean phenotype of GF mice, revealing the role of the microbiota as modulator of the energy balance (Bäckhed et al., 2007). More recently, the association between gut microbiota and obesity was also observed in humans. Several lines of evidence have revealed that, obese people are characterized by a persistent imbalance of gut's microbial community (dysbiosis) when compared to healthy normal weight subjects (Cotillard et al., 2013). Furthermore, low fecal bacteria diversity resulted to be associated to a marked overall adiposity/dyslipidemia, impaired glucose homeostasis and higher low-grade inflammation, which are anomalies frequently observed in overweight/obese subjects (Le Chatelier et al., 2013). Moreover, metabolic dysfunctions in overweight/obese people, as well as in animal models of obesity, may be caused by increased intestinal permeability and diffusion of lipopolysaccharide through circulation promoting low-grade inflammation and insulin resistance. It has been shown that the intraluminal microbial detection requires the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) that are distributed on the

cell surface and within the cytosol of innate immune cells. Toll-like receptors and nucleotide-binding oligomerization domain receptors families function as extracellular and intracellular PRR, respectively, that trigger innate immune responses (Cani et al., 2007; Amar et al., 2008).

Although, it was already known that the diet is the major modulator of the bacterial composition and function, an elegant study showed that the beneficial effects, induced by a healthy diet, are strongly dependent on the individual background. In a randomized crossover study, several biometric and blood parameters were recorded in 39 healthy individuals who were subjected to a fiber rich diet for a few days. Two distinct groups were identified as non-responders and responders, i.e., individuals who present very low or high improvements of the clinical parameters, respectively. The 16S rRNA sequencing of their gut microbiota showed that the responders had an abundance of *Bacteroidetes versus* the non-responders mainly due to a bloom of *Prevotella*. Furthermore, the direct role of the beneficial effects due to *Prevotella* abundance was testified by transplantation of responders and non-responders' feces in GF mice: an improved glucose metabolism and an increased liver glycogen content were observed in the mice exclusively colonized with the responders' microbiota. This study highlights the potential importance of individual members of the gut microbiota, both in terms of abundance and in terms of their interactions with other microbial species and with the host metabolism in response to diet changes (Kovatcheva-Datchary et al., 2015). Consistently, it was also shown that higher weight gain could be induced through gut microbiota transfer from human twins discordant for obesity to GF mice. In addition, it was observed that the cohousing of lean and obese mice prevented the obesity development (by coprophagia). This rescue was correlated to the invasion of *Bacteroidetes* from lean to obese microbiota correlated to a lean-like metabolic profile (Ridaura et al., 2013). Clear evidence of the direct involvement of the gut microbiota in the control of obesity was provided by the relevant changes of microbiota composition associated with weight and mass fat loss observed in obese patients after bariatric surgery (BS). GF mice colonized with stools obtained from bariatric patients showed a reduced fat mass gain versus the counterpart colonized with pre-surgical feces (Tremaroli et al., 2015). In addition, several clinical studies reported that BS significantly reduces body weight and fat mass over time. BS also ameliorates or even leads to the remission of T2DM and MS and has profound effects on the cardiovascular system (Le Chatelier et al., 2013). Presently, BS results to be the most effective weight loss strategy in severe obese patients (Buchwald et al., 2009).

The changes in the food intake, the retrieval of the gut hormone secretion, the gastric emptying, the bile acids secretion and the changes in intestinal gluconeogenesis seem to be the factors which mainly concur to the improvements in body composition, the restoration of metabolic functions and the reduction of inflammation which are the major improvements frequently observed after BS (Mulla et al., 2018; Isbell et al., 2010). However, the benefits of weight loss following bariatric procedures are still debated regarding the gut

microbial gene richness, pro-inflammatory and metabolic profile of obesity (Aron-Wisnewsky et al., 2019; Engin, 2017).

1.7 Oral microbiota and obese phenotype

The oral microbiota is the second most complex microbial community in the human body after the colon. Previous studies have highlighted the important role of these microorganisms in maintaining oral and systemic health through colonization resistance (Wade, 2013), nutriment digestion (Moye et al., 2014) and immunity response (Slocum et al., 2016). While in the gut microbiome most of the bacterial population is anaerobic, the oral cavity harbors a big quantity of aerobic bacteria (Verma et al., 2018). Recently, the role of the oral-gut microbiome connection in pathologies, which are characterized by chronic inflammation, like inflammatory bowel disease (IBD), has been found (Takahashi, 2015). *Campylobacter concisus* and *Fusobacterium nucleatum* were amongst the first oral bacteria to be implicated in IBD. In fact it has been shown that both of these oral bacteria, in particular conditions can translocate to the intestine and to colonize the mucosal niche suggesting that this phenomenon may be a common feature of microbial dysbiosis, frequently observed in IBD patients (Strauss et al., 2011; Kirk et al., 2016). Transient and commensal populations that often form biofilms on soft and hard surfaces in the mouth, compose the oral microbiome (Avila et al., 2009). In healthy conditions, it prevents the colonization of the oral cavity by pathogenic microbes (Walker, 2016). For example the genus *Streptococcus* secretes bacteriocins able to prevent colonization of pathogenic gram-negative bacterial species (Wang & Kuramitsu, 2005). The most up-to-date information on taxa of the oral microbiome may be found in the Human Oral Microbiome Database (HOMD) (http://www.homd.org/), (Dewhirst et al., 2010). Cultivation-independent analyses indicate that the most common genus is *Streptococcus*; other genera, such as *Neisseria, Gemella*, *Granulicatella,* and *Veillonella*, have been found, although not present in all individuals (Aas et al., 2005). Furthermore, the taxa present in the oral cavity appear to be dependent on positive and negative interactions within the microbial community as well as interactions with the host (Levy & Borenstein, 2013; Diaz & Valm, 2020).

The oral microbiome has been associated with obesity, similarly to the gut microbiome. The oral microbiome also shows differences in the overall composition between obese and lean subjects. It has been observed that with an increase in BMI, the diversity in bacterial species, in saliva and the sublingual plaque, decreases (Tam et al., 2018). In a recent case-control study, de Andrade and coworkers have observed a significant association between obesity and salivary microbiome structure and composition in adolescents. In detail, obesity may be related to the occurrence of higher abundance of microbes such as *Neisseria* spp. and *Rothia* spp. when compared to the lean counterpart, that can be related to the maintenance of high BMI (de Andrade et al., 2020). Preclinical and clinical studies have highlighted the key role of oral/gut bacteria in the etiology of obesity (Degruttola et al., 2016). Furthermore, it has been shown that saliva microbiome profiles

significantly correlate with those in the stool, even though the oral and stool communities shared little taxonomic resemblance (Ding & Schloss, 2014). Therefore, saliva is a valuable biological matrix to detect bacterial dysbiosis in systemic diseases. Very recently, salivary microbiome has been successfully used to describe salivary microbiome changes during body weight loss on an individual-specific level showing the effect of BS (Džunková et al., 2020). Due to the complexity of the oral microbiome, which encompasses more than 700 bacterial species, 32% of which has not yet been cultivated (Zhao et al., 2017), a comprehensive analysis afforded by next generation sequencing of 16S rRNA gene, allows for the discovery of new taxa potentially related to obesity. Importantly, this experimental approach does not exclude species that might have a crucial role in disease onset (Gupta et al., 2019). Several lines of evidence showed that obesity could have an impact both in gut and oral cavity microbiota. However, little is known about the oral/gut microbial dynamics in post-surgery perturbation and the effects in mitochondrial functionality. In this study we analyzed the microbiota changes, both in saliva and stool samples and the association of mitochondrial functions in peripheral blood mononuclear cell (PBMC) in a cohort of severe obese patients, undergoing BS, without any overt co-morbidities. PBMC from venous blood samples were selected for analysis because blood is an accessible tissue that is used in many clinical and nutritional studies. PBMC are often used as surrogates for predicting potential effects in inaccessible tissues. Their value in this respect is likely to be highly dependent on the context of the research and thorough evaluation is still required to further validate this potential role. Definition of the normal degree of variation, as well as the responsiveness of gene expression in these cells to environmental factors, will provide a key component in this process.

2. Materials and Methods

2.1 Study Design

This was a non-randomized longitudinal study. A cohort of 36 severe obese patients, undergoing BS, has been enrolled at Department of Systems Medicine, Tor Vergata University, (Rome). The inclusion criteria were as follows: age > 18 years < 60; BMI > 35 Kg/m^2 < 50; no common obesity-associated comorbidities (diabetes, hypertension, liver steatosis) whereas the exclusion criteria were as follows: antibiotics, prebiotics and probiotics intake; any drug intake with a known major impact on gastrointestinal tract in the 3 months prior to study enrollment; pregnant or lactating women; chronic and degenerative diseases. All patients signed an informed consent for participation in the study and all relevant information on family and medical history, current drug consumption, dietary, smoking habits, physical activity and psychological state were recorded by a validated detailed questionnaire. The surgery day, an interview was carried out for the collection of anamnestic data; anthropometric (weight, height, waist, and hip circumference) and clinical (blood pressure, heart rate) parameters were recorded. Furthermore, bioimpedance analysis was performed

to determine the body composition. Immediately after surgery, patients followed a liquid diet from day 1 to 3 and then a semi-liquid diet for 2 to 4 weeks. At the first follow-up visit (1 month after surgery) the patients gradually reintroduced solid foods with a low protein intake (1.5 g/kg of ideal weight), prescribed a polyvitamin and advised to carry out at least 30 min/day of aerobic and endurance physical activity, to limit the loss of lean mass. At the follow-up visits foreseen by the protocol (6 and 12 months after surgery), a re-evaluation of the aforementioned clinical, anthropometric and biochemical parameters, as well bioimpedance analysis, was carried out and all biological matrices were collected again (Fig. 3). This study was approved by the ethical committees of Istituto Superiore di Sanità (prot. PRE 173116 of 15 March 2016; PRE-BIO-CE 10938 of 6 April 2018) and of Tor Vergata University (protocol of the study connecting DNA 169/15).

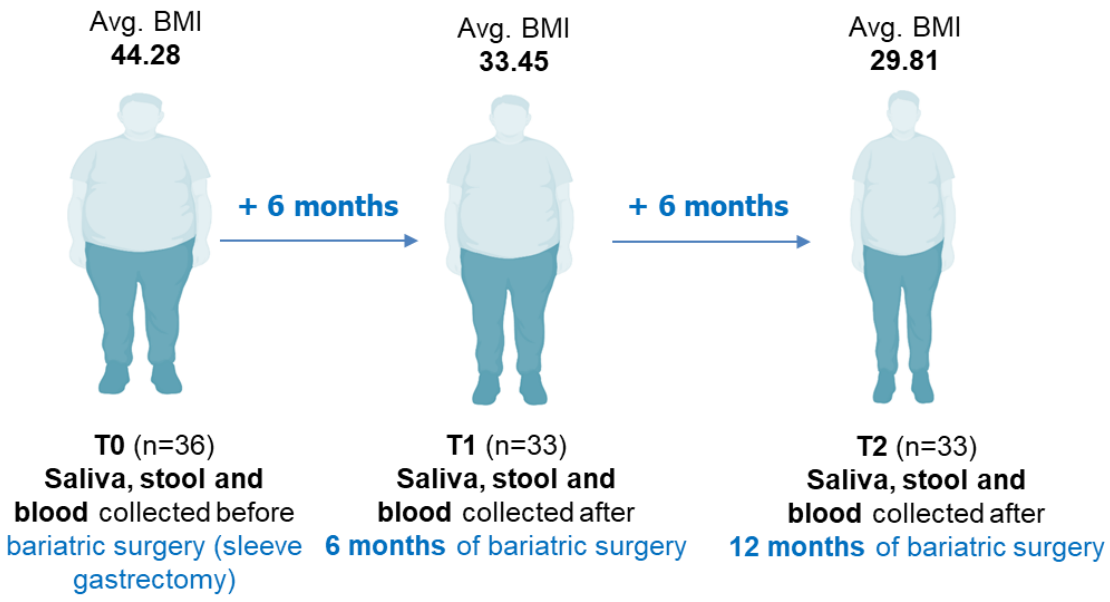

Fig. 3 Study design and follow-up procedures.

2.2 Sample collection

Different biological matrices were collected and properly processed and stored. At each medical examination, stool samples were freshly collected in sterile containers and immediately frozen at -80°C. Saliva samples were collected through oral rinse. For the oral rinse specimens, we use 15 ml of sterile saline solution (0.9 % NaCl). The patients swirl it in the oral cavity for 15 seconds reclining the head backwards. Afterwards, they spit into a 50 ml sterile falcon tube containing 7.5 ml of PreservCyt Solution (Hologic, Inc,

Marlborough, USA); total ratio 2:1. The samples were centrifuged at 3,000 g for 10 minutes at 4°C. The cell pellet was resuspended in 1 ml of the same solution and frozen at -80°C. None of the subjects had eaten or brushed their teeth for at least 1 h before they provided their sample. PBMC were isolated immediately after collection of blood using a protocol designed to minimize sample processing time and maximize the consistency of processing between samples.

For the separation of PBMC from whole blood, BD Vacutainer® CPT™ (8-ml draw volume each), containing sodium citrate as anti-coagulant, was used accordingly to the manufacturer instruction (in average 5 to 8 X 10^6 cells were isolated). Moreover, for RNA purification, blood was also collected into PAXgene® Blood RNA Tubes (PreAnalytix QIAHEN, Inc., Germantown, MD, USA) (2.5 ml of whole blood) following the manufacturer instructions.

2.3 DNA Isolation from stool, saliva and PBMC samples

MagNALyser Green Beads (Roche) were used for optimal homogenization and lysis of the stool and saliva samples before DNA isolation. The MagNALyser Instrument was used with the following protocol: tubes were placed in the MagNALyser Instrument and processed two times for 30 seconds at 6000 rpm. Subsequently, DNA was extracted using EZ1 Advanced XL Qiagen (EZ1® DSP Virus Kit Handbook) for saliva samples and EZ1 Advanced XL Qiagen (EZ1® DNA Tissue Handbook) for stool samples, according to the manufacturer's instruction. DNA concentration was determined by the Qubit ds DNA HS Assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The same extraction protocol was applied to Milli-Q Ultrapure Water and a mixture of known bacterial species (ZymoBIOMICS™ Microbial Community Standard), as negative and positive controls, respectively.

2.4 16s rRNA gene sequencing

Genomic DNA was amplified using fusion primers targeting the variable V3 and V4 regions of the 16S rRNA gene with indexing barcodes (Klindworth et al., 2013). The PCR product size was checked on a 0.8% agarose gel and the DNA concentration estimated using a Qubit fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and equimolar libraries were pooled and purified using AMPure XP beads according to manufacturer's recommendations (Beckman Coulter, Stockholm, Sweden). The pool 16s rRNA library was validated by the Agilent 2100 Bioanalyzer system. Purified pooled amplicons were adjusted to 4 nM, spiked with 5% PhiX, denatured and diluted as described by Illumina before loaded into MiSeq cartridges (Illumina, San Diego, CA, United States). The paired-end sequencing (2x300bp paired-end) was performed using an Illumina MiSeq sequencer with the Illumina MiSeq reagent kit 600 version 3, according to the Illumina 16S metagenomic library preparation protocol (Part # 15044223 Rev. B).

2.5 Bioinformatics analysis of 16s rRNA sequencing data

Quantitative Insights Into Microbial Ecology version 2 (QIIME2) was used for the raw data analysis in order to generate a comprehensive taxonomic profile of the microorganism present in stool and saliva samples and to perform diversity analysis (version 2019.7, https://qiime2.org). First, we performed quality control checks on raw sequence data using FastQC, following this pair-end reads were fused and primers, ambiguous and chimeric sequences were removed. Divisive amplicon denoising algorithm 2 (DADA2) was run with the following parameters: —p-trunc-len-f 290—p-trunc-len-r 260—p-trim-left-f 20—p-trim-left-r 0 and found the trimming of the first 20 nucleotides from the forward reads with low quality scores avoided excessive sequence loss during chimera removal. Truncating reads at positions 290th for the forward and 260th bp for the reverse sequence read, allowed the removal of low-quality sequences while maintaining sufficient length to merge matching paired ends (Bolyen et al., 2019) with resolution of amplicon sequence variants (ASVs), (Callahan et al., 2016; Callahan et al., 2017). For the taxonomic assignment to the ASV, we used the Naïve Bayes classifier trained on the Greengenes database version 13.8 (http://greengenes.lbl.gov) for stool samples. For saliva samples, we used the HOMD (version 15.2) (http://www.homd.org/55). Finally, ASVs multiple sequence alignment (MAFFT), masking of highly variable positions, generation of a phylogenetic tree with FastTree method and the root tree was generated and used as input to perform phylogenetic-diversity analysis with QIIME2.

2.6 Droplet digital PCR and Real time PCR

Norgen genomic DNA isolation kit (Norgen Biotek Corp. cat. n. 24700) was used to isolate total DNA from PBMC accordingly to the manufacturer instructions. Relative mitochondrial DNA copy number was analyzed by both absolute QPCR (Life Technologies 7500 Fast Real Time System) and droplet digital PCR (ddPCR) (Biorad QX 200). Mitochondrial and nuclear DNA were detected by dehydrogenase 2 (MT-ND2) and ribosomal protein lateral stalk subunit P0 (RPLP0) TaqMan® probes, respectively (cat. no. 4331182; Life Technologies, Austin, TX, USA). Peripheral blood was collected using PAX gene blood RNA tubes (Qiagen/BD company cat. no.762174) and cryopreserved at -80°C. This methodology guarantees the stability of intracellular RNA for years. Total RNA was purified accordingly to the manufacturer instructions. Complementary DNA was retro-transcribed by the high-capacity cDNA reverse transcription kit (cat. no. 4368813; Life Technologies) and the gene expression analysis was carried out by Real-Time PCR. The gene expression levels of SIRT3, Drp1 and PGC1-α, were calculated using specific Taqman assays (FAM labeled) by comparative method ($2^{-\Delta\Delta Ct}$). rRNA 18S assay (VIC labeled) was used as housekeeping reference gene.

2.7 Statistical analysis

For diversity analysis, the feature tables were rarefied to 9111 sequences for stool samples and 11837 for saliva samples by random subsampling in QIIME2. To evaluate alpha diversity, we computed Faith's phylogenetic diversity (PD), the number of ASVs observed in each sample, Pielou's evenness and Shannon index accounting for both evenness and richness (Faith & Baker, 2006). The Kruskal–Wallis test was used as a non-parametric statistical test to evaluate pairwise differences. To measure beta diversity, we used the unweighted and weighted UniFrac distance (Lozupone et al., 2011), to estimate dissimilarity among time groups by incorporating the phylogenetic distances between ASVs. Pairwise permutational multivariate analysis of variance (PERMANOVA) with 999 random permutations was used to test the significance of differences between time groups. The Principal Coordinate Analysis (PCoA) plots of microbial diversity were depicted using the ggplot2 package (version 3.3.3) in the R Studio (version 1.3.1073, Boston, MA, USA).
Next, for the relative frequency statistical analysis at different taxonomic levels, the data obtained by the gut and saliva microbiota characterization had a non-normal distribution; therefore, median values were used to describe continuous variables. Non-parametric statistics (Wilcoxon signed-rank test) were applied to compare the levels of repeated measurements (T0, T1 and T2). All analyses were performed using Stata software version 16 (Stata Corporation, College Station, TX, USA). Next, we graph the significant results through GraphPad Prism version 8.0.2 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.
In order to predict the functional profiles of the microbial community in stool and saliva samples before and after the BS, we conducted Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) (Douglas et al., 2020), with ASVs according to the instructions published at (https://github.com/picrust/picrust2.wiki). ASVs are placed into a reference tree, which is used as the basis of functional predictions. This reference tree contains 20,000 full 16S rRNA genes from prokaryotic genomes in the Integrated Microbial Genomes database (Markowitz et al., 2012). Phylogenetic placement in PICRUSt2 is based on the output of three tools: HMMER (http://www.hmmer.org) to place ASVs, EPA-ng (Barbera et al., 2019) to determine the optimal position of these placed ASVs in a reference phylogeny, and Genesis Applications for Phylogenetic Placement Analysis (GAPPA), to output a new tree incorporating the ASV placements (Czech et al., 2019). This results in a phylogenetic tree containing both reference genomes and environmentally sampled organisms, which is used to predict individual gene-family copy numbers for each ASV. The PICRUSt2 predictions based on several gene family databases are supported by KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology (KO) (Kanehisa et al., 2016) and enzyme commission numbers (EC numbers) (Alderson et al., 2012) database. PICRUSt2 KOs predictions from 16S rRNA marker gene data were produced for stool and saliva samples in the three different periods, T0, T1 and T2.

Generalized linear models implemented in microbiome multivariable associations with linear models 2 (MaAsLin2) R package version 1.4.0 (http://huttenhower.sph.harvard.edu/maaslin2) (Mallick et al., 2021) was used to compare the pathways in the three different periods. Results were visualized as a heatmap of the top 50 pathways with significant associations between the study times. All predictions were corrected for multiple testing (Benjamini–Hochberg method, FDR $q<0.05$).

3. Results

3.1 Study population

In this section are reported the results obtained by a longitudinal study on severe obese patients who had to undergo BS and the main characteristics of the selected population are described in Table 1. For the study 36 patients, 32 of whom completed the study, have been recruited; 91.6% (33/36) were women, with an age range of 37–59 years and a median BMI of 44.28 ($39.74<BMI<48.32$) Kg/m^2. Each patient has been re-evaluated at 6 and 12 months after BS. As expected, after surgery, a significant decrease of glycemic index, insulin, homeostatic model assessment for insulin resistance (HOMA-IR), glycated hemoglobin (HbA1c), low-density lipoprotein cholesterol (LDL-C), triglycerides and a significant increase in high-density lipoprotein (HDL) levels were recorded in all patients. Consistently, a significant decrease of the levels of C-reactive protein, as a standard inflammation marker, was also observed. Measurement of the anthropometric parameters revealed a high significant decrease of body weight (median value; T0= 115.5 kg vs T2 = 76.5 kg; p-value <0.0001), BMI (median value; T0= 44.28 kg/m^2 vs T2= 29.81 kg/m^2 ; p-value <0.0001), waist circumference (median value; T0= 122 cm vs T2= 93.25 cm; p-value <0.0001), hips circumference (median value; T0= 141 cm vs T2= 110.5 cm; p-value <0.0001), and W-H ratio (median value; T0= 0.86 vs T2= 0.83; p-value 0.0058), 12 months after BS. At the enrolment time, 52.7% (19 of 36) of the individuals had metabolic alteration such as dyslipidemia, insulin resistance that usually herald the onset of MS. After BS, only 15% (5 of 33) of patients were still affected by several metabolic alterations. Moreover, the analysis by high sensitivity magnetic luminex assay in plasma samples also showed a significant decrease of TNF-α and IL6, testifying a reduction of the obesity associated-chronic inflammation (Table 1).

Characteristics	T0 Median (IQ range)	T1 Median (IQ range)	T2 Median (IQ range)	*p-value T0 vs T1	*p-value T0 vs T2	*p-value T1 vs T2
Number	36 (3M/33F)	33 (2/31)	33 (2/31)			
Weight (kg/m2)	115.5 (103-130.5)	86.7 (77.3-96)	76.5 (68.05-91.6)	<0.0001	<0.0001	<0.0001
BMI	44.28 (39.74-48.32)	33.45 (30.6034.80)	29.81 (27.10-32.21)	<0.0001	<0.0001	<0.0001
Waist circumference (cm)	122 (114-129)	100 (90-107)	93.25 (83.75-101.5)	<0.0001	<0.0001	<0.0001
Hips circumference (cm)	141 (132-150)	120 (110-127)	110.5 (103.75-119)	<0.0001	<0.0001	<0.0001
W-Hratio	0.86 (0.83-0.91)	0.83 (0.80-0.89)	0.83 (0.79-0.88)	0.0316	0.0058	0.0997
Current smoker (%)	16.6	16.6	14.28	>0.999	>0.999	>0.999
Glycemia mg/dl	93 (87-102.5)	81 (77-87)	85 (80-91.5)	<0.0001	<0.0001	0.0636
Insulin (uU/ml)	17.95 (14.78-22.13)	7.8 (6.1-8.7)	7.4 (5.4-9.6)	<0.0001	<0.0001	0.4404
HOMAIR	4.32 (3.00-5.21)	1.51 (1.17-1.98)	1.50 (1.05-2.07)	<0.0001	<0.0001	0.5052
HbA1c	38 (36-43)	34 (32-37)	34 (32-36)	<0.0001	<0.0001	0.5831
Total cholesterol (mg/dl)	200.5 (168.5-224.5)	195 (163-230)	202 (176.5-234.5)	0.5857	0.1635	0.0444
HDL-C (mg/dl)	50 (41-57)	55 (47-62)	55.5 (49.5-67)	0.0293	0.0013	0.0016
LDL-C (mg/dl)	123.6 (95.8-148)	120.4 (96.8-147.2)	122.6 (105.3-154.8	0.8094	0.5434	0.5435
Triglycerides (mg/dl)	124 (96-179)	95 (73-126)	84.5 (75-110.5)	<0.0001	<0.0001	0.0937
ALT (U/l)	29 (22.5-38.5)	18 (14-22)	18 (13-21.5)	0.0002	0.0007	0.3340
AST (U/l)	20 (16-24)	18 (16-21)	20 (15-24.5)	0.2517	0.4539	0.1229
C-reactive Protein (mg/l)	7.64 (4.03-12.05)	2.4 (0.9-3.9)	1.4 (0.6-2.2)	<0.0001	<0.0001	0.0002
TNF-α (pg/ml)	9.04 (7.65-11.49)	9.12 (7.37-10.29)	7.80 (5.64-8.85)	0.6088	0.0061	0.0033
IL6 (pg/ml)	1.74 (1.178- 2.765)	1.55 (0.69-2.35)	0.75 (0.34-1.11)	0.0455	<0,0001	0.0001

Table 1. Median values and interquartile ranges of studied variables measured in obese patients before (T0) and 6 months (T1) and 12 months (T2) after bariatric surgery. These data were obtained by routine clinical analysis with the exception for TNF-alpha and IL6 which were measured by High Sensitivity Magnetic Luminex Assay. The p-value for difference between T0, T1 and T2 groups were calculated by Wilcoxon rank-sum test.

3.2 Characterization of fecal and saliva bacterial communities before and after gastrectomy

3.2.1 Fecal alpha and beta diversity analysis

Measurements of alpha diversity, i.e., the measures of the variability of species within a sample, (this measure considers both the richness "number of taxonomic groups" and the evenness "distribution of abundances of the groups") and the beta diversity i.e., the variation of microbial communities between samples, have been carried out in stool and saliva samples (Wagner et al., 2018). In addition, analysis of microbial relative abundance taxa has also been determined in both biological matrices.

The sequencing depth ranged from 9,111.0 to 66,332.0 (mean 31,302) and the number of features was 6,964 in 82 subjects after the DADA2 denoising step. After rarefying the features tables to 9,111 sequences per sample, we noticed that all the samples present a good coverage. We compared the taxonomic diversity using different metrics of alpha diversity that incorporate species richness and evenness. A significant increase in alpha diversity as observed by Faith's PD was found at both 6 and 12 months after BS (T0 vs T1 p = 0.044036 and T0 vs T2 p = 0.002437, Kruskal–Wallis test) (Fig. 4A). Conversely, no significant variations were observed with ASVs, Shannon's index and Pielou's evenness in feces samples (Fig. 4 B, C and D).

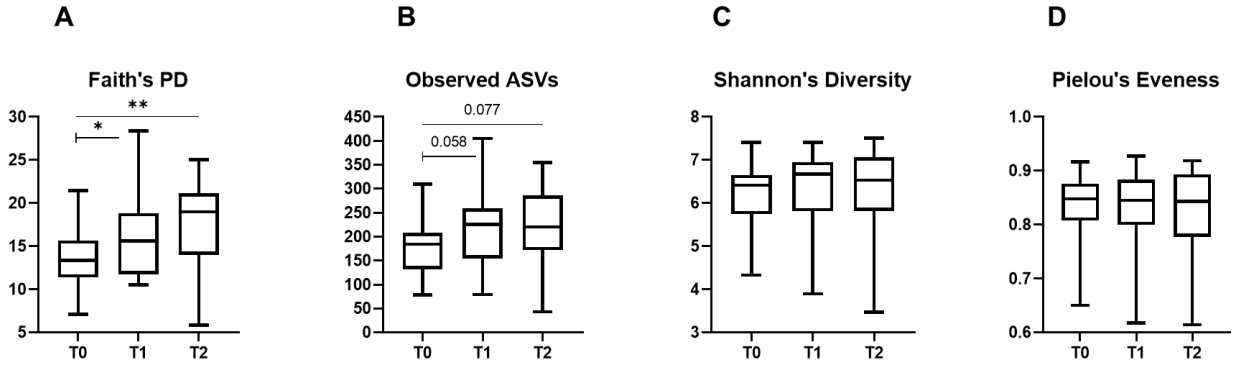

Fig 4. Alpha diversity among groups. Diversity for: A) Faith PD (T0 vs T1 p-value= 0.044, T0 vs T2 p-value= 0.0024, Kruskal–Wallis all groups p-value=0.005); B) ASVs (p-value=0.104, Kruskal–Wallis all test); C) Shannon index (p-value=0.434, Kruskal–Wallis test); D) Pielou's evenness (p-value=0.988, Kruskal–Wallis test). *p<0.05, **p<0.01. The number of samples at different time point was: T0= 27, T1= 30 and T2= 25.

In order to quantify the beta diversity, phylogenetic methods (UniFrac distance) were used. PCoA is used for visualization of the data in the distance matrix in a 3D plot. The distance matrix is transformed into a new set of orthogonal axes where the first axis (PC1) can be used to explain the maximum amount of variation present in the dataset, followed by the second axis (PC2), third (PC3), etc. PCoA of microbial ASVs has revealed a difference in the microbial community composition after BS by both the unweighted (T0 vs T1 Pseudo-F=1.296, p=0.074 and T0 vs T2 Pseudo-F= 1.680, p= 0.013, PERMANOVA) and weighted UniFrac distance (T0 vs T1 Pseudo-F=2.258, p=0.010 and T0 vs T2 Pseudo-F= 1.984, p= 0.036, PERMANOVA). Although high interindividual variation is observed, the fecal microbiota shows a significant increase as early as 6 months after BS and this trend remains constant even 12 months after surgery (Fig. 5).

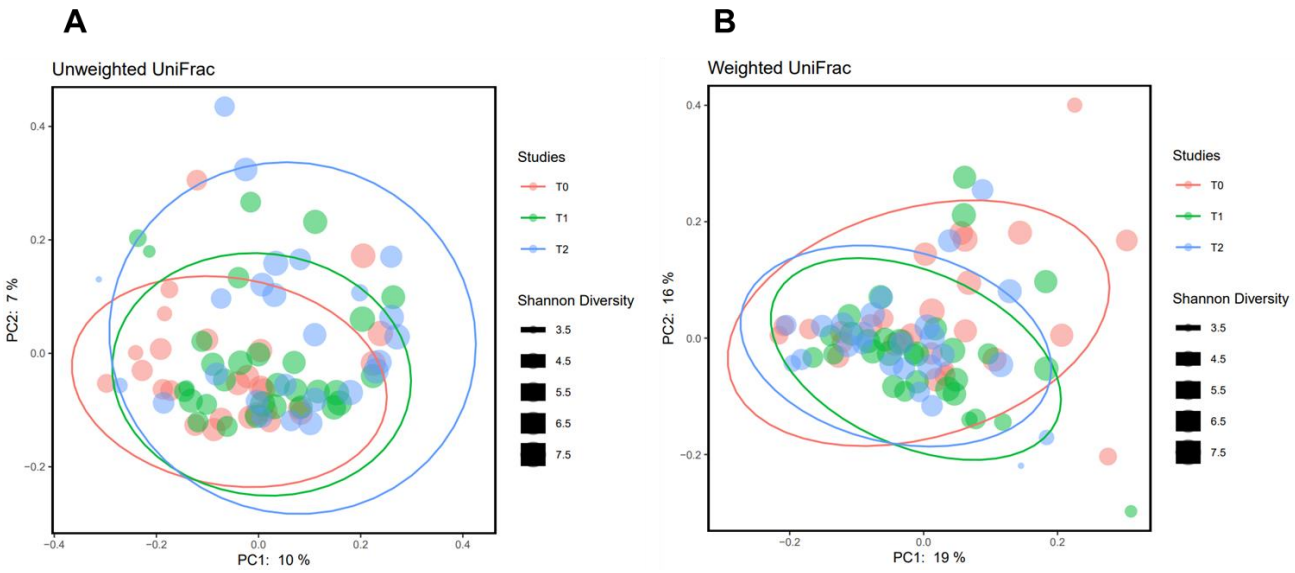

Fig. 5 Principal Coordinate Analysis (PCoA) plots of beta diversity. Statistical significance between T0 vs T1 and T0 vs T2 groups using distance matrices for beta-diversity: (a) unweighted UniFrac distance, (b) weighted UniFrac distance. Statistics were calculated using pairwise PERMANOVA with 999 permutations. *p<0.05. The ellipses represent the 95% confidence interval for each time. The size of the dots represents the values of Shannon's diversity.

3.2.2 Fecal microbial community composition before and after BS

In our study, the most representative phyla which are modulated after BS are *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Verrucomicrobia* and *Actinobacteria*. The modulation of some less represented phyla, such as *Tenericutes*, *TM7* and *Synergistetes* has also been observed (Fig. 6A).

After surgery, the gut microbiota composition changed at different taxonomic levels. A significant reduction of *Firmicutes* both after 6 and 12 months, and a significant increase of *Bacteroidetes* one year after BS have been observed. Interestingly, the phylum *Verrucomicrobia* is almost absent before the intervention but significantly increases after BS, accordingly to an improvement of gut healthy state (Dao et al., 2016). Furthermore, although poorly represented, the phylum *Actinobacteria* decreases after 12 months in contrast to what observed for the phylum *Synergistetes* which increases 6 months after BS. It is also of note the significant decrease of *TM7* phylum after 1 year post-BS. Interestingly, the abundance of the *TM7* phylum, known to be associated with the microenvironment inflammation, resulted to be reduced after BS (Brinig et al., 2003) (Fig. 6B and C).

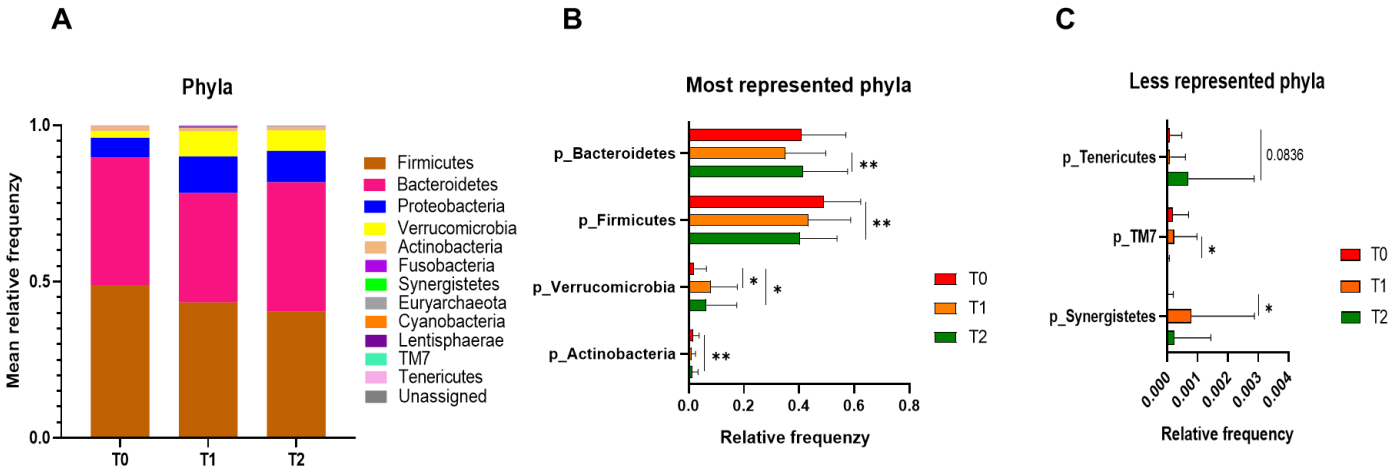

Fig. 6 Effect of bariatric surgery at the phylum level on gut microbiota composition of patients with severe obesity. A) Relative frequency average of the total phyla detected in gut microbiota composition before (T0) and 6 months (T1) and 12 months (T2) after bariatric surgery. B) Relative frequency of the most represented phyla which resulted significantly modulated after surgery. C) Relative frequency of the less represented phyla showing a significantly modulation after BS. Statistics were calculated using nonparametric Wilcoxon signed-rank test. * $p < 0.05$; ** $p < 0.01$. T0 vs T1, T0 vs T2 and T1 vs T2.

The most remarkable differences were observed at the genus level (Fig. 7A and B). Interestingly, a strong abundance of *Bacteroides, Akkermansia (muciniphila), Alistipes (onderdonkii and indistinctuss), Acidaminococcus, Oscilospira, Phascolarctobacterium, Ruminococcus (lactaris), Barnesiella (intestinihominis)* and a significant decrease of the genus *Roseburia (faecis), Faecalibacterium (prausnitzii), Dorea (longiatena), Dialister, Gemminger (formicillis), Streptococcus, Bifidobacterium, Collinsella (aerofacens), Butyricicoccus (pullicaecorum),* and *Catenibacterium* were observed after the surgical procedure.

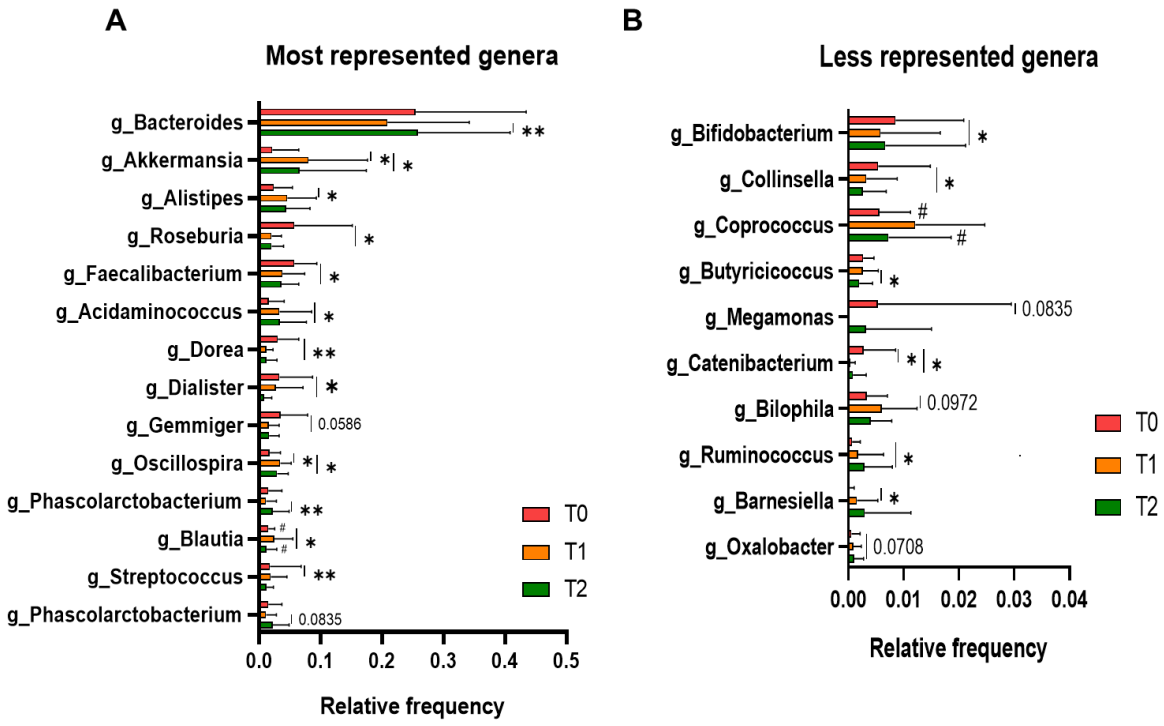

Fig. 7 Effect of bariatric surgery at the genus level on gut microbiota composition of patients with severe obesity. A) Relative frequency of the most represented genera, which resulted significantly modulated after surgery. B) Relative frequency of the less represented phyla showing a significantly modulation after BS. Statistics were calculated using nonparametric Wilcoxon signed-rank test. # and * $p < 0.05$; ** $p < 0.01$. T0 vs T1, T0 vs T2 and T1 vs T2.

3.2.3 Saliva alpha and beta diversity analysis

To characterize the microbiome in saliva samples an ASV-based analysis using QIIME 2 was performed. We studied the optimal parameters for sequence denoising, using DADA2. The sequencing depth ranged from 1,782.0 to 58,981.0 (mean 30,269.0), and the number of features was 7,408 in 99 subjects after the DADA2 denoising step. After rarefying the feature tables to 11,837 sequences per sample, we noticed that all the samples present a good coverage. We found a significant increase in alpha diversity at T1 with Faith's PD (T0 vs T1 $p = 0.028184$, Kruskal–Wallis test) (Fig. 8A), while no significant variations were observed with ASVs, Shannon's index and Pielou's evenness in the post-surgery time (Fig. 8B, C and D).

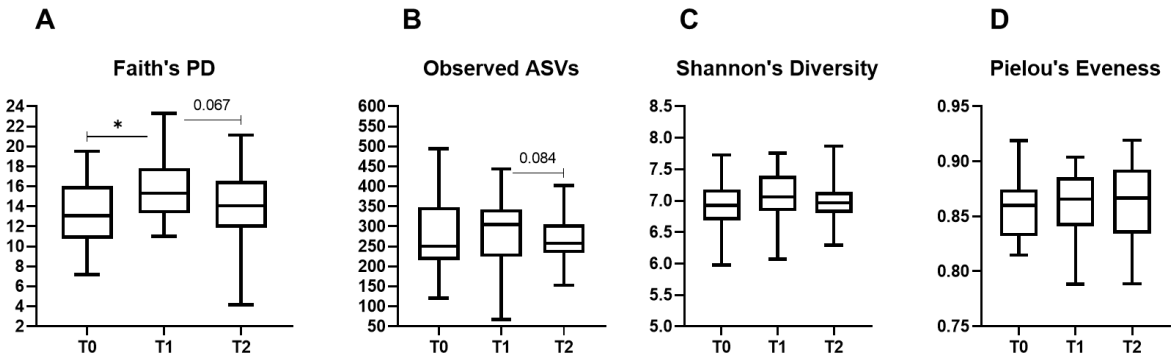

Fig. 8 Alpha diversity among groups. Diversity for: A) Faith PD (T0 vs T1 p-value= 0.028, Kruskal–Wallis all groups p-value= 0.059); B) ASVs (p-value= 0.268, Kruskal–Wallis all test), C) Shannon index (p-value= 0.628, Kruskal–Wallis test); D) Pielou's evenness (p-value= 0.449, Kruskal–Wallis test). *p<0.05. The number of samples at different time points was T0=34, T1=33 and T2=30.

Phylogenetic methods (UniFrac distance) were used to analyse the beta diversity. A change to the limit of significance is observed only six months after bariatric surgery when using the unweighted (T0 vs T1 Pseudo-F=1.405, p=0.060, PERMANOVA) and weighted UniFrac distance (T0 vs T1 Pseudo-F=2.221, p=0.050, PERMANOVA) (Fig. 9A and B). Moreover, similarly to what reported for the feces, also in saliva microbiota, the different time groups (T0, T1 and T2) could not be clearly separated by principal coordinates analysis. However, interesting changes are observed in a small number of samples after BS.

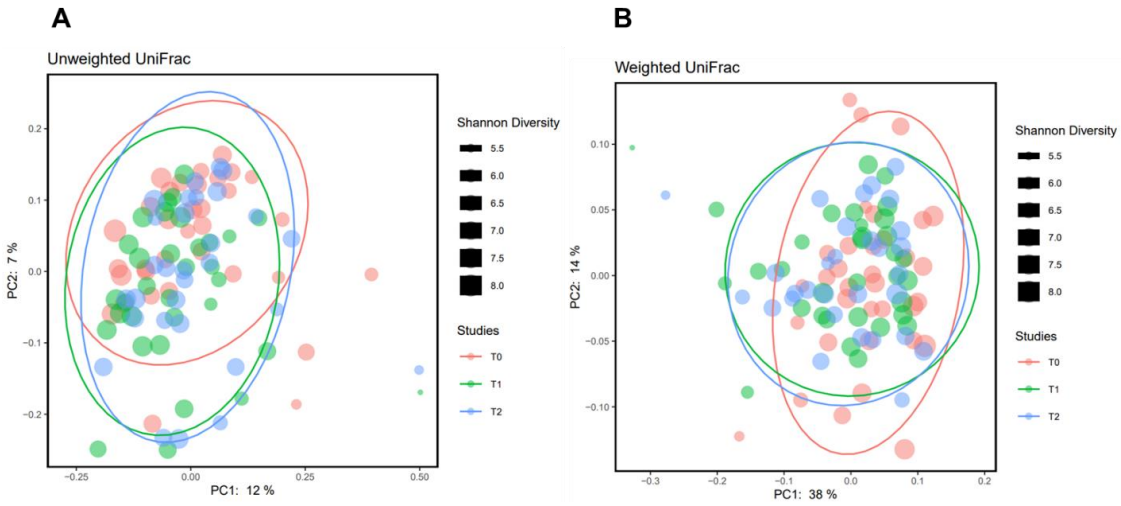

Fig. 9 Principal Coordinate Analysis (PCoA) plots of beta diversity. Statistical significance between T0 vs T1 and T0 vs T2 groups using distance matrices for beta-diversity: (a) unweighted UniFrac distance, (b) weighted UniFrac distance. Statistics were calculated using pairwise PERMANOVA with 999 permutations. The ellipses represent the 95% confidence interval for each time. The size of the dots represents the values of Shannon's diversity.

3.2.4 Saliva microbial community composition before and after BS

In saliva samples, at the phylum level, the main genera were *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, which occupied about 80% of the oral microbial community composition (Fig. 10A). *Firmicutes* one of the most represented phyla present a significant increase 6 months after BS. In contrast, *Bacteroidetes* and *Proteobacteria* showed a time-dependent decrease after BS (Fig. 10B). Interestingly, significant changes have been observed even in less represented phyla such as *Saccharibacteria (TM7)*, *Spirochaetes* and *Synergistetes* that significant increase after the intervention, while *Absconditabacteria (SR1)* showed a significant decrease only 6 months after BS (Fig. 10C).

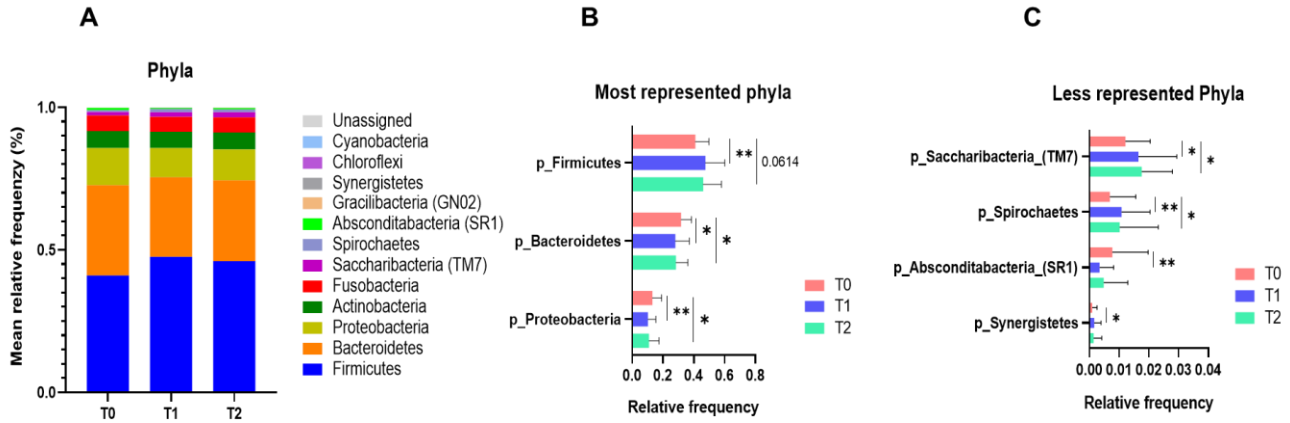

Fig 10. Effect of bariatric surgery at the phylum level on saliva microbiota composition of patients with severe obesity. A) Relative frequency average of the total phyla detected in saliva microbiota composition before (T0) and 6 months (T1) and 12 months (T2) after bariatric surgery. B) Relative frequency of the most represented phyla which resulted significantly modulated after surgery. C) Relative frequency of the less represented phyla showing a significantly modulation after BS. Statistics were calculated using nonparametric Wilcoxon signed-rank test. * $p < 0.05$; ** $p < 0.01$. T0 vs T1, T0 vs T2 and T1 vs T2.

Also, in the case of the saliva microbiota the most remarkable differences were observed at the genus level (Fig. 11). The most abundant genera are shown in figure 11A. Interestingly, the genus *Streptococcus* increases significantly after BS. In contrast, the genus *Prevotella* and *Neisseria* decrease consistently after the intervention. In figure 12B are represented the less represented genera which are modulated by BS. In particular, the genus *Saccaribacteria_(TM7)_(G-1 and G-6)*, *Selenomonas*, *lactobacillus*, *Treponema* and *Lachnoaerobaculum* significantly increase after BS. In the other hand, *Absconditabacteria (SR1)*, *Tennerella* and *Peptostreptococcus* involved in the onset of oral inflammatory disease, like periodontitis, showed a reduction after surgery (Jepsen et al., 2021).

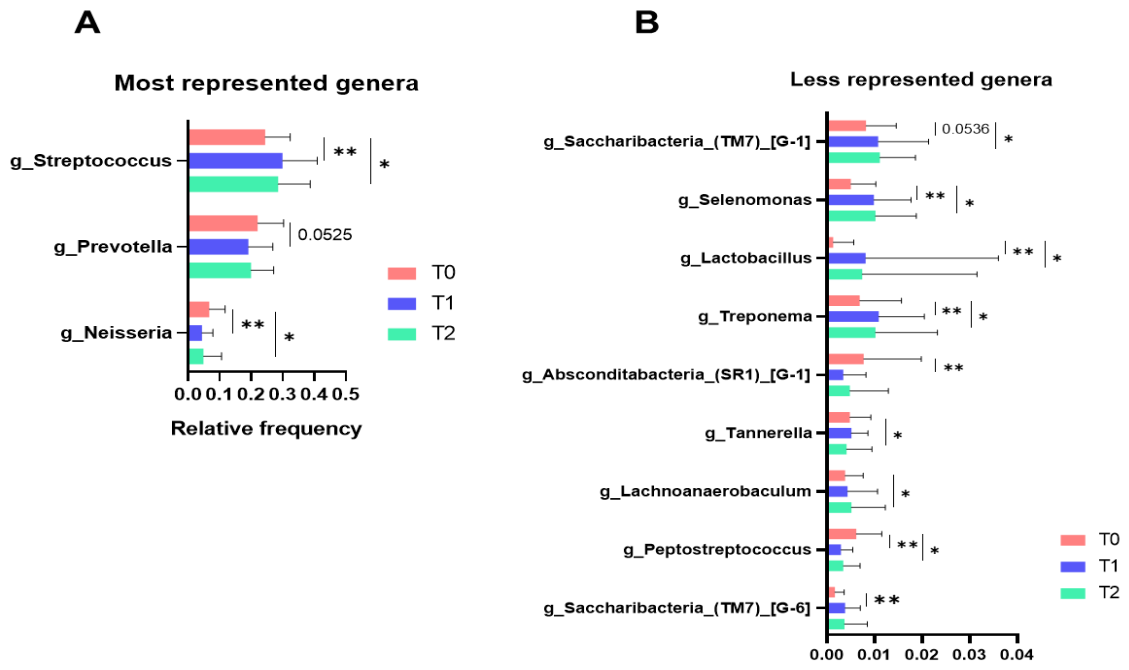

Fig 11. Effect of bariatric surgery at the genus level on saliva microbiota composition of patients with severe obesity. A) Relative frequency of the most represented genera which resulted significantly modulated after surgery. B) Relative frequency of the less represented phyla showing a significantly modulation after BS. Statistics were calculated using nonparametric Wilcoxon signed-rank test. * $p < 0.05$; ** $p < 0.01$. T0 vs T1, T0 vs T2 and T1 vs T2.

3.3 Functional prediction of the feces and saliva microbiome before and after BS.

In order to characterize, at the biological level, the microbiota composition changes observed after BS, PICRUSt2 was used to predict functional pathways. This method is based on KEGG annotations, KO was predicted at T0, T1 and T2 groups in feces and saliva samples. MaAslin2 was applied to identify the distinct KOs in the comparison between T0 vs T1 and T0 vs T2 groups. Among the predicted KO pathways, we found 204 KOs with significant associations after BS (T0 vs T1 and T0 vs T2) in feces samples (FDR $q<0.05$). In Fig.12, the top 50 KOs with the highest q-value have been reported. Conversely, no significant surgery-related changes among the predicted KO pathways were observed in saliva samples. In stool samples, we used the KO database of molecular functions (http://www.kegg.jp/)(Kanehisa et al., 2016). First of all, it should be emphasized that the majority of KOs, modulated after BS, resulted to be strictly related to microbial pathways involved in the metabolism of carbohydrates, lipids, amino acids and nucleotides, as well as responsible for signal transduction, cell growth and cell death processes. Among the top 50 KOs, significantly modulated 6 months after BS, only 11 (K12283, K00688, K02674, K05602, K06992, K07540, K07540, K07543, K07545, K07605 and K11741) maintained their status versus the T0 counterpart, up to one year after intervention. Then, we focused our attention to these 11 KOs. For carbohydrate metabolism the changes of the pathway known to be involved in starch and sucrose metabolism (K00688 glycogen

phosphorylase), testify a significant negative association after BS. Interestingly, this KO is also linked to the modulation of the insulin signaling pathways in agreement with the restoration of the insulin sensitivity which we observed in our cohort after intervention. Inversely, the pathways involved in histidine metabolism (K05602 histidinol-phosphatase) and in toluene degradation (K07540 benzylsuccinate synthase, K07543 benzylsuccinate CoA-transferase BbsE subunit and K07545 (R)-benzylsuccinyl-CoA dehydrogenase) were positively associated. Furthermore, we have found KOs, positively associated, which are directly involved in specific bacterial functions, such as, electrochemical potential-driven transport (K11741 quaternary ammonium compound-resistance protein SugE), secretion system (K02674 type IV pilus assembly protein PilY1 and K12283 mshM; MSHA biogenesis protein MshM), bacterial proteins motility (K02674 type IV pilus assembly protein PilY1) and cytoskeleton formation (K07605 type II keratin, basic).

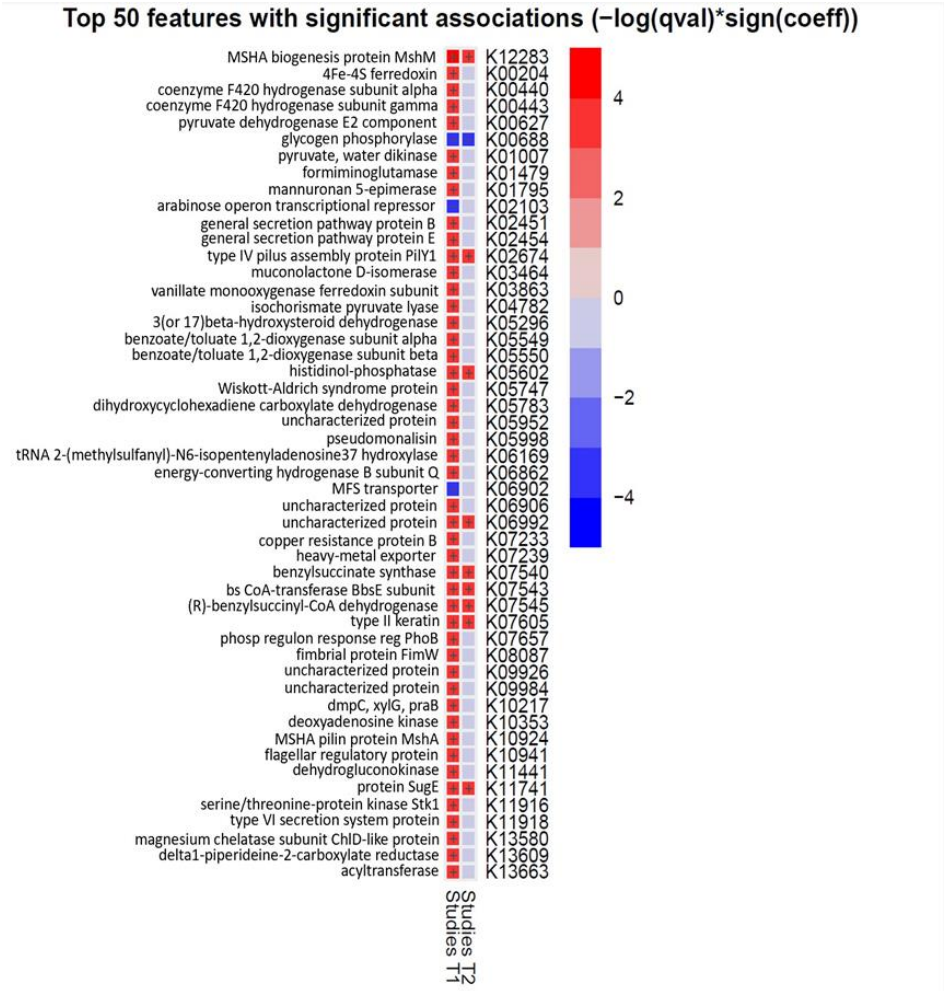

Fig 12. PICRUSt2, KEGG Orthology (KO) prediction in T0, T1 and T2 groups. The Heatmap shows the top 50 pathways with significant associations before and after the bariatric surgery (T0 vs T1 and T0 vs T2) detected by MaAsLin 2's, calculating using the logarithm of the p.value corrected with the Benjamini-Hochberg method and the sign of the coefficient of the contrast variables.

3.4 Analysis of molecular mitochondrial markers

The mitochondria play a vital role in metabolism, producing energy (ATP) from carbohydrates, lipids and proteins. Moreover, the excess of nutrients leads to an increased mitochondrial production of ROS, which causes mitochondrial dysfunction. In this study, we followed the expression level of i) SIRT3, a known key regulator of mitochondrial activity; ii) PGC1-α, the master regulator of mitochondrial biogenesis and iii) Drp1 a key regulator of the mitochondrial fission which is a crucial step for the removal of damaged organelles (mitophagy). In addition, the analysis of the absolute mtDNA-CN in PMBC has been also carried out during the follow up. As shown in figure 13 a significant increase in the expression levels of SIRT3, PGC1-α and Drp1 has been observed both after six months and one year after the intervention. Interestingly, the ratio between mitochondria DNA and nuclear DNA (mitDNA/nuclDNA) showed a significant reduction one year after the BS.

These data suggest that, after the weight loss intervention, there is at least a partial restoration of both functionality and mitochondrial dynamics in parallel to the restoration of eubiosis in oral and gut microbiome.

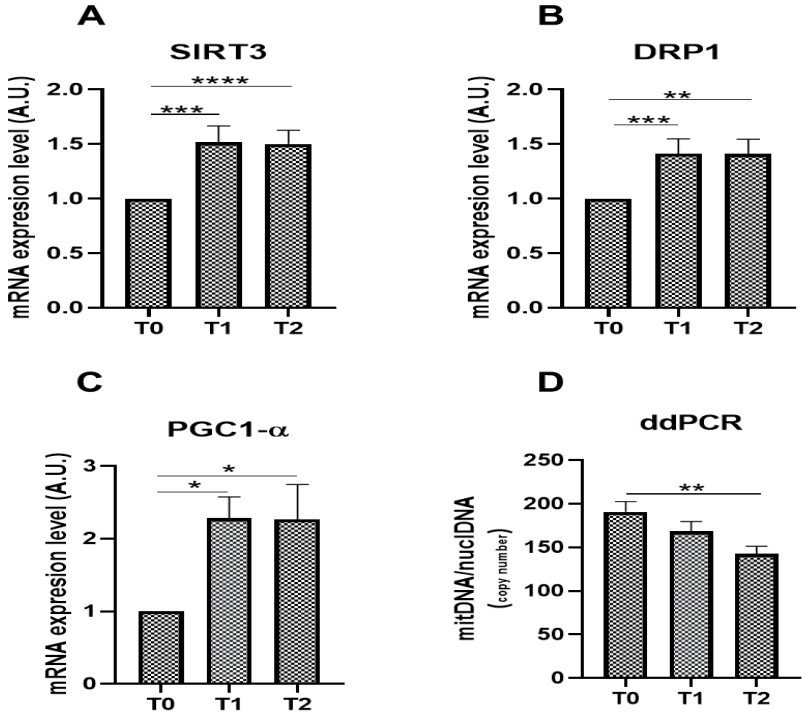

Fig. 13 Analysis of gene expression levels and mtDNA/nuclDNA in PBMC by RT-PCR and ddPCR, respectively. A) SIRT3, B) PGC-1α and C) DRP-1 expression level at the baseline (T0) and after six (T1) and twelve (T2) months of BS. C) ddPCR analysis of the ratio between mitochondrial DNA and nuclear DNA (mtDNA/nuclDNA). The number of patients analyzed at different time points are: T0 (36), T1(33), T2 (32). Statistics were calculated using two-sample tests Mann Whitney test for gene expression analysis and nonparametric Wilcoxon signed-rank test for ddPCR analysis (mean and SEM). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. T0 vs T1, T0 vs T2 and T1 vs T2.

4. Discussion

It is well known that dietary behavior plays an important role in the balance and composition of the intestinal microbiota and the dysbiosis is a common feature of several NCD such as, T2DM and obesity. NCD are also characterized by a sterile chronic low-grade inflammation. In this longitudinal study, a cohort of severe obese patients without any comorbidities, who were undergoing a BS (sleeve gastrectomy) intervention, has been enrolled and the analysis of the gut and oral microbiome have been carried out. Furthermore, in searching for predictive biomarkers and in order to investigate the molecular mechanisms which could underlie this metabolic disease, the 16s metagenomics approach has been integrated with clinical and molecular analysis. A complete panel of clinical and anthropometric analysis has been collected and the modulation of molecular players, known to have a crucial role in the mitochondria homeostasis at different levels (genome integrity, dynamics and biogenesis), have been analyzed in peripheral blood. In general, the modulation of clinical-anthropometric parameters and the stool and saliva microbiome composition in obese patients showed an important recovery to a healthier profile after BS, although with some notable exceptions. An early improvement in anthropometric and systemic inflammation parameters has been observed, whereas LDL-Chol levels did not vary and surprisingly total cholesterol showed a modest, tardive increase. Interestingly, a similar trend has been already reported and mainly ascribed to a specific typology of BS, in particular to the purely restrictive interventions, like sleeve gastrectomy (Heffron et al., 2016). Furthermore, the early changes in circulating levels of adiponectin and leptin, indicating an improvement of the adipose tissue function, were associated to the restoration of the insulin sensitivity in agreement with what we observed in our cohort (Rafey et al., 2020). Since the main effect of leptin, *via* hypothalamic signaling, is to suppress the appetite, thus contrasting the weight gain, it might seem counterintuitive that after sleeve gastrectomy we have observed a reduction of this adipokine rather than an increase. However, this phenomenon is well described and may be due to 'leptin resistance', whereby obese individuals paradoxically have higher levels of circulating leptin but reduced leptin sensitivity (Gruzdeva et al., 2019). Another consideration is the interaction between leptin and adiponectin. In normal-weight people, leptin enhances the adiponectin secretion whereas this effect is lost in patients with obesity due to the action of caveolin-1, which attenuates leptin-dependent adiponectin increase (Singh et al., 2016).

It is interesting to note that the healthier phenotype observed after BS is associated to a relevant remodeling in oral and gut microbiota. In order to investigate the dynamics of the oral/gut microbiome associated with BS and to identify potential bacterial biomarkers, we performed various statistical analyses. First, it should be stressed that analysis of microbial diversity shows different trends for the oral and gut microbiome diversity. A significant increase of stool microbiome diversity has been observed, both six months and one year after BS, whereas no significant changes are observed in saliva microbiota up to the end of the follow

up. The changes in the gut microbiome diversity are in agreement with previous studies conducted both in humans and in animal models (Kong et al., 2013; Guo et al., 2017; Aron-wisnewsky et al., 2019). However, a reduced diversity of the oral microbiota has been reported to indicate a good oral health status including less number of decayed teeth, periodontal pockets and bleeding (Takeshita et al., 2016). Furthermore, in contrast to what is observed in the gut microbiome, the saliva microbiome is likely not directly affected by the bariatric surgery, but its composition can be altered indirectly. Consistently, it was previously reported that the hyperglycemia, found in obesity, causes elevated levels of glucose in the saliva that promote the growth of bacteria associated with dental caries and gingivitis, while the reduction of glycemia, observed after weight loss, could increase the growth of beneficial bacterial species (Goodson et al., 2017).

The second important message comes out from the taxonomic analysis. At the baseline, the severe obese patients are characterized by both an increase in the proportion of bacteria belonging to the *Firmicutes* phylum and a reduction in those belonging to the *Bacteroidetes* one. It is known that higher *Firmicutes*/*Bacteroidetes* can contribute to gut dysbiosis and the related systemic metabolic dysfunctions. In agreement, in our cohort we observed a significant decrease in the *Firmicutes*/*Bacteroidetes* one year after intervention. Noteworthy, it has been clarified that a high proportion of *Firmicutes* induces a major metabolic degradation of several sources of energy, increasing the caloric absorption and the consequent weight gain (Kallus & Brandt, 2012). The taxonomic analysis at a deeper level, i.e., at the genus level, further highlights the complexity of the rearrangements induced by this intervention. In agreement with previous studies, a significant increase of *Akkermansia* and a decrease of butyrate-producing genera, such as *Roseburia*, *Dorea and Faecalibacterium* have been observed in our patients, at both six and twelve months after BS (Liu et al., 2017; Ilhan et al., 2020). Although, the impact of specific microbial assets on the obesity-related comorbidities is still under investigation, recent studies have already shown that an increase of *Akkermansia* may be associated to a healthier profile in BS patients (Shen et al., 2019). Mechanistic studies have shown that *Akkermansia muciniphila* has anti-inflammatory and antidiabetic properties (Cani & de Vos, 2017). In a recent randomized trial has been shown that supplementation of *Akkermansia muciniphila* for 3 months can restore insulin sensitivity, improve the blood lipids asset and reduce inflammation in obese subjects, suggesting a potential therapeutic use of this probiotic (Depommier et al., 2019). Although there is a general consensus about the crucial role of the butyrate producers' bacteria in the obesity related comorbidities recover, contrasting data have been collected (Aron-wisnewsky et al., 2019). It could be of great interest to extend the follow-up for longer time after BS to understand the role of these bacteria, as short chain fatty acids (SCFA) producers, both in the maintenance of the weight loss and the healthier phenotype. PICRUSt2 analysis has been applied to predict the potential functional role of the observed microbiome changes in severe obese patients. It should be noted that at six months after surgery there is a large rearrangement of

the various bacterial pathways; most of them revert to the initial status, except for some pathways whose modulation persists after twelve months. In particular, one year after surgery, we observe a reduction of a pathway involved in starch and sucrose metabolism (glycogen phosphorylase) and directly involved in blood glucose levels, which is in line with the restoration of insulin sensitivity and the normal glycemic level observed in our patients (Aller et al., 2011).

There are many factors that can influence the saliva microbiome composition of an individual, such as dental hygiene, oral health status, nutritional composition of the food and immunological factors. Furthermore, the individual-specific resident bacteria may have a protective role preventing the colonization by harmful bacterial species. Although several studies have shown that the saliva microbiota from obese people significantly differs from normal weight subjects, it is still an open question whether the saliva microbiota can concur to the healthier phenotype observed after BS (Wu et al., 2018).

Mitochondrial functionality is the result of a fine-tuned balance between biogenesis, morphology and mitophagy. The impairment of this quality control and the consequent engulfment of the cells with dysfunctional organelles lead to the release and accumulation of DAMPs able to trigger inflammatory response. Several mitochondrial components, such as cell free mitochondrial DNA, cardiolipin, ATP, mitochondrial transcription factor and N-formyl peptides are known as DAMPs. Therefore, mitochondrial dysfunction induces inflammation and the inflammation itself causes mitochondrial dysfunction creating a vicious cycle.

In our study a significant reduction of the mitDNA-CN was observed during the follow up in PBMC samples, inversely associated to an increase of the gene expression levels of i) PGC1-α, the master regulator of mitochondrial biogenesis, ii) SIRT3, a deacetylase at mitochondrial localization and iii) Drp1, a crucial factor of mitochondrial fission. The apparently discordance between the PGC1-α activation and the reduction of the mitDNA-CN might suggest a concomitant activation of the removal of dysfunctional organelles and the induction of mitochondrial biogenesis. Interestingly, this hypothesis is consistent with the activation of Drp1, which is essential for the degradation of mitochondria and the subsequent elimination of them by mitophagy (Kleele et al., 2021). Furthermore, the significant up regulation of SIRT3, which is a well-known regulator of energy metabolism, is perfectly in agreement with animal and human studies which testified that a reduced activity of this sirtuin is strongly associated to a higher risk of obesity and metabolic syndrome development (Hirschey et al., 2011). In addition, recent studies suggest that the increase of the mitDNA-CN may be a sort of compensatory response due to the accumulation of damaged mitochondrial genomes. In particular, the presence of 8-oxoguanine residues in the D-loop region increases mitochondrial transcription factor A binding inducing mitochondrial genome replication. Thus, the decrease of the mitDNA-CN observed after BS could be due to the reduction of oxidative DNA damage of the

mitochondrial genome (Pastukh et al., 2016). All together, these molecular data strongly suggest that the recovery of the mitochondrial homeostasis might be, at least partially, responsible for the healthier profile observed after BS.

5. Conclusions and future perspectives

The present study assessed the impact of the BS on fecal and saliva microbiome in a cohort of severe obese patients. The 16s metagenomics analysis has revealed the high complexity and diversity of microbial communities living in the oral cavity and intestine. A clear restoration of eubiosis, at both gut and oral microbiota level, has been observed after BS concomitantly to the restoration of a healthier status as testified by an integrative analysis of the clinical and anthropometric profiles. In addition, after BS a significant recovery of the mitochondrial homeostasis at the systemic level has been observed. All together these data strongly suggest a causal role of the microbiota in obesity as well as in the obesity related comorbidities onset.

Further metagenomics and metabolomics association studies are necessary to understand how gut/saliva microbiota composition and derived microbial metabolites can affect the host health and, in particular, how can concur to the development of the co-morbidities frequently associated to the obese phenotype. Since oxidative stress, mitochondrial dysfunction and gut dysbiosis seem to be closely interconnected in the pathogenesis of obesity, a better understanding of the mechanisms underlying this crosstalk could allow the development of new potential therapeutic strategies in obesity management as well as the identification of predictive biomarkers of metabolic syndrome development.

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