

Investigation of the Duodenojejunal Microbiome in Human Obesity

Emilie Steinbach

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Sorbonne University

École Doctorale de Physiologie, Physiopathologie et Thérapeutique

UMR-S 1269 – Nutrition and Obesities: Systemic Approaches (NutriOmics)

Investigation of the Duodenojejunal Microbiome in Human Obesity

Doctoral Thesis

Presented by Emilie STEINBACH

Directed by Prof K. CLEMENT, Supervised by Dr T. LE ROY

Presented and publicly defended on September 29th 2023, to the Jury composed of:

Prof Harry SOKOL, MD, PhD, PU-PHPresidentProf Julie-Anne NAZARE, PhDRapporteurProf Gabriel PERLEMUTER, MD, PhD, PU-PHRapporteurDr Benoit CHASSAING, PhDExaminerDr Tiphaine LE ROY, PhDThesis SupervisorProf. Christine POITOU, MD, PhD, PU-PHThesis co-director, InvitedProf Karine CLEMENT, MD, PhD, PU-PHThesis Director

In the presence of my peers,

With the completion of my doctorate in science, in my quest for knowledge, I have carried out demanding research and demonstrated intellectual rigour, ethical reflection, and respect for the principles of research integrity.

As I pursue my professional career, whatever my chosen field, I pledge, to the greatest of my ability, to maintain integrity in my relationship to knowledge, my methods and my results.

Je tiens à remercier l'ensemble des membres de mon jury.

Merci au Professeur Harry Sokol d'avoir accepté d'en être le président.

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LIST OF ABBREVIATIONS

ALAT:	ALAnine Transaminase
ALAI: AMPs:	
INSERM:	AntiMicrobial peptides
	Institut National de la santé et de la Recherche Médicale
ANSM:	Agence Nationale de Sécurité du Médicament et des produits de santé
ASAT:	ASpArtate Transaminase
AT:	Adipose Tissue
AUDIT :	Alcohol Use Disorder Test
BAT:	Brown Adipose Tissue
B6:	Black six mice (C57BL/6)
BAs:	Bile Acids
BDI:	Beck Depression Inventory
BMI:	Body Mass Index
BS:	Bariatric Surgery
CCK:	CholeCystoKinin
CFUs:	Colony-Forming Units
CRP:	C-Reactive Protein
Ctrl group:	ConTRoL group
CVD:	Cardio-Vascular Disease
DEBQ:	Dutch Eating Behaviour Questionnaire
DEXA:	Dual-Energy X-ray Absorptiometry
DJ:	DuodenoJejunal
DJF:	DuodenoJejunal Fluid
DMEM:	Dulbecco's Modified Eagle Medium
DNA:	DeoxyriboNucleic Acid
DPP-IV:	DiPeptidyl Peptidase-4
DPP-IVi:	DPP-IV Inhibitor
e-CRF:	electronic-Case Report Form
EEC:	Entero-Endocrine Cells
FDR:	False Discovery Rate
BH method:	Benjamini Hochberg
FM:	Faecal Microbiome
FMT:	Faecal Microbiota Transplants
FXR:	Farnesoid X Receptor
GF:	Germ Free
GGT:	Gamma-Glutamyl Transferase
GI:	GastroIntestinal
GIP:	Gastric Inhibitory Polypeptide
GIT:	GastroIntestinal Tract
GLP-1:	Glucagon-Like Peptide-1
GM:	Gut Microbiome
H2RA:	Histamine Type-2 Receptor Antagonists
HAD:	Hospital Anxiety and Depression scale
Hba1c:	Glycated Haemoglobin / A1c
HDL:	High-Density Lipoprotein
HFD:	High Fat Diet

HOMA-ir:	Homeostatic Model Assessment for Insulin Resistance		
IBD:	Irritable Bowel Disease		
IBS:	Irritable Bowel Syndrome		
IL-6:	InterLeukin-6		
IL-8:	InterLeukin-8		
IL-3:	Type 3 Innate Lymphoid Cell		
Inc.	Imidazole Propionate		
Je-MiMe:	-		
	Jejunal Microbiota in Metabolic Disease		
Je/Col-MiMe:	Jejunal or Colonic mucosa-associated -MiMe		
LDL:	Low-Density Lipoprotein		
LPS:	LipoPolySaccharides		
LZ:	LysoZyme		
MCP-1:	Monocyte Chemoattractant Protein-1		
NASH:	Non Alcoholic SteatoHepatitis		
NEB:	New England Biolabs		
NOD2:	Nucleotide Binding Oligomerization Domain Containing 2		
Ob group:	Obese group (obese participants without type 2 diabetes)		
ObD group:	Obese and type 2 Diabetes group (Ob with type 2 diabetes)		
OGTT:	Oral Glucose Tolerance Test		
OM:	Oral Microbiome		
OMS/WHO:	Organisme Mondial de la Santé/World Health Organisation		
ONT:	Oxford Nanopore Technologies		
OSA:	Obstructive Sleep Apnea		
PCR:	Polymerase Chain Reaction		
PHQ-9:	Patient Health Questionnaire		
PP:	Pancreatic Polypeptide		
PPI:	Proton Pump Inhibitor		
PSS-10:	Perceived Stress Scale		
Reg3:	Regenerating islet-derived protein (i.e., $\text{Reg}3\gamma$)		
RNA:	RiboNucleic Acid		
rRNA:	ribosomal RNA		
SAA:	Serum Amyloid A		
SCFAs:	Short Chain Fatty Acids		
SGB :	Species Genome Bin (Metaphlan4 catalogue)		
SI:	Small Intestine		
SIBO:	Small Intestinal Bacterial Overgrowth		
SPF:	Specific Pathogen-Free		
T2D:	Type 2 Diabetes		
TLR(s):	Toll Like Receptor(s)		
TNFα:	Tumour Necrosis Factor-alpha		
TSH(-us):	Thyroid Stimulating Hormone (ultrasensible)		
UCP-1:	UnCoupling Protein-1		
USI:	Upper Small Intestine		
USIM:	USI Microbiome		
WAT:	White Adipose Tissue		
W test:	mann-whitney-Wilcoxon non-parametric test		
WT:	Wild Type		

INTRODUCTION

1. Obesity

Introduction to Obesity: Definition and Epidemiology

Humanity is facing a constantly rising epidemic of obesity and inter-related metabolic disorders, of which type 2 diabetes mellitus (T2D) and cardiovascular diseases (CVD) have the highest mortality rates (Flegal et al., 2013, p. 201; Nathan, 2008; Sturm and Hattori, 2013; WHO, n.d.). Not only is there an increase in the total number of individuals with obesity, with a prevalence as high as 30% in adults over the age of 50 in Europe, but also an increase in the average body weight in the world population (Figure I.1.1). In France, in 2020, 17% of adults were obese (Odoxa et ObEpi-Roche, n.d.). Linear time trend forecasts suggest that 51% of the world population will be obese by 2030 (Finkelstein et al., 2012).

This epidemic is a significant public health threat and pressures healthcare systems and economies worldwide (WHO, n.d.). Moreover, obesity is a personal threat with high economic, physical and mental health costs. However, there is a clinical need to find a solution for effectively preventing and treating these 'nutrition-related' diseases. It is thus a top public health priority and a significant challenge in dire need of investigation.

Overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health (WHO, n.d.). The most widely used measure to characterise an individual's body size is the Body Mass Index (BMI). It is calculated by taking the body weight in kilograms and dividing it by the square of the height in metres (BMI = weight (kg) / height (m)²). This index allows for determining thresholds to define overweight, obesity, and its various degrees of severity. An individual with a BMI over or equal to 25 kg/m² is considered overweight, a BMI over or equal to 30 kg/m² defines a class I obesity, over or equal to 35 kg/m² corresponds to class II obesity, and beyond or equal to 40 kg/m² defines a class III obesity (WHO, 2010).

BMI is simple to calculate and cost-free. Although BMI remains the most frequently used tool for classifying patients with obesity, it does not reflect body composition or the distribution of fat mass (Salmón-Gómez et al., 2023). However, visceral fat mass or ectopic

adipose tissues (AT) increase health risk compared to subcutaneous and gynoid fat mass, which are thought to play beneficial roles as energy sources. When the storage capacities of subcutaneous AT are exceeded, and the energy balance stays positive, subcutaneous AT accumulates together with an accumulation at the visceral level and as ectopic deposits. Visceral fat corresponds to the adipose deposits that accumulate around the internal organs in the abdominal cavity, and an "android morphology" (as opposed to "gynoid morphology") is associated with an altered metabolic profile and the development of obesity-related complications in numerous studies (Chait and den Hartigh, 2020; Item and Konrad, 2012; Ziegler et al., 2017). Ectopic deposits correspond to increased lipid storage in organs such as the liver, the muscles or the heart and are associated with T2D, liver diseases such as non-alcoholic steatohepatitis (NASH), atherosclerosis and CVD (Britton and Fox, 2011; Krssak and Roden, 2004; Lettner and Roden, 2008; Neeland et al., 2019).

Technologies that are more expensive and more challenging to implement routinely, even in clinical settings, have been developed, such as bioelectrical impedance or Dual-energy X-ray Absorptiometry (DEXA), which determine an individual's lean and fat mass proportions and their location. A complementary measurement can be performed simply: waist circumference, which partially characterises abdominal obesity and the accumulation of visceral fat mass. A waist circumference over 94 cm for men and 80 cm for women indicates an increased risk of developing comorbidities, such as T2D or hypertension, increases (WHO, 2008).

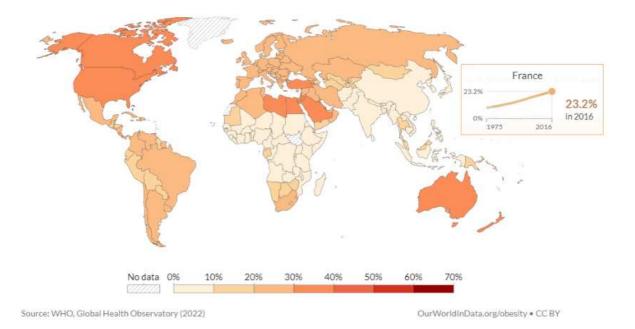


Figure I.1.1 - Epidemiology of obesity worldwide and in France.

Obesity is one of the leading risk factors for premature death. It was linked to 4.7 million deaths globally in 2017. In other words, 8% of global deaths were attributed to obesity in 2017. 13% of adults in the world are obese, 39% of adults in the world are overweight, and one in five children and adolescents globally are overweight. In France, there has been a +155% increase in the prevalence of obesity, with 9.10% in 1975 and 23.20% in 2016. Figure adapted from <u>Ritchie and Roser (2017</u>).

Pathophysiology of Obesity

Obesity is a complex multifactorial disease. In this section, I will briefly summarise some aspects of the pathophysiology of obesity that are relevant to my PhD project. This section is not exhaustive.

Both environmental and (epi)genetic factors are causal determinants in the development of metabolic diseases. The most frequent form of genetic obesity (monogenic obesity) is explained by single or multiple alterations in the leptin/melanocortin pathway, which regulates food intake and energy expenditure (Clément et al., 1998), but it concerns a tiny percentage of obesity, representing only 2 to 3% of childhood and adult obesity (Bell et al., 2005; Hinney et al., 2006; Lubrano-Berthelier et al., 2003). In common forms of obesity, multiple genetic factors were identified, albeit with small effects, thanks to large-scale analyses of genetic variations. Although the heritability of obesity is estimated between 40

and 70% (Elks et al., 2012), the increase in prevalence over the last decades is mainly explained by environmental factors (Malik et al., 2013; Mutch and Clément, 2006).

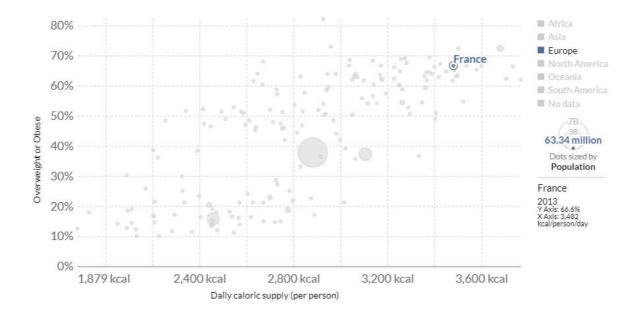


Figure I.1.2 - <u>Share of adult men overweight or obese vs daily supply of calories</u>. In 2013 in France, the daily caloric supply per person, per day, was estimated at 3,482 kcal, and 66.60% of the French population is overweight or obese. <u>Figure adapted from Ritchie and Roser (2017)</u>.

Indeed, industrialisation and economic growth of our "Western Civilizations" transformed the human lifestyle and favoured a positive energy balance that has likely contributed to the rise of metabolic diseases (Heymsfield and Wadden, 2017; McAllister et al., 2009). The two most obvious causes are decreased energy expenditure and increased energy intake. Our modern lifestyle is sedentary (i.e., seated work conditions) with decreased physical activities for leisure (i.e., watching television), resulting in an overall insufficient physical activity (Guthold et al., 2018), and there is an increase in the consumption of high-calorie food with a rise in availability, marketing and perceived convenience. In France, the average caloric intake per person per day was estimated at 3,482 kcal per day, and 66.60% of the population was overweight or obese (Figure I.1.2.) (Ritchie and Roser, 2017). However, in terms of nutrition, there are not only quantitative modifications, with the increase in caloric density, but also qualitative modifications that must be taken into account in the pathophysiology of obesity.

Indeed, traditional, primarily plant-based diets have been replaced by high-fat diets, particularly saturated ones, by high simple and low unrefined carbohydrates diets and transformed foods (Baker and Friel, 2014; Popkin et al., 2012; Swinburn et al., 2011). Some transformed energy-dense foods are sometimes called "empty calories" as, despite their high caloric density, they are known to provide few micronutrients essential to health. Paradoxically, undernutrition is frequent in patients with obesity and exposes these individuals to various health complications (Barazzoni and Gortan Cappellari, 2020; WHO, n.d.).

Traditional diets were rich in fibres, known to promote satiety and, thus indirectly, weight gain and overall metabolic health by reducing food intake (Astrup et al., 1990; Poutanen et al., 2017; Rigaud et al., 1987; Wanders et al., 2011). Moreover, fibres are also proposed to blunt the digestibility of proteins and fatty acids and to increase faecal excretion (Baer et al., 1997; Miketinas et al., 2019), to promote gut health and gut microbiome and host symbiosis (Bretin et al., 2023; Menni et al., 2017; Zhong et al., 2022).

Obesity is a complex multifactorial disease not only due to a dysregulation of the food quantity or quality (<u>Blüher, 2019</u>). Several other pathophysiological elements are associated with the development and maintenance of obesity with time.

For instance, hallmark psycho-emotional factors, such as increased psychological stress and depression, but also inappropriate sleep, result in neuroendocrine perturbations that impact energy homeostasis, appetite control, glucose tolerance, and energy expenditure (Knutson et al., 2007; Mullington et al., 2003; Spiegel et al., 2004). It is important to note that these psycho-emotional factors can be causes *and* consequences of metabolic diseases. Positive feedback loop mechanisms further aggravate mental and physical conditions (Faith et al., 2011; Heymsfield and Wadden, 2017). In this line, the cohort I constituted to answer my PhD hypothesis was extensively phenotyped, including sleep and psycho-socio-emotional questionnaires in addition to lifestyle questionnaires.

Along with this myriad of environmental triggers perturbing energy balance (Heymsfield and Wadden, 2017), biological maladaptation of organs and their altered communication contribute to the chronic aspect of obesity, meaning the maintenance of obesity and its resistance to treatment.

Many studies depict an altered molecular and cellular cross-talk between the immune system, AT depots and organs, such as the liver, muscle, intestine and brain (DeFronzo et al., 2015; Kwon and Pessin, 2013; Touch et al., 2017). Recently, the gut microbiome (GM) has also been increasingly discussed and recognised as an important factor in the development and chronicity of obesity; this will be discussed later in this introduction (subsection: <u>Gut</u> <u>microbiome in Obesity</u>).

In the following section, I will further develop the concept of chronic, low-grade inflammation and the AT remodelling observed in obesity.

Chronic Low-Grade Inflammation

Obesity is characterised by chronic low-grade inflammation. Unlike acute infections, which trigger a short and localised immune response, inflammation in obesity involves a moderate increase in inflammatory markers concentrations that persist over time (Hotamisligil, 2017a; Kotas and Medzhitov, 2015; Medzhitov, 2008).

There is more than one cause and, or one origin of inflammation. Amongst many others, lifestyle changes, such as physical activity or diet, can trigger inflammation (Calder et al., 2011). Western-transformed diet has been shown to alter intestinal homeostasis and gut epithelial integrity, thus triggering a local inflammation that can subsequently and independently lead to low-grade peripheral inflammation, notably due to the passage of bacterial fragments in the circulation (Calder et al., 2011; Cani et al., 2008; Chassaing et al., 2017b, 2017b; Monteiro-Sepulveda et al., 2015; Winer et al., 2016). In addition, inflammation has multiple origins within the body, such as the heart, liver, muscles or particularly the AT (discussed below). Local stress tissue will further recruit inflammatory markers and contribute to tissue dysregulation and metabolic deterioration.

Indeed, chronic inflammation is associated with the infiltration of immune cells such as macrophages, dendritic cells, and mast cells, which contributes to the progressive dysfunction of critical tissues such as the heart, muscles, liver or particularly the AT and the development of related comorbidities, such as insulin resistance, T2D, and CVD (Hotamisligil, 2006; Kotas and Medzhitov, 2015; Nathan, 2008; Tilg and Moschen, 2006).

Chronic inflammation alters the organ's cross-talk between organs. It contributes to insulin resistance in AT (discussed below) and other insulin-target tissues and has systemic repercussions that contribute to functional alterations in other organs. An increase in proinflammatory signals is observed, amongst others, in the liver, muscles (Fink et al., 2014), pancreatic islets, central nervous system, and intestine (Winer et al., 2016).

Metabolic regulation and immune response are highly interconnected, and the function of each is dependent on the other (Hotamisligil, 2006). Disruption of this interaction supports the development of chronic pathologies, including obesity and T2D (Kotas and Medzhitov, 2015). It is essential to highlight the vicious circle: metabolic alterations trigger inflammation, further aggravating their function and thus increasing metabolic alterations and exacerbating inflammation.

Adipose Tissue Inflammation and Remodelling in Obesity

Adipose tissue mainly comprise adipocytes. Briefly, we can differentiate two types of adipocytes: white adipocytes making up the White Adipose Tissue (WAT) and brown or beige adipocytes, making up the Brown Adipose Tissue (BAT) <u>(Sakers et al., 2022)</u>.

BAT partially contributes to thermogenesis through increased uncoupling protein-1 (UCP-1) expression. UCP-1 is located in the inner mitochondrial membrane and enables a leak of protons down the electrochemical gradient generated by the Electron Transport Chain, bypassing ATP synthase. This uncoupling process dissipates the potential energy of the gradient as heat instead of storing it via ATP synthesis. UCP-1 is activated under cold conditions leading to increased lipolysis and fatty acid oxidation in mitochondria. BAT adipocytes thus require higher energy expenditure and display promising metabolic functions in the study of obesity. However, BAT is less present in adult humans, and most studies have been carried out in rodents (Brandão et al., 2021; Chouchani et al., 2019).

WAT is mainly located in subcutaneous and visceral areas and contains a large vacuole and fewer mitochondria than BAT. WAT plays several functions besides energy storage, including a secretory function. Lipogenesis corresponds to the process of fatty-acid storage (i.e., available from ingested nutrients) in the form of triglycerides. This energy source can be released from adipocytes under energy demands (i.e., fasting, throughout prolonged physical activity) in the form of free fatty acids and glycerol. This process is called lipolysis. The released free fatty acids can be recruited as an energy source from muscles, while the liver can recruit glycerol for gluconeogenesis.

AT also plays an essential endocrine role, secreting numerous adipokines. Adipokines, or adipocytokines, are a diverse group of biologically active substances, including hormones, enzymes, cytokines, and growth factors (i.e., leptin, adiponectin, monocyte chemotactic protein-1 - MCP-1, - IL-6, TNF α). These substances are produced by adipocytes and the stromal-vascular component of AT, predominantly in subcutaneous AT. Adipokines play a crucial role in regulating metabolism. In particular, adipokines significantly impact insulin sensitivity, chronic inflammation linked with metabolic syndrome, eating behaviour, and energy expenditure.

Additionally, adipokines establish functional connections between AT and various organs and systems, including the cardiovascular, reproductive, skeletal, endocrine, autoimmune, and coagulation systems (Balistreri et al., 2010; Kershaw and Flier, 2004; Marcelin et al., 2019; Ouchi et al., 2011; Sahin-Efe et al., 2012).

AT shows significant plasticity and adapts to allow energy source storage depending on availability (Sakers et al., 2022). In obesity, AT remodelling occurs due to increased energy intake, resulting in modifications to the architecture of WAT. As introduced earlier, the localisation of WAT is also increased in the visceral area and as ectopic deposits. These changes contribute to the chronic nature of obesity and the emergence and worsening of interrelated comorbidities.

Expansion of AT can occur through two mechanisms: an increase in adipocyte size, called hypertrophy, and an increase in the number of mature adipocytes after differentiation of preadipocytes, called hyperplasia. In obesity, there is a shift towards hypertrophy compared to hyperplasia, which is more detrimental from a metabolic standpoint due to associations with decreased insulin sensitivity and increased blood sugar levels (Yang et al., 2012). Larger adipocytes have a higher basal lipolytic capacity, reduced stimulated lipolysis, and contribute

more to inflammation in AT than smaller adipocytes (Laurencikiene et al., 2011; Marcelin et al., 2022, 2019; Poitou et al., 2005).

Because of excessive AT accumulation and subsequent insufficient vascularisation (and low-grade inflammation), there is a development of an inflammatory state (interrelated and also independent from low-grade inflammation) within AT which may further alter organ cross-talk communication, favouring insulin resistance. Resident macrophages mainly populate AT in a balanced and metabolically healthy state. In contrast, in obesity, there is significant recruitment of newly differentiated macrophages, strongly contributing to inflammation (Zatterale et al., 2019).

Finally, these factors (hypoxia, low-grade inflammation, local inflammation of AT) favour fibrosis of AT, which can be characterised by an accumulation of extracellular matrix components, further impairing the tissue's function and plasticity. These mechanisms contribute to the vicious cycle of obesity as fibrosis increases inflammation, further increasing the alterations associated with obesity (Calder et al., 2011; Hotamisligil, 2017b, 2006). There is, thus, in obesity, a complex association of different phenomena that will further alter several mechanisms, including satiety signalling (leptin), energy expenditure (decreased lipolysis) and favour a vicious cycle of obesity (Marcelin et al., 2022; Sun et al., 2013).

The Burden of Obesity and the Historical Lack of Effective Therapies

Obesity presents heavy consequences on physical and mental health. The physical complications associated with obesity include CVD (Britton and Fox, 2011), hypertension, atherosclerosis, and heart failure (Powell-Wiley et al., 2021), which account for the majority of global deaths (Institute for Health Metrics and Evaluation (IHME), 2022). Additionally, obesity is linked to increased prevalence of lung damage and sleep apnea syndrome (Almendros et al., 2020), as well as higher incidence rates of 13 types of cancers, including colon, breast, and bile duct cancers (Basdevant, 2006; Institute for Health Metrics and Evaluation (IHME), 2022; WHO, n.d.). Cancer-related death was the second-leading cause worldwide in 2019 (IHME, 2020). Furthermore, obesity is a significant risk factor for liver diseases, including NASH (Kim et al., 2016), and contributes to the development of interrelated metabolic disorders, such as insulin resistance leading to T2D.

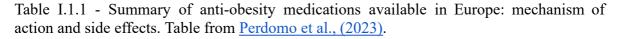
Regarding mental health, obesity is correlated with self-esteem impairment, social exclusion, discrimination, and chronic depression (Wadden and Stunkard, 1985). These factors have economic consequences, such as employability difficulties, reduced access to education, and increased medical costs (Finkelstein et al., 2005). It is crucial to consider the psychological, social, and economic impacts of obesity, as they may serve as both causes and consequences of the condition and further contribute to psychological stress.

Obesity is a major public health issue, and it is necessary to continue the search for effective treatments. There is a need for therapeutic strategies addressing as many targets and causes of obesity as possible. The primary goal is to limit weight gain and, if possible, achieve weight loss, which can result in metabolic improvements with just a 5-10% loss (Bassaganya-Riera et al., 2021; Ochner et al., 2015).

First, lifestyle modifications and psychological support can be implemented to promote weight loss. These include nutritional, physical activity and dietary advice from professionals, encouraging regular physical activity, and psychological support. These interventions can result in a moderate but significant weight loss of 5-10% (Bray et al., 2018; Fock and Khoo, 2013; Oppert et al., 2021; Williamson, 2017). Lifestyle interventions are interesting as behaviour can be modulated to improve health. However, compliance with the interventions and maintenance over time can be difficult for the patients to sustain (Bray et al., 2018; Bray and Ryan, 2021; Ochner et al., 2015).

For patients with moderate to severe obesity, medication can be considered in addition to lifestyle interventions. In Europe, a few drugs are currently available such as Orlistat, Naltrexone-Bupropion, Liraglutide, and Semaglutide (Table I.1.1). These drugs target different modes of action. Briefly, Orlistat inactivates gastric and pancreatic lipase, which limits fat absorption; Naltrexone-Bupropion acts on the central control of appetite (naltrexone is an opioid receptor antagonist, and bupropion is a dopamine and noradrenaline reuptake inhibitor); Liraglutide and Semaglutide also act on the central control of appetite (GLP-1 receptors agonist) and also targets peripheral mechanisms (i.e., slow down GIT transit and gastric emptying, increases satiety, reduces glucose levels) (Perdomo et al., 2023).

	Orlistat	Naltrexone-bupropion	Liraglutide or Semaglutide
Mechanism of action	Inactivation of gastric and pancreatic lipase	Central control of appetite; opioid receptor antagonist (naltrexone); and dopamine and noradrenaline reuptake inhibitor (bupropion)	Central control of appetite (through GLP-1 receptor agonism in hypothalamus and hindbrain) and peripheral actions—eg, slowing gastrointestinal transit and reducing glucose levels
Adverse effects	Steatorrhoea, oily faecal spotting, faecal urgency, and fat-soluble vitamin deficiency	Dry mouth, nausea, vomiting, irritability, diarrhoea, constipation, headache, insomnia, dizziness, hypertension, seizures, and precipitation of mania	Nausea, vomiting, diarrhoea, constipation, dyspepsia, abdominal pain, and headache



These medications have side effects, from steatorrhea for Orlistat to the precipitation of mania for natrexone-bupropion, making the latter less suited for neuropsychiatric patients (Bray et al., 2018). GLP-1 receptors agonists mainly present adverse gastrointestinal effects, although other more serious adverse effects have been reported such as an increased risk of thyroid cancer (Bezin et al., 2023), although findings are contradicted (Hu et al., 2022). Additional studies are required to address the concerns regarding the use of GLP-1 receptor agonists and serious adverse effects including pancreatitis and thyroid cancers (Drab, 2016; Thompson and Stürmer, 2023). Combining two or three therapies targeting various metabolic pathways involved in obesity has also been investigated. Combination therapies can lead to lower doses of each drug and reduce the risk of significant side effects. Several combination treatments are available in Europe, resulting in a weight loss of 3.8-12.7 % through various mechanisms (Perdomo et al., 2023).

In case of severe obesity (patients with a BMI $\ge 40 \text{ kg/m}^2$ or $\ge 35 \text{ kg/m}^2$ and having at least one obesity comorbidity), or if there was a failure to lose weight with the different treatments described above, bariatric surgery (BS) could be performed. BS patients undergo a thorough and comprehensive evaluation of the patient's physical and psychological health and are subject to pre- and post-operative multidisciplinary management (Haute Autorité de Santé, 2009). There are several types of surgical procedures, all of which achieve weight loss by inducing dietary restriction with or without malabsorption after surgical alterations of the

Upper Small Intestine (USI). Four of these procedures are currently included in the recommendations of the HAS in France (Figure I.1.3.) such as the Roux-en-Y gastric bypass (RYGB) and the longitudinal gastrectomy or "Sleeve" (SG) (Rubino et al., 2016).

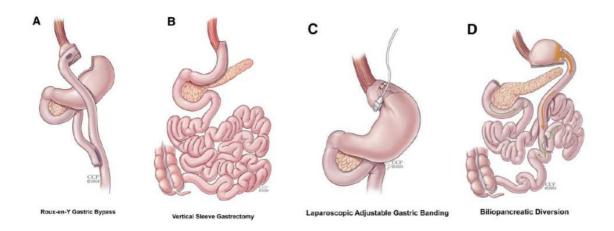


Figure I.1.3 - <u>Diagrams of the four bariatric and metabolic operations included in the</u> recommendations of the HAS in France.

There are several types of surgical procedures, all of which achieve weight loss by inducing dietary restriction with or without malabsorption after surgical alterations of the USI. Four of these procedures are currently included in the HAS recommendations in France. These procedures are the Adjustable gastric banding; Roux-en-Y gastric bypass (RYGB); the Longitudinal gastrectomy or "Sleeve" (SG); the Biliopancreatic diversion with Y-loop. Figure reused from a reprinted version from the Cleveland Clinic Foundation (CCF) from <u>Rubino et al., (2016)</u>.

Over the past decade, Sleeve Gastrectomy (SG) and Roux-en-Y Gastric Bypass (RYGB) have been the most common BS techniques. SG and RYGB achieve approximately 30% body weight loss at twelve months after BS. However, long-term studies revealed a higher efficacy of RYGB compared to SG (27% vs 23% weight loss over 5-7 years of follow up after BS) (Hu et al., 2020; Nielsen et al., 2022; Perdomo et al., 2023). These procedures lead to significant weight loss and improvements in comorbidities like T2D. Studies have shown that BS increases life expectancy, especially for patients with T2D (Adams et al., 2023). However, it is an invasive procedure with potential complications, and patients require life-long medical follow-up and nutritional monitoring due to possible micronutrient deficiencies (Robert, 2016; Wolfe et al., 2016). An interesting study showed that bariatric surgery decreases mortality but increases the risk of suicide commitment by patients after BS.

This risk has been estimated to be 2,4 higher than patients with obesity who did not receive BS (Adams et al., 2023). A comparative study showed that life expectancy was three years longer in patients from the surgery group compared to obese patients that were candidates for BS but did not undergo BS. Patients who underwent BS have a life expectancy 5,5 years shorter than the general population (Carlsson et al., 2020).

Despite the burden of obesity, the current management, although essential, remains insufficient to improve the health and quality of life of patients with obesity. Each participant has an individual response to these treatments, and we still lack the knowledge to predict which patients will respond best to each intervention (Perdomo et al., 2023). The choice of treatment relies on the patient's preference (i.e., based on the cost of medications) and the clinician's evaluation of the benefits-risks of each intervention. In this line, innovative approaches are required, and the GM is now recognised as an actor in the physiopathology of obesity and associated comorbidities. Moreover, the microbiome is thoroughly investigated as a novel potential actor in the treatment of obesity.

2. Introduction to the Gut Microbiome and Methods of Analysis

The human body has co-evolved with and harbours billions of microorganisms, including bacteria and archaea, viruses, fungi, and yeasts in a mutual and beneficial relationship (figure I.2.1). Depending on the physicochemical properties of the site or microhabitat (i.e., lumen, mucosa) they inhabit and their localisation on the human body (i.e., skin, lung, vagina) we can distinguish different populations of microorganisms or ecosystems, which are referred to as different microbiomes (Donaldson et al., 2016). The gut microbiome (GM) refers to the ecosystem colonising the gastrointestinal (GI) tract (GIT) from the mouth to the anus. Compared to other human microbiomes, the GM is the most abundant in terms of colony-Forming Units (CFUs) per millilitre (Aron-Wisnewsky et al., 2012; Marsland and Gollwitzer, 2014). The number of human cells is outnumbered by bacterial cells by a ratio of at least 1:1 (Rosner, 2014; Savage, 1977; Sender et al., 2016a, 2016b). Recently, the amount of bacterial species identified in the human gut has been estimated to be 4,616 (Almeida et al., 2021).

The faecal microbiome (FM) has been the most studied amongst the GM due to its relative accessibility. The human FM is mostly dominated by Firmicutes (now named Bacillota) and Bacteroides (now named Bacteroidota) (Oren and Garrity, 2021), and there is a high intra and inter-individual variability (Arumugam et al., 2011; Eckburg et al., 2005; Franzosa et al., 2015; Lay et al., 2005; Qin et al., 2010). Several factors can account for GM variability between individuals or within individuals over time. These factors may particularly shape the GM during the first years of life and include the delivery mode, exposition to certain drugs such as antibiotics, diet, exercise, age or geography such as genetics (Claesson et al., 2012; Clarke et al., 2014; De Filippo et al., 2010; Long and Swenson, 1977; Vangay et al., 2015; Wu et al., 2011). Various diseases have been linked to the GM. This is the case of obesity which will be discussed in the following chapter.

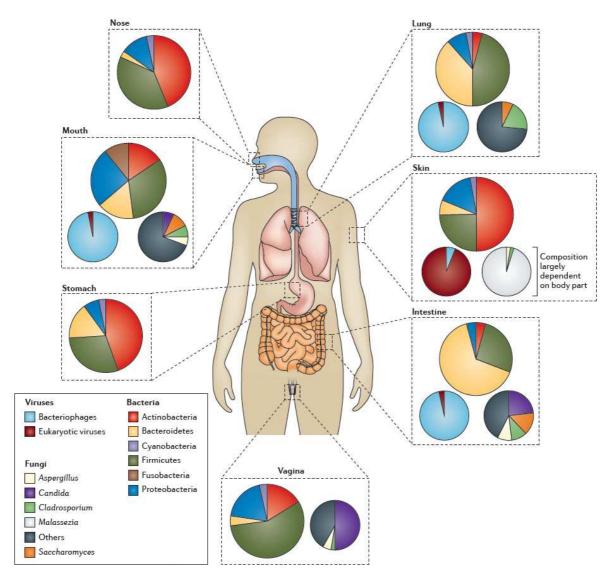


Figure I.2.1 - <u>The human body harbours distinct microbiomes on specific body sites</u>. This figure shows the relative abundance of bacterial, fungal, and viral communities at various body sites exposed to the external environment, including the nose, mouth, skin, stomach, intestinal tract, vagina, and lungs. The bacterial composition is illustrated by the six most commonly detected phyla: Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, and Proteobacteria. The fungal composition encompasses the most prominent genera, such as Aspergillus, Candida, Cladosporium, Malassezia, and Saccharomyces, with additional types of fungi summarised as "Others." The viral composition is categorised into two main types: bacteriophages and eukaryotic viruses. Figure Reused from Marsland and Gollwitzer (2014)

Over the last several decades, major advances in microbiology have been made possible thanks to the development of methods to study microbial ecosystems, from bacterial culture to next-generation sequencing techniques and even third-generation sequencing techniques. This increased access to high-throughput sequencing has led to the description of the gut microbiome with the MetaHit and the Human Microbiome Projects (Huttenhower et al., 2012; Qin et al., 2010) and the discovery of alterations in the compositions and functions of intestinal microbiome in many chronic diseases, including metabolic diseases.

In the following section, I will briefly describe some techniques from a historical perspective. I will particularly develop next-generation (Illumina) and third-generation (Nanopore) sequencing, two techniques I used in my PhD. This section is not exhaustive and will focus on GM research.

Fluorescence In Situ Hybridisation

Introduced in 1980, Fluorescence in situ hybridisation (FISH; Figure I.2.2.) is a cytogenetic technique that enables the localisation, quantification, and identification of specific DNA sequences and location on a chromosome in a (fixated) sample through labelling with fluorescent probes and visualisation through microscopy or flow cytometry (Amann et al., 1990; DeLong et al., 1989; Wagner et al., 2003). A major limitation of this method is that it only enables the detection of bacterial species that have been specifically targeted with probes, thus not suitable for studying the full diversity of the GM, as it only provides information on a small subset of bacteria (Amann and Fuchs, 2008).

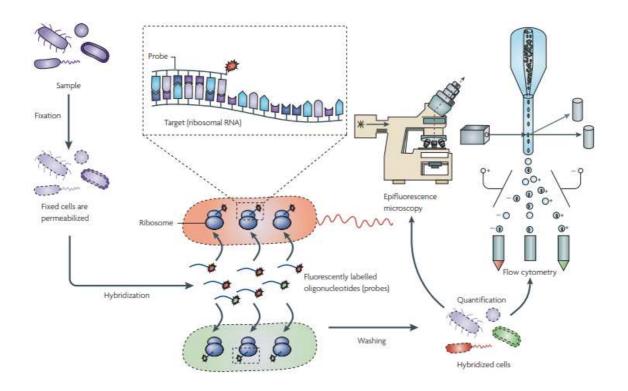
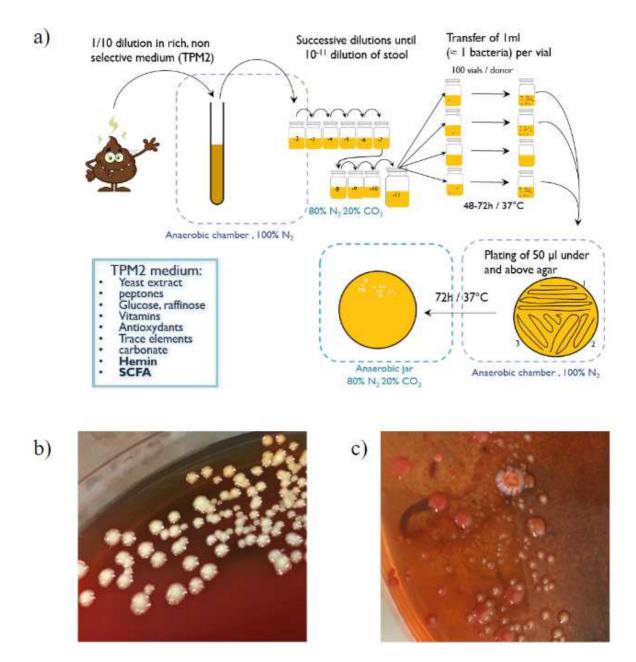


Figure I.2.2.: Fluorescence in situ hybridisation.

Fluorescence in situ hybridisation (FISH) is a cytogenetic technique that uses fluorescent probes to locate, identify and quantify specific chromosome sequences in a fixed sample. Figure reused from <u>Amann and Fuchs (2008)</u>.

Bacterial Culture

Bacterial culture has been used for many decades and is still a widely used technique in microbiology to grow and study individual bacterial cells. Growth is performed on various nutrient media (i.e., agar plates, liquid media), providing the conditions and nutrients for bacteria to thrive. Bacteria are first isolated through subsequent dilutions and then spread onto the surface of the selected growth medium (Figure I.2.3). The bacteria then grow into visible colonies on the medium, which can then be further isolated, if necessary, to be studied and characterised. One of the main limitations of bacterial culture is that it only allows for the growth of a subset of species that can be cultured under the specific conditions used in the laboratory. This is a significant limitation in the study of the full diversity of the GM, and this is particularly the case for anaerobic strains. It is thus proposed that only 30% of the bacteria composing the GM can be cultured (Doré and Corthier, 2010). Additionally, bacterial culture is time-consuming and labour-intensive. The advantages of this technique are, amongst others, that it enables the growth of strains that were sampled 'alive' and, potentially, to the discovery of new unidentified strains. Growing live strains isolated from patients, for example, is a powerful tool for studying their causal and mechanistic impact on various models. This can lead to the development of experimental trials, for example, in mice models, with the administration of a specific taxon.





a) This figure shows the serial dilutions method to isolate bacteria from stool or duodenojejunal fluid (as used for the present work). b) This image shows a pure culture colony type and c) a mixed culture colony type. Images are reused from Dr Tiphaine Le Roy's observations.

Since 1980, high throughput molecular approaches, including 16S rRNA sequencing, have been developed and are being widely used to study the GM.

16S ribosomal RNA Sequencing

<u>Woese et al. (1975)</u> discovered that a fraction of the gene encoding the small subunit 16S ribosomal RNA (16S rRNA) is highly conserved amongst bacteria, enabling the identification up to the genus level, while other fractions are highly variable enabling distinction between bacteria up to the strain level (<u>Woese et al., 1975, 1974</u>). 16S rRNA sequencing involves amplifying through polymerase chain reaction (PCR) and sequencing a specific ultra-variable region of the 16S rRNA gene (Figure I.2.4).

Although 16S is the most widely used method for studying GM, it presents limitations. Significant limitations are: First, this method does not allow accurate identification up to the species level (Christensen et al., 1998); Moreover, 16s rRNA only enables the detection and study of bacteria of an ecosystem and not of the other microorganisms that do not have a 16sRNA gene (i.e., fungi, viruses); Additionally, 16S rRNA does not provide information about whole genomes and their functions, although Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST) analysis can be used to infer functions with caution (Langille et al., 2013). PICRUST is a software designed to predict functional potential from marker genes; Finally, 16S rRNA is always combined with amplifications. This method can be thus affected by PCR biases which can affect the accuracy and reproducibility of the results (Lee et al., 2012; Morgan and Huttenhower, 2014; Woese et al., 1975, 1974).

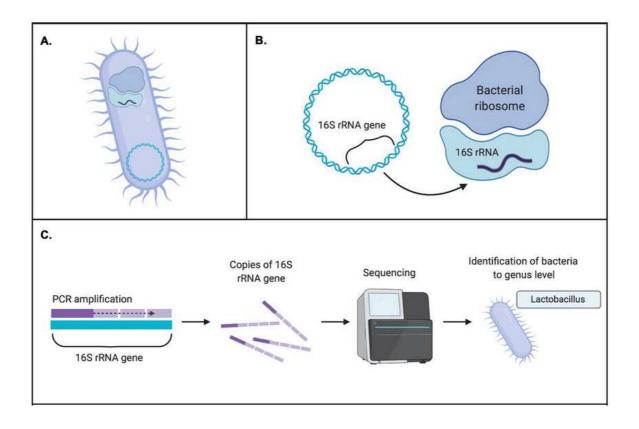


Figure I.2.4 - <u>16S ribosomal RNA sequencing</u>.

The circular DNA of the bacterium (A) contains the 16S rRNA gene coding for the ribosomal subunit (B), which is highly conserved among bacteria. This gene can be amplified and sequenced to identify the bacteria up to the genus level (C). Figure adapted and reused from <u>Patel et al., (2022)</u>.

While 16s rRNA sequencing approaches are based on a single gene, metagenomics, on the other hand, corresponds to the analysis of the entire genomic content of the collected sample providing accurate species-level identification and the study of their function.

Metagenomics

Metagenomics corresponds to a set of high-throughput DNA sequencing technologies that allow for the rapid and efficient sequencing of large amounts of DNA, such as a whole ecosystem (i.e., the whole genome of the microbiome in faeces). This allows for the identification and characterisation of most bacteria in the sample, providing a more complete picture of the GM than 16S rRNA gene sequencing.

I will present three generations of DNA sequencing (figure I.2.5). First-generation sequencing is the slowest and least accurate of the three and can be used to sequence long DNA fragments. Next-generation sequencing is much faster and more efficient than first-generation sequencing and is better suited for shorter DNA fragments. Third-generation sequencing enables the sequencing of long and intact DNA fragments. However, it is still a relatively new technology and has yet to be as widely used as first- and second-generation technologies.

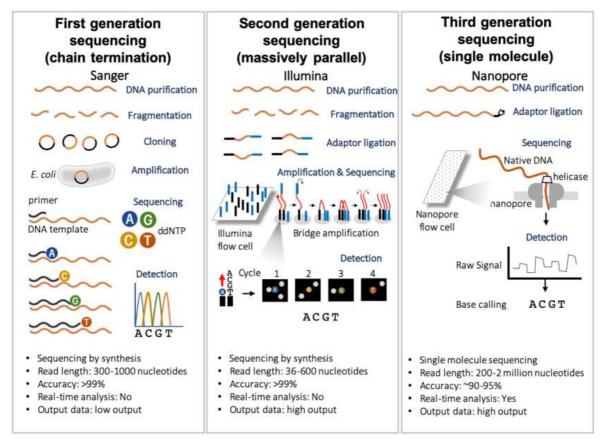


Figure I.2.5.- Metagenomic Sequencing.

Schematic Representation of First-, Second- and Third-generation Sequencing. For each method, an example is given (i.e., Sanger, Illumia, Nanopore). However, these examples are not exhaustive. Figure reused from <u>Patterson et al., (2020)</u>.

First-Generation Sequencing

Sanger sequencing was the first widely used method of DNA sequencing and was developed by Frederick Sanger in the late 1970s (Sanger et al., 1977). It is also known as the dideoxy chain termination method. This method uses dideoxynucleotide triphosphates and DNA polymerase to synthesise a complementary DNA strand in the presence of a template strand.

Next-Generation Sequencing

Next-generation sequencing, on the other hand, refers to a group of high-throughput DNA sequencing technologies that were developed in the mid-2000s. These technologies, including Illumina sequencing, Ion Torrent sequencing, and PacBio sequencing, use various approaches to sequence DNA quickly and efficiently. Compared to Sanger sequencing, NGS analysis enables much greater coverage depth, allowing the identification of undiscovered taxa (Lazarevic et al., 2009; Margulies et al., 2005).

One of the most commonly used next-generation sequencing methods is shotgun sequencing. Shotgun sequencing, a laboratory technique to determine the DNA sequence of a genome, involves fragmenting the organism's genome into small DNA fragments and sequencing them individually. In this method, the fragments are randomly broken up, and software is employed to identify overlaps within the DNA sequences. These overlaps serve as a basis for reassembling the fragments in the correct order, thereby reconstructing the complete genome ("Shotgun Sequencing," 2022).

The Illumina sequencing method is a commonly used platform for performing shotgun sequencing. In this approach, single molecules of DNA are attached to a flow cell and amplified *in situ* through 'bridging amplification' (massively parallel sequencing) and used as templates for synthetic sequencing using fluorescent reversible terminator deoxyribonucleotides (Bentley et al., 2008). This method enables metagenomic sequencing but is still often used for 16S rDNA sequencing (Degnan and Ochman, 2012).

Third-Generation Sequencing

Third-generation sequencing, also known as single-molecule sequencing, allows for the direct, real-time sequencing of individual DNA molecules. Unlike first- and second-generation sequencing technologies, which rely on amplification and fragmentation of DNA molecules, third-generation sequencing allows for the direct sequencing of long, 'intact' (unfragmented) DNA molecules.

One example of a third-generation sequencing technology is the MinION, developed by Oxford Nanopore Technologies (ONT). The MinION is a portable, handheld sequencing device that uses nanopore technology to sequence DNA molecules in real time. The device contains microscopic pores coated with proteins in a membrane that can selectively bind to specific DNA bases. As the DNA molecule passes through the pore, the protein binding events, detected as changes in the electrical current flowing through the membrane, determine the molecule's sequence.

Third-generation sequencing has several advantages over first- and second-generation sequencing technologies. It allows for the direct sequencing of long, intact DNA molecules, providing more accurate and comprehensive sequence data (Alili et al., 2021b; Morgan and Huttenhower, 2014; Petersen et al., 2019).

The technologies mentioned in this section have significantly improved the speed and accuracy of traditional Sanger sequencing, allowing for the rapid and cost-effective analysis of large genomic datasets. NGS technologies use various approaches to sequencing DNA, including sequence-by-synthesis, single-molecule real-time sequencing, and reversible terminator-based methods. These technologies are commonly used in a variety of applications, including genomic sequencing, metagenomics, transcriptomics, and epigenomics.

A large work of my thesis project focused on optimising and developing bacterial DNA extraction protocols, preparation of DNA libraries and choosing the most appropriate sequencing technology for the duodenojejunal microbiome. All of these steps can be sources of bias in the study of the microbiome. These aspects and the related literature will be detailed in the "Result" section of this manuscript (Part I. Technical development).

Metatranscriptomics, Metabolomics, and Metaproteomics

Metagenomics provide information on the genetic potential of the community of microbes. At the same time, metatranscriptomics, metaproteomics, and metabolomics allow researchers to investigate the functional aspects of the community, including gene expression, protein synthesis, and metabolite production. Combining metagenomics with metatranscriptomics, metaproteomics, and metabolomics offers a more comprehensive understanding of microbial communities' structure, function, and dynamics. This integrative approach can help elucidate complex interactions between microbes and their environment, reveal the ecological roles of individual community members, and provide insights into the mechanisms driving community structure and function (Franzosa et al., 2014, p. 201; Morgan and Huttenhower, 2012).

My PhD project combined metagenomics and metabolomics on duodenojejunal fluid and stools. We were limited by the amount of duodenojejunal fluid we could sample to allow complementary 'omics' studies.

Challenges of processing multi-omics data set

Such NGS and 3GS sequencing methods generate data sets with millions of genes. There are various methods and approaches for classifying genes obtained from NGS. Some common approaches include: 1) Sequence alignment and comparison to known reference sequences: i.e., after obtaining the sequences from Illumina sequencing, they can be aligned to known reference sequences using sequence alignment algorithms, such as Basic Local Alignment Search Tool ("BLAST: Basic Local Alignment Search Tool," n.d.). This allows for the identification of genes and can provide information about the function and potential role of the gene in the organism; 2) Gene annotation is the process of identifying functional elements and assigning biological information to genes, such as their function and the pathways in which they are involved. This can be done using a variety of computational tools and databases, such as the Gene Ontology (GO) database ("Gene Ontology Resource," n.d.), which provides standardised terms for gene annotations; 3) Functional analysis involves studying the function of genes and the pathways in which they are involved. This can be done using a variety of computational tools and techniques, such as gene expression analysis, protein-protein interaction analysis, and pathway analysis.

The choice of a database can influence the outcomes when annotating the genes obtained through any sequencing method mentioned above. Indeed, each catalogue has its characteristics (Li et al., 2014; Qin et al., 2010). For example, they can differ in terms of the number and diversity of genomes they include. Specific databases might be biased towards certain groups of organisms or geographical locations (i.e., specific to the faecal microbiome). They may not represent the diversity of a specific ecosystem (i.e., the duodenojejunal microbiome). In this case, the analysis may miss community members.

Description of the Microbial Ecosystems

Genomic microbiome studies produce enormous data sets containing millions of genes. To summarise, visualise and interpret such data, several key metrics and indexes are commonly used (Morgan and Huttenhower, 2012). In microbiology, we can differentiate diversity within a sample (alpha diversity) or diversity between samples (beta diversity) (Lozupone and Knight, 2008).

Alpha diversity is a measure of the diversity within a single sample or community. A variety of metrics are frequently used, such as 1) metagenomic richness, which is a measure of the total number of unique features present in a sample (operational taxonomic units, genus, species or even genes in shotgun experiments) (Chazdon et al., 1998; Morgan and Huttenhower, 2012); 2) diversity, which is similar to the metagenomic richness and also takes into account the evenness of the distribution of features depending on different calculation methods (Simpson, 1949); 3) the relative abundance of each taxon within a sample can also be reported and compared between samples.

Conversely, Beta diversity is a measure of the difference in composition between different samples or communities of samples. It is typically calculated using a variety of metrics, such as the number of shared or unique species or types between different samples, the relative abundances of different species or types, or the overall dissimilarity between samples. Commonly used approaches are dissimilarity matrices (Bray-Curtis index or Euclidean distances), which are used to quantify the compositional dissimilarity between samples. Distance between samples can be visualised through principal component analysis (Zuur et al., 2007).

These indexes and results are essential for characterising and comparing the diversity, composition, and phylogenetic relationships of different bacterial communities in the gut microbiome. They are commonly used in GM studies to identify patterns and trends in the data and to gain insights into the function and health implications of the gut microbiome. I used some of these approaches in my PhD project, and I will detail the methods later in this manuscript.

3. The Gut Microbiome in Obesity

The GM and its host live in symbiosis, maintaining a delicate balance. This complex microbial ecosystem that resides in the human's gut plays essential beneficial roles in the host physiology, including immune system maturation, promoting the barrier function of the gut, digestion, and metabolite production (vitamins, short-chain fatty acids, etc.).

However, in metabolic diseases, the composition, richness and functions of faecal microbiome (FM) are altered (Cotillard et al., 2013; Le Chatelier et al., 2013; Yatsunenko et al., 2012). These alterations of FM – sometimes encompassed under the term 'dysbiosis' - can be associated with low-grade inflammation, which is frequent in these metabolic diseases (Hotamisligil, 2006; Nathan, 2008; Tilg and Moschen, 2006). In recent years, several diseases and disorders have been associated with FM alterations, including mental health disorders, inflammatory bowel diseases, obesity, and diabetes (Hou et al., 2022; Jostins et al., 2012; Vijay and Valdes, 2022).

It is now suggested that immune-related pathologies or metabolic disorders can be triggered or exacerbated by the characteristics of the microbiota we harbour. Indeed, the alterations of the FM are thought to promote obesity and inflammation (Dao et al., 2016; Everard et al., 2013; Schneeberger et al., 2015).

Association studies suggest a link between FM, energy homeostasis (carbohydrate and lipid metabolism), inflammatory homeostasis and obesity. Studies have demonstrated that the characteristics of the FM can be associated with an individual's susceptibility to developing obesity-related metabolic diseases.

In twin studies, certain producers of SCFAs, such as *Eubacterium ventriosum* and *Roseburia intestinalis* were particularly positively associated with obesity (<u>Tims et al., 2013</u>). Other bacteria, such as *Bacteroides thetaiotaomicron* were found to be reduced in obesity and to protect mice under High Fat Diets (HFD) from obesity (<u>Liu et al., 2017</u>). In obesity, there is a predominance of *Bacteroides, Subdoligranulum, Faecalibacterium, Dialister*,

Bifidobacterium, Pseudobutyrivibrio, and *Blautia* genera and with the previous characteristics, a higher Firmicutes/Bacteroidetes ratio had also been observed (Ley et al., 2006; Riva et al., 2017). However, the validity of this ratio is highly debated, as results are contradictory across studies (Duncan et al., 2008; Jumpertz et al., 2011). Furthermore, clinical studies have also reported reduced bacterial diversity in a subset of obese subjects, which has been associated with more pronounced metabolic dysfunctions, insulin resistance, and higher blood triglyceride levels compared to obese subjects with preserved bacterial diversity (Cotillard et al., 2013; Le Chatelier et al., 2013).

The composition and also the localisation of gut microbes are shown to associate with or even play a role in the onset of obesity. For example, microbial encroachment of the intestinal mucus (Chassaing et al., 2017a; Viennois et al., 2017) associates with an alteration of the intestinal barrier by disrupting both the protective mucus and tight-junction proteins. This can trigger an inflammatory cascade, with intestinal barrier disruption causing leakage of bacterial fragments, such as lipopolysaccharides (LPS), into the host system, driving low-grade systemic inflammation CD14/TLR4 activation. This inflammatory cascade further disrupts the GM and the gut barrier (Cani and Delzenne, 2009). Interestingly, Chassaing and colleagues propose that inflammation does not necessarily initiate after caloric excess and insulin resistance, showing that, for instance, diet by itself could be an initial proinflammatory trigger (Chassaing et al., 2017b, 2017a, 2016; Viennois et al., 2017, 2017).

Interventions tried to modulate the GM to treat obesity and associated comorbidities. Several approaches, such as nutritional interventions with a diet rich in fibre, fruits, vegetables, and fermented foods (Cotillard et al., 2013; Dao et al., 2016; Seganfredo et al., 2017; Zhang et al., 2015), using probiotics and prebiotics (Belda et al., 2022; Cani and de Vos, 2017; Plovier et al., 2017; Vallianou et al., 2020), bariatric surgery (Aron-Wisnewsky et al., 2012, 2019b; Debedat et al., 2019) or preventing encroachment in the mucus (Tran et al., 2019) were shown to ameliorate metabolic phenotype and/or inflammation, respectively.

Beyond correlations, studies tried to investigate the causal effect of the FM. Causal investigations of GM can be performed with germ-free mice living in sterile environments or Specific Pathogen-Free (SPF) mice. Using these models, studies showed that mice displayed less body fat and were protected from obesity and associated metabolic disorders.

The causal effect of the FM has also been explored with Faecal Microbiota Transplants (FMT) in metabolic diseases, for example, in mice depleted from their microbiota with antibiotics and laxatives (Le Roy et al., 2018). Such studies showed the role of GM in these diseases (Kootte et al., 2017; Vrieze et al., 2012). FMT from conventionally raised mice to germ-free mice resulted in significant increases in body fat despite reduced food intake. Similar results were observed in experiments involving FMT from obese subjects to germ-free mice (Bäckhed et al., 2004; Turnbaugh et al., 2006).

In an FMT experiment involving obese subjects, improvements in hepatic and peripheral insulin sensitivity were observed and persisted for at least six weeks after the transfer. The FM of obese subjects was characterised by lower microbial diversity, higher quantities of Bacteroidetes, and reduced quantities of Clostridium cluster XIV compared to lean, healthy donors (Vrieze et al., 2012). Following the transfer, the intestinal microbial diversity of obese subjects increased significantly.

To conclude, the importance of the FM in metabolic diseases has been demonstrated (Aron-Wisnewsky et al., 2019a, 2019b, 2012; Cani, 2018; Cotillard et al., 2013; Dao et al., 2016; Wu et al., 2011). The microbiome can be seen as an integrator of environmental triggers, further altering the host's immune response and metabolic state.

Its exploration can provide not only cues for patient stratification but also a better understanding of metabolic health, ultimately resulting in the development of effective treatments (Aron-Wisnewsky et al., 2019a, 2019b; Dao and Clement, 2018; Plovier et al., 2017).

Further research is needed to understand better the complex relationships between gut microbiome, obesity, and metabolic diseases.

Limitations Pertaining to the Faecal-microbiome Field of Research

As introduced above, perturbation of microbial composition is thought to contribute to metabolic diseases such as obesity and T2D (Debedat et al., 2019).

Gut microbiology is thus a promising research field for understanding and treating metabolic diseases. It is, however, questionable whether the extrapolated conclusions of studies exclusively on faeces, probably reflecting the microbiome of the last segments of the colon, are sufficient. Indeed, most studies have overlooked the upper digestive tract microbiome in metabolic diseases and particularly in humans.

4. The Upper Small Intestinal Microbiome

Studies have shown that the alterations of composition and functions of the faecal microbiome (FM) can lead to the onset and the chronic aspect of metabolic perturbations, and FM is associated with metabolic diseases such as obesity and type 2 diabetes (T2D). However, most studies focused on FM and knowledge of whole gastro-intestinal microbiome cannot be extrapolated from faeces (Savage, 1977; Sundin et al., 2017). FM is not a proxy for the microbiome of the small intestine (SI) and, thus, particularly for the upper SI (USI). This can be explained by the major changes in physicochemical properties along the GIT, which account for the changes in microbial communities being able to colonise these segments.

The USI is particularly interesting in metabolic research as it is the main site of nutrient absorption, appetite control or glucose homeostasis through neuroendocrine signalling.

Anatomy of the Upper Small Intestine

The SI is an approximately 6 to 7 metres long tubular and hollow-like organ of the GIT that extends between the pyloric sphincter and the ileocecal valve. Compared to the colon, the small intestinal mucosa is 15 times larger (Helander and Fändriks, 2014). The SI is further constituted of three sub-segments: the duodenum, the jejunum and the ileum.

The duodenum is the first segment of the SI. The majority of the duodenum is located in the retroperitoneum, proximal to the stomach and receiving the chyme. It is approximately 25 cm long and is divided into four sections: the first (bulb), second (descending), third (transverse), and fourth (ascending). The bulb is located at the pylorus and is about 5 cm long, and the posterior wall is in direct contact with the gastroduodenal artery, common bile duct, and portal vein. The major papilla is located between the first and second sections of the duodenum. The second section, the descending duodenum, is about 10 cm long and in front of the right kidney and ureter and the lateral border of the inferior vena cava. The third section, the transverse duodenum, is bordered by the uncinate process of the pancreas and the hepatic flexure of the colon. The fourth section courses in a cephalad direction to the left of the aorta and inferior to the neck of the pancreas (Carlson, 2018; Lopez et al., 2022). The duodenum corresponds to the section receiving primary bile acids (BAs), essential for the digestion of lipids. The Angle of Treitz or ligament of Treitz corresponds to the separation between the duodenum and the jejunum. This localisation corresponds to the sampling site of duodenojejunal fluid in our cohort.

The jejunum is the middle segment of the SI. The jejunum and ileum are located within the peritoneal cavity. They are anchored to the retroperitoneum by a broad-based mesentery and have an average length of 6 metres, with the jejunum comprising 40% and approximately 2,5 meters and the ileum comprising 60% or approximately 3,5. The jejunum begins at the ligament of Treitz, and the ileum ends at the ileocecal valve. The jejunum is located in the central abdomen, while the ileum is mostly located in the hypogastric region and pelvic cavity.

The jejunum and ileum can be distinguished by characteristics such as a 1) thicker mucosal lining, thicker wall, larger diameter, less fatty mesentery, and longer and straighter vasa recta (blood vessels arising from arterial arcades in the mesentery of the jejunum and ileum) in the jejunum; 2) as well as the presence of plicae circulares and villi in the mucosa of the SI and particularly the jejunum. Plicae circulares are transverse folds of mucosa and submucosa, visible on gross inspection (i.e., during endoscopy). Villi are finger-like projections of the mucosa, containing microvilli that and only visible through microscopy. Both optimise nutrient absorption by increasing the SI's surface area. They are prominent in the proximal intestine and diminish throughout the SI, indicating a decrease in nutrient absorption in more distal areas. The epithelium of the SI is composed of crypts and villosities. In contrast, the large intestine is devoid of plicae circulares, and has crypts but no villosities. There is not a precise anatomical region corresponding to the separation between the jejunum and the ileum. The ileum is the final and longest segment of the SI (Carlson, 2018; Lopez et al., 2022).

The SI has a complex lumen architecture composed of structures that increase its surface area to facilitate digestion and absorption. As for the rest of the GIT, the wall of the SI is made up of four main layers: the mucosa, the submucosa, the muscularis propria, and the serosa.

The mucosa is the innermost layer responsible for nutrient and water absorption. The submucosa is a dense connective tissue layer containing blood vessels, lymphatics, and the Meissner plexus of the enteric nervous system. The muscularis propria consists of two smooth muscle layers and the myenteric plexus. The serosa is the outermost layer and is a single layer of mesothelial cells. The epithelium shows high enteroplasty with renewals/extrusion of enterocytes every 3-6 days, making it the most rapidly renewing tissue in adult mammals (Amerongen, 2018; Campbell et al., 2019).

Functions of the Upper Small Intestine Compared with the Colon

The USI is an essential site for neuroendocrine signalling, nutrient sensing, production of entero-hormones and activation of different pathways that may induce energy expenditure, thermogenesis, promote satiety and lower food intake or regulate glucose homeostasis by promoting insulin secretion or by lowering hepatic glucose production (Berthoud et al., 1995; Duca et al., 2015). It is, therefore, a site of utmost importance in the study of obesity and associated cardiometabolic diseases.

Digestion, Sensing and Absorption of Nutrients

The primary site for digestion and absorption of proteins, lipids, and carbohydrates is the USI, while the colon is responsible for fermenting non-digestible carbohydrates. The digestive system facilitates food digestion, nutrient absorption, and waste elimination through various organs, including the mouth, oesophagus, stomach, SI, colon, rectum, and anus. Additionally, accessory organs such as the liver, gallbladder, and exocrine pancreas contribute to the digestive process and nutrient uptake (Amerongen, 2018).

Starting in the mouth, digestion involves food breakdown during mastication and under salivary enzymes' action. The food bolus travels through the oesophagus, approximately 20 cm long in humans, before reaching the stomach (Meyer et al., 1986), where food is combined and converted into chyme. Peristaltic contractions of the stomach facilitate the controlled transfer of chyme to the SI through the pyloric sphincter, located at the stomach's distal part (Ramkumar and Schulze, 2005).

In the SI, pancreatic enzymes and BAs, released in the duodenum, help transform food into nutrients, which are subsequently absorbed by enterocytes. The portion of chyme not absorbed proceeds to the colon, where water and specific minerals are absorbed, GM ferments non-digestible proteins and polysaccharides, and GM transforms BAs into secondary BAs that are later reabsorbed in the distal SI and colon by the host. The remaining substances from faeces are eliminated through the anus (Carlson, 2018).

Carbohydrates Digestion, Sensing and Absorption

Carbohydrates come in complex starches, disaccharides, and monosaccharides (simple sugars). Starch exists as amylose or amylopectin. Sucrose and lactose are common disaccharides, while glucose, galactose, and fructose are monosaccharides that do not require further digestion for absorption. Starches and disaccharides must be broken down into monosaccharides before they can be absorbed in the SI.

Digestion of starch begins in the mouth with salivary amylase. However, most carbohydrate digestion occurs in the SI with the help of pancreatic amylase, which breaks down starches into short-chain sugars called oligosaccharides. These oligosaccharides are then broken down into monosaccharides by saccharidases in the brush border of the SI. The monosaccharides are then absorbed via active transport or facilitated diffusion into the enterocyte and can be used for energy or transported into the venous system (Koepsell, 2020).

Carbohydrates are sensed and absorbed as mono- and disaccharides via transporters, primarily SGLT1, GLUT2, and GLUT5 in the proximal SI (Mueckler and Thorens, 2013). Nutrient sensing through SGLT1 and GLUT transporters can lead to GLP-1 signalling. SGLT1 is implicated in glucose sensing and facilitates glucose and galactose absorption. There are 14 GLUTs transporters (Mueckler and Thorens, 2013). Amongst these, GLUT2 and GLUT5 are mainly involved in glucose, and galactose and fructose transport, respectively (Gorboulev et al., 2012; Poulsen et al., 2015).

The translocation of GLUT2 to the apical membrane of enterocytes for glucose absorption is influenced by glucose concentration and is associated with diabetes and insulin resistance (Kellett et al., 2008; Leturque et al., 2012). A recent study demonstrated a relationship between enteroendocrine cells and enterocytes that couples sugar detection and

absorption. Glucose detection by the T1R2/T1R3 receptor on enteroendocrine cell surfaces causes membrane depolarisation and calcium influx into enterocytes (Koepsell, 2020; Smith et al., 2018). Interestingly, the GM has been shown to influence the expression of SGLT1 and whole-body glucose homeostasis (Bauer et al., 2018b). This will be discussed in the literature review on the USIM in metabolic diseases inserted further.

Fatty Acids Digestion, Absorption and Sensing

Lipid digestion begins in the mouth with lingual lipases and continues in the stomach and intestine (Iqbal and Hussain, 2009). Triglycerides are hydrolysed into 2-monoacylglycerol (2-MAG) and free fatty acids. Free fatty acids are absorbed by enterocytes through passive diffusion or facilitated transport. Transporters involved in facilitated transport include fatty actid translocase FAT/CD36 (Poirier et al., 1996), fatty actid transport protein FATP4 (Cifarelli and Abumrad, 2018), and fatty acid binding protein FABPpm (Chabowski et al., 2007; Masson et al., 2010).

Lipids sensing is partly mediated by the ACSL gene family (Bauer et al., 2018a). The ACSL gene family has five members, numbered 1, 3, 4, 5, and 6 (Masson et al., 2010; Soupene and Kuypers, 2008). Research on mRNA expression of genes encoding the five ACSLs in various rat tissues revealed that all isoforms are expressed in the small intestine (Bowman et al., 2016; Mashek et al., 2006). Amongst them, ACSL3 and ACSL5 are the most highly expressed isoforms (Bowman et al., 2016). Interestingly, the GM has been shown to influence the expression of genes related to lipid metabolism in both the distal small intestine and the colon (Derrien et al., 2011; Hooper et al., 2001).

In the proximal small intestine, one study showed the alteration of jejunal expression of several genes involved in metabolic control, including glucose and energy homoeostasis in germ-free mice after FMT (Aidy et al., 2013). Another study showed that an HFD reduces hepatic protein expression of ACSL isoforms ACSL3 and ACSL4 (Bowman et al., 2016), which are preferentially expressed in the ileum. However, they did not study whether these effects also occur in the USI and whether they depend on diet-induced changes or USIM changes.

The potential impact of these GM-induced changes in ACSL expression on lipid sensing and glucose regulation needs to be explored. In the review we submitted, I will further discuss the role of USIM on fatty-acid sensing and GLP-1-dependent glucoregulatory lipid-sensing pathway (Bauer et al., 2018a).

Once in the enterocytes, fatty acids are re-esterified and form lipid complexes in the endoplasmic reticulum. Fatty acids and MAG bind to FABP1 (L-FABP) and FABP2 (I-FABP) (Cifarelli and Abumrad, 2018). Chylomicrons, lipoproteins rich in TAG and cholesterol esters, are formed in enterocytes (Miller and Small, 1983). Chylomicrons are then secreted into the lymphatic system and enter the bloodstream (Cifarelli and Abumrad, 2018). The majority of TAG biosynthesis in enterocytes occurs via the MAG pathway. TAG is synthesised through the actions of monoacylglycerol acyltransferases (MGAT) and diacylglycerol acyltransferase (DGAT) (Iqbal and Hussain, 2009). Triglycerides are then transported via the lymphatic or venous routes, depending on the administered fatty acids and monoacylglycerols (Lecleire, 2008).

If we briefly compare the USI to the Colon, in terms of digestion, absorption and excretion, the absorptive surface of the colon is decreased and colonic functions include 1) forming and propelling faeces toward the rectum for elimination (motility); 2) Absorption of the remaining (10%) water and electrolytes by osmosis and; 3) Microbial production and host absorption of microbially-produced vitamins (vitamin K and B vitamins, including biotin) (Azzouz and Sharma, 2022).

For metabolic research, there is a significant interest for the SI and particularly for the proximal segments: the duodenum and the proximal jejunum. Indeed it is the main site of nutrient sensing and absorption and the interconnected neurohormone production, which will be discussed below.

Finally is also interesting to note that, compared to the colon, the USI (particularly the duodenum and the proximal jejunum) is the first site to be exposed to nutrients (i.e., glucose, fatty acids, proteins) but also to orally administered drugs (i.e., metformin). Metformin is orally prescribed, and its effects take place preferentially in the USI. In mice, after oral and even intravenous (IV) administration, metformin is found to accumulate preferentially in the SI mucosa compared to other tissues, including the liver and plasma (Jensen et al., 2016;

<u>Wilcock and Bailey, 1994</u>). The same was observed in humans after oral administration, where the concentration of metformin was 30-300 times higher in the jejunum compared to plasma levels (<u>Bailey et al., 2008</u>). Interestingly, the glucose-lowering effects of metformin are increased with intraduodenal administration compared to IV administration (<u>Stepensky et al., 2002</u>).

Endocrine Function

The intestine is a major endocrine organ. The entero-endocrine cells (EEC) are not grouped in glands but are dispersed along the intestinal epithelium. The enteroendocrine cells in the intestine are of the "open" type, featuring microvilli on the apical pole, which increases the surface area for nutrient detection in the intestinal lumen. Their basal pole is more prominent than their apical pole, allowing exocytosis of secretory vesicles containing hormones into the bloodstream. In the stomach, endocrine cells are of the "closed" type and are not in contact with the lumen (Gribble and Reimann, 2019). Enteroendocrine cells are diverse and secrete at least twenty different hormones (Figure I.4.1). It has been shown that a single enteroendocrine cell can secrete multiple enteroendocrine hormones (Gehart et al., 2019; Haber et al., 2017; Habib et al., 2012). These hormones may display orexigenic or anorexic effects, influence food intake and play incretin roles. They can alter intestinal motility and modulate the secretion of various acids involved in digestion.

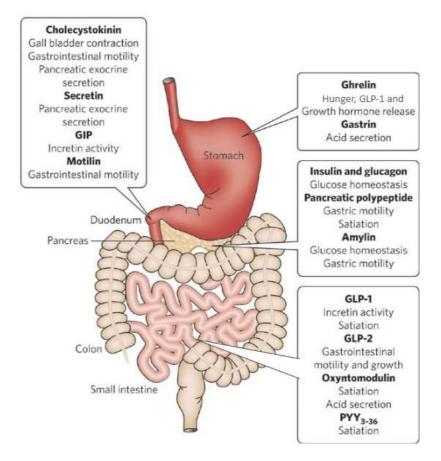


Figure I.4.1 - <u>Gut hormones and the regulation of energy homeostasis</u>. Enteroendocrine cells are dispersed throughout the GIT and secrete various enterohormones displaying different functions. Figure reused from <u>Murphy and Bloom (2006)</u> and adapted with Dr Celine Oskinsi PhD manuscript (Osinski, 2022).

The stomach is where Ghrelin and Gastrin are mainly secreted. Ghrelin displays an orexigenic effect (Cummings et al., 2001; Wren et al., 2001). Fasting leads to an increase in blood ghrelin concentration, and food intake leads to a decrease. Gastrin is also secreted in the stomach in response to food intake. It stimulates insulin secretion, BAs secretion and histamine secretion, another enterohormone (Kaneto et al., 1969; Rehfeld and Stadil, 1973; Zeng et al., 2020). Gastrin has been shown to act as a growth factor for the proximal stomach.

Duodenum is the main site of secretion of Secretin, an anorexigenic hormone which plays various other functions, such as delaying gastric emptying, inhibiting gastrin secretion in the stomach and protecting the mucosa by stimulating mucus secretion in the intestine (Cheng et al., 2011; Siu et al., 2006). Moreover it also stimulates bile secretion by the gallbladder and bicarbonate and insulin secretion by the pancreas (Sekar and Chow, 2013). The duodenum is also the main site of 5-HT synthesis, storage and secretion through enterochromaffin cells (Banskota et al., 2019), which may be implicated in intestinal motility (Bulbring and Lin, 1958) but mainly in immune cell activation, intestinal inflammation generation, bone remodelling, and metabolic homeostasis (Banskota et al., 2019).

In more distal regions, in the jejunum, neurotensin (NT) is secreted by the detection of lipids. This hormone participates in the regulation of intestinal motility, pancreatic secretions, and fatty acid absorption (Li et al., 2020).

Peptide YY (PYY) is primarily secreted in the distal part of the USI, stimulated by the arrival of food in the ileum and functioning as an anorexigenic hormone (Gribble and Reimann, 2019; Rigaud, 2014). This negative feedback on food intake contributes to the "ileal brake," characterised by reduced acid secretion, pancreatic secretions, gastric emptying, and transit speed.

Throughout the gastrointestinal tract, enteroendocrine cells secrete incretin hormones (incretin: INtestine seCREtion INsulin), such as GIP and GLP-1, which potentiate insulin secretion in response to oral glucose intake (GIP and GLP-1) and lipid (for GIP) and contribute to 50% of postprandial insulin secretion (Gribble and Reimann, 2019). GLP-1 is also implicated in regulating food intake (Gupta and Raja, 2023). As discussed in the literature review, it has been shown that the USIM can alter SGLT-1 sensing and whole-body glucose homeostasis through GLP-1 mediated effect (Bauer et al., 2018b).

Barrier Function

As an introduction to this section, the intestinal epithelial cells play crucial roles in maintaining a healthy relationship between the GM and host immunity through two major functions: 'segregation' and 'mediation' (Okumura and Takeda, 2017).

'Segregation' involves the construction of a physical and a chemical mucosal barrier by intestinal epithelial cells, which spatially separates GM in the intestinal lumen and immune cells in the lamina propria, preventing potential conflicts and inflammation.

'Mediation' refers to the process wherein intestinal epithelial cells respond to gut microbes or their metabolites. The epithelial cells can produce mediators such as cytokines and chemokines, inducing T-cell immune responses and delivering antigens to antigen-presenting cells in lymphoid tissues. This contributes to antigen-specific IgA responses and oral tolerance to food antigens. Activated T cells produce cytokines like IL-17 and IL-22, promoting antimicrobial molecule production by intestinal epithelial cells and regulating pathogenic opportunistic microbes' overgrowth (Okumura and Takeda, 2017).

I will add a third 'mechanic' function which is crucial in USI and will be discussed last in this section.

The immune system as a segregator and mediator

The intestine and its mesentery form the largest compartment of the immune system, containing the most extensive collection of lymphoid tissue in the body. Approximately 70% of peripheral lymphocytes and 40% of the body's lymph nodes are located in close proximity to the intestine (Brandtzaeg et al., 1989; Mowat and Agace, 2014). In humans, about 70-80% of all Ig-producing cells are found in the intestinal mucosa (Brandtzaeg and Baklien, 1976), and at least 70% of all immunoglobulins (Ig) produced by mammals are IgA produced by mucosal B-cells (Macpherson et al., 2008).

In proximal GIT regions, the SI's immune system protects the surface epithelium's ability to digest and absorb nutrients by defending it from infection and preventing bacterial overgrowth in this region. Immune mechanisms include IL-17- and IL-22-producing T cells, innate lymphoid cells and intraepithelial T cells with innate and cytolytic effector functions (Mowat and Agace, 2014). Regulatory T cells help prevent hypersensitivity reactions to dietary antigens.

Mucosal lymphocyte cells educate the immune system to develop tolerance toward commensal microbes (Johansson et al., 2013; Shan et al., 2013). Microfold cells (M-cells) actively engulf and present antigens derived from luminal microorganisms and diet to sub-epithelial immune cells (Ohno, 2016; Ohno et al., 2012).

In addition to the regional differences and specialisations across different segments of the GIT discussed earlier, there are also differences in immune system response and barrier functions.

About 50% of Peyer's patches are scattered in the USI, along the distal 25 cm of the ileum in humans (Cornes, 1965), and follicle-associated epithelium with M-cells covers these patches (Owen and Jones, 1974).

Peyer's patches are the primary sites for initiating adaptive immune responses to luminal antigens derived from bacteria and food (Cornes, 1965; Reis and Mucida, 2012). Lymph from various intestinal segments drains into lymph node aggregates in the mesentery, including gastric lymph nodes, duodeno-pancreatic lymph nodes, mesenteric lymph nodes, and caudal lymph nodes, forming the largest lymphoid aggregates in the body (Mowat and Agace, 2014). Mesenteric lymphatic nodes collect bacterial and antigenic material derived from the adjacent intestine and regulate the migration of relevant immune cells to the associated intestinal mucosa (Coffey and O'Leary, 2016).

In contrast, the large intestine (colon) is a reservoir for numerous commensal microorganisms essential for health which can also ferment non-digestible polysaccharides and produce various metabolites influencing our health. The colonic immune system recognises these microorganisms as potential hazards and keeps them "at arm's length" without expelling them. This process involves the production of a thick mucus layer, the generation of IgA antibodies, and the presence of large numbers of regulatory T cells (Mowat and Agace, 2014).

Knowledge from FM showed that most bacteria in human faeces are coated with specific IgA (van der Waaij et al., 1996). The massive IgA secretion is crucial for immunologic homeostasis within the lamina propria and its protective function.

In the proximal region of the GIT, the microbiome has been studied less regarding local and chronic low-grade inflammation.

A study showed that, in obesity, jejunal mucosa shows increased infiltration of immune and adaptive T cell infiltration and notably $CD8\alpha\beta$ T cells in the jejunal epithelium

of patients with obesity. This infiltration of T cells impaired insulin sensitivity in enterocytes. The underlying mechanisms include the mislocation of GLUT2 induced by T cells' cytokine secretion, while this was not the case with T cells from lean subjects (Monteiro-Sepulveda et al., 2015).

Another study found that jejunal permeability increased in participants with obesity with a reduction of occludin and tricellulin. This was associated with increased circulating levels of LPS binding protein and zonulin, two markers of intestinal barrier dysfunction. Quantification of urinary lactitol/mannitol (L/M) confirmed intestinal barrier permeability to be increased (for small molecules) in subjects with obesity and associated with systemic inflammation. Finally, the authors showed this increased permeability to increase by two folds in obese subjects compared to lean subjects after a lipid challenge (Genser et al., 2018).

It would thus be interesting to investigate the role of the proximal small intestinal microbiome regarding these mechanisms and the potential protective or harmful role of specific pathogens in this region.

Mucus as a main segregator and habitat in the colon

Physical barriers encompass the mucus layer, glycocalyx on microvilli of absorptive intestinal epithelial cells, and cell junctions. These barriers physically prevent mucosal invasion by the GM.

Mucus, a viscous fluid secreted by goblet cells, is enriched in mucin glycoproteins, forming large net-like polymers. Mucus secretion is regulated by the host sensing gut microbes or their metabolites, such as SCFAs or cytokines (e.g., IL-5, IL-13) (Okumura and Takeda, 2017).

If we compare the SI to the colon in terms of mucosal architecture, the colon has a much higher number of goblet cells, producing mucus. In the colon, mucus is organised as a thick bilayer: the outer layer being looser and the inner layer being dense and firm, which prevents colonisation by gut microbes (Figure I.4.2). This inner mucus plays an important role as a physical barrier between the host and its intestinal epithelium and the microbiome. The outer mucus layer may be a substrate for bacteria in certain conditions (Okumura and Takeda,

<u>2017</u>). MUC2 is a highly O-glycosylated protein crucial in mucus organisation (Shan et al., 2013). In the colon, various antimicrobial molecules, including IgA and defensins, help protect against bacterial invasion of the inner mucus layer. A highly glycosylated GPI-anchored protein, Lypd8, contributes to segregating intestinal bacteria and intestinal epithelia in the large intestine (Okumura and Takeda, 2017).

Antimicrobial Peptides as a Main Segregator in the Upper Small Intestine

The epithelium of the SI has a much higher number of Paneth cells that produce antimicrobial molecules, including antimicrobial peptides (AMPs). In contradiction, the colon has almost no Paneth cells.

In the SI, the regenerating islet-derived protein 3 (Reg3) family of proteins produced by intestinal epithelial cells, particularly Paneth cells, also play a crucial role as a chemical barrier. These barriers play critical roles in segregating intestinal bacteria and epithelial cells. AMPs, including defensins and cathelicidins, disrupt microbial cell membranes by forming pore-like structures. The Reg3 family of proteins, especially Reg3 γ , is active against Gram-positive bacteria and is crucial for spatial separation in the SI (Okumura and Takeda, 2017; Shin et al., 2022). The gut peptide Reg3g links the SI microbiome to the regulation of gut function, energy balance and glucose levels. This will be discussed in the review inserted further in this manuscript.

The production of antimicrobial molecules by Paneth cells is regulated by TLR4/MyD88 and NOD2 signalling driven by gut microorganisms. Gut immune cells influence mucosal barriers through cytokine production or direct cell-cell contact. IL-17 and IL-22 upregulate AMPs and Reg3 family proteins secretion, while IL-6 enhances intestinal epithelial cell proliferation and contributes to mucosal injury healing. Pro-inflammatory cytokines, such as TNF- α and IFN- γ , inhibit epithelial cell proliferation. Th2 cytokines, such as IL-5 and IL-13, promote colonic wound healing (Lueschow and McElroy, 2020; Okumura and Takeda, 2017; Sicard et al., 2017). These molecules act as a chemical barrier between the host and pathogens, reducing colonisation of the mucus and mucosa by microbes (Chelakkot et al., 2018; Daniel et al., 2021; Hayes et al., 2018).

The intestinal mucosal barrier system is crucial for host health, and defects in these functions may lead to intestinal inflammation and irritable bowel disease (IBD), a group of diseases characterised by the chronic inflammation of parts or all of the digestive tract. Symptoms include, amongst many others, chronic diarrhoea, chronic fatigue, weight loss or to give another example, the presence of blood or mucus in stools. In USI research, the mucosal barrier has particularly been studied in regard to IBD, which includes ulcerative colitis and Crohn's disease (Okumura and Takeda, 2017). Some IBD patients exhibit reduced production of mucus or AMPs, and mice with genetic defects in mucosal barrier components demonstrate increased sensitivity to intestinal inflammation. Muc2-dependent microbial colonisation of the jejunal mucus layer is diet sensitive and confers local resistance to enteric pathogen infection (Birchenough et al., 2023). This is also further discussed in the review.

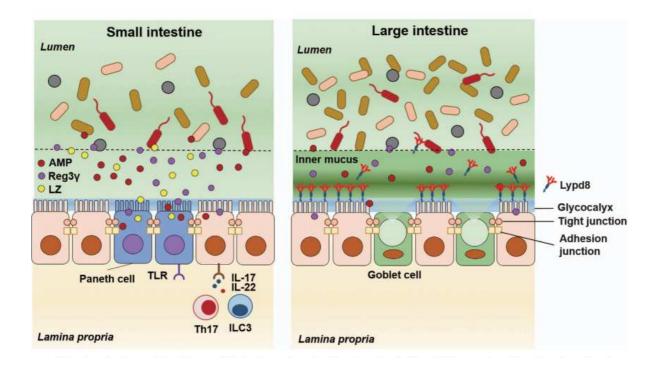


Figure I.4.2 - Differences of mucosal barriers in the small intestine and the large intestine. In the small intestine, chemical barriers, including AMPs produced by Paneth cells, have major roles in segregating intestinal bacteria and intestinal epithelial cells. In the large intestine, inhabited by a tremendous number of bacteria, intestinal bacteria and intestinal epithelial cells are separated by the inner mucus layer containing polymerised MUC2. Lypd8, a GPI-anchored protein expressed in the epithelial cells, promotes the segregation of the two by binding to intestinal bacteria, especially flagellated bacteria. Legend: AMPs, antimicrobial peptides; LZ, lysozyme; TLR, toll-like receptor; ILC3, type 3 innate lymphoid cell. Figure and legend reused from Okumura and Takeda (2017). Finally, peristaltism also plays a role as a 'mechanical' barrier against GM, particularly in the SI. In healthy patients, peristaltic activity is increased in the SI compared to the colon. This activity is crucial for preventing bacterial colonisation by pathogens or overgrowth in the SI. For example, a study showed that gram-negative bacilli in gastric and duodenal samples of women with late radiation enteropathy were associated with abnormal motility (Husebye et al., 1995). The SI undergoes two types of peristaltic activity: postprandial motility and fasting migrating motor complex motility pattern (Husebye, 1999; Vantrappen et al., 1977).

Characteristics of the Upper Small Intestinal Microbiome

Bacterial density increases progressively from the mouth to the colon. Spatial physico-chemical differences between the SI and the colon account for such changes. In the USI there is a decreased mucus production which may serve as a substrate for certain bacteria, there is an increased production of AMPs or BAs production, increased luminal flow rapidity and downward peristalsis flow preventing colonisation. In the USI, compared to the colon, there is a progressive increase in pH, from 6.6 in the proximal small intestine to 7.5 in the terminal ileum and 7.0 in the distal colon (Evans et al., 1988). There is also a progressive decrease in oxygenation. These changes affect the distribution of microorganisms along the length of the GIT but also the composition of the microbiome (Ahmed et al., 2007; Crespo-Piazuelo et al., 2018; Dicksved et al., 2009; Hayashi, 2005; Scheithauer et al., 2016; Sundin et al., 2017; Wilkinson et al., 2017).

Indeed, these distinct physicochemical properties account for changes in microbial composition along the GI tract (Figure I.4.3): at the phylum level, the stomach and the SI are dominated by Actinobacteria (renamed Actinomycetota) and Firmicutes (renamed Bacillota), while the colon shows higher proportions of Bacteroidetes (renamed Bacteroidota) (Scheithauer et al., 2016; Sommer and Bäckhed, 2016). The acidic pH and oxygen-rich environment of the proximal SI favours microbial colonisation with acid- and oxygen-tolerant bacteria (e.g. *Lactobacillus, Streptococcus, Veillonella*), whereas in the colon, oxygen-poor conditions and slower transit results in the fermentation of complex polysaccharides, resulting

in greater taxonomic diversity and dominance of saccharolytic anaerobic Bacteroidales and Clostridiales.

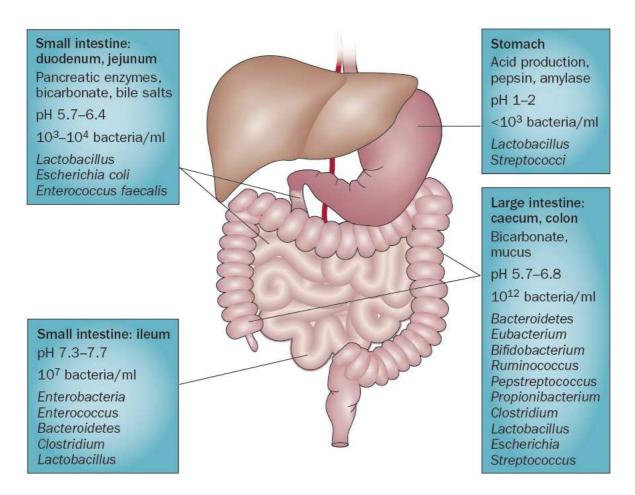


Figure I.4.3 - <u>The Gut Microbiome varies along the Gastro-Intestinal Tract</u>. The different segments of the GIT harbour different microbiomes regarding bacterial load and composition. Changes in physicochemical properties such as pH and oxygen availability, bile acids and antimicrobial peptide secretion, luminal flow rapidity, downward peristalsis flow, and nutrient availability explain these microbiome changes. Figure reused from Aron-Wisnewsky et al., (2012).

Investigations of the human SI microbiome showed in health and diseases that the duodenal microbiome is estimated to harbour $10^1 - 10^4$ CFU/mL (Booijink et al., 2007; Cotter, 2011; Donaldson et al., 2016; Hao and Lee, 2004; Macfarlane and Dillon, 2007; Sekirov et al., 2010). Studies reported to find in great abundance: *Lactobacillus, Streptococcus* (Hao and Lee, 2004; Macfarlane and Dillon, 2007), *Lactobacillus/Lactobacillaceae, Escherichia coli/Enterobacteriales* (Aron-Wisnewsky et al.,

2012; Donaldson et al., 2016), Enterococcus faecalis (Aron-Wisnewsky et al., 2012), Bacteroides, Clostridium, Streptococcus, Candida, Saccharomyces (Hillman et al., 2017) Bifidobacteria, Prevotella, Streptococcus, Clostridium, Lactobacillus, Enterococcus, Veillonella, Neisseria (Martinez-Guryn et al., 2019), Veillonellaceae, Lactobacillales, Pseudomonadales, Candida and Saccharomyces (Piewngam et al., 2020).

In the more distal area, the jejunal microbiome is estimated to contain $10^3 - 10^7$ CFU/mL (Aron-Wisnewsky et al., 2012; Booijink et al., 2007; Cotter, 2011; Lema et al., 2020; Martinez-Guryn et al., 2019; Piewngam et al., 2020; Sekirov et al., 2010). Studies report that this microbiome is dominated by *Lactobacillus, Streptococcus, Staphylococcus, Bifidobacteria, Enterococcus, Enterobacteriales*. Some studies report that strict anaerobes may be part of the normal jejunal microbiome (Aron-Wisnewsky et al., 2012; Donaldson et al., 2016; Hillman et al., 2017; Kennedy and Chang, 2020; Lema et al., 2020; Martinez-Guryn et al., 2017; Kennedy and Chang, 2020; Lema et al., 2020; Martinez-Guryn et al., 2019) but this is conflicted while others do not report these genera (Booijink et al., 2007; Piewngam et al., 2020).

To conclude, the SI is a challenging environment for the microorganisms colonising the GIT due to the presence of digestive enzymes and BAs, the shorter transit time, and thus the intermittent food substrate delivery. Consequently, the bacterial population of the USI have lower biomass and was found to have less diversity but greater dynamism in response to the rapidly changing luminal conditions. The proportion of gram-positive to gram-negative bacteria, facultative anaerobic to strict anaerobic species, increases from proximal to distal segments of the SI and colon, partly due to oxygen use by proximal aerobic and facultative anaerobic communities.

The source of the USI microbiome is not known. This ecosystem could be composed of transient microorganisms or endogenous resident organisms. The bacterial population of the USI could be continuously replenished by bacteria passing by the oral cavity (i.e., ingested with the food, contaminated through contact with our hands, contaminated from oral microbiome, OM). However, the acidic and enzymatic secretions of the stomach have a bacteriocidal effect on microbes from the oral cavity or food, and fewer than 10¹-10² CFUs per millilitre typically pass down the GIT to the duodenum and jejunum. The peristalsis and the ileocaecal valve prevent the retrograde migration of bacteria from the colon into the SI.

Due to the small population of bacteria in the jejunum, even a small retrograde migration from the colon could significantly increase the bacterial population of the jejunum (Sundin et al., 2017).

Dynamics of the Upper Small Intestinal Microbiome

The few studies on the small intestinal microbiome have generally employed either invasive sampling procedures or material from sudden death victims (Ahmed et al., 2007; Hayashi, 2005; Wang et al., 2005, 2003; Willing et al., 2010) preventing the study of the USIM over time or as a function of diet. Ileostomists provide an alternative to this problem (Gorbach et al., 1967; Zoetendal et al., 2012). Indeed, studies with ileostomies enable longitudinal effluent sampling directly from the SI. Some biases to take into account are potential contamination from the skin and external environment and oxygen penetration through the stoma increasing facultative anaerobe population (Hartman et al., 2009; Kastl et al., 2019; Zoetendal et al., 2012).

Bacterial populations in the USI have lower biomass and are less diverse, but are more dynamic in response to rapidly changing luminal conditions. Booijink et al., (2010) studied seven patients with IBD who underwent ileostomies and revealed significant interindividual differences. There were also intraindividual temporal fluctuations between morning and afternoon profiles throughout 9–28 days. Another study also found these rapid dynamics over a more extended study period (Zoetendal et al., 2012). Another study focused on *Streptococcus* and *Veillonella* species in ileostomies, showing considerable strain-level richness and temporal variation in the SI (van den Bogert et al., 2013). They showed a total of 160 *Streptococcus* and 37 *Veillonella* isolates, with temporal variance in 7 predominant isolates within 72 hours. This temporal variation contrasts with the relatively stable composition in the colon (Zoetendal et al., 2008). As discussed below, dietary influences drive some of these findings (Kastl et al., 2019).

Carbohydrate fermentation plays a vital role in GM function. Zoetendal and colleagues (2012) demonstrated that the small intestinal metagenome of ileostomists is significantly enriched with carbohydrate metabolism-related genes compared to the faecal metagenome in the same patients (Figure I.4.4). The small intestinal microbiome rapidly metabolise simple carbohydrates for community maintenance (Turnbaugh et al., 2010).

Streptococci are enriched in genes for energy generation and are thought to contribute to primary digestion in the SI, supporting the growth of secondary fermenters like Veillonella and Clostridium (Zoetendal et al., 2012). The cohabitation of these species occurs in the intestine, stomach, oesophagus, throat and oral cavity (Andersson et al., 2008; Bik et al., 2006) and is probably due to their metabolic interaction with lactic acid production and utilisation, respectively (Egland et al., 2004).

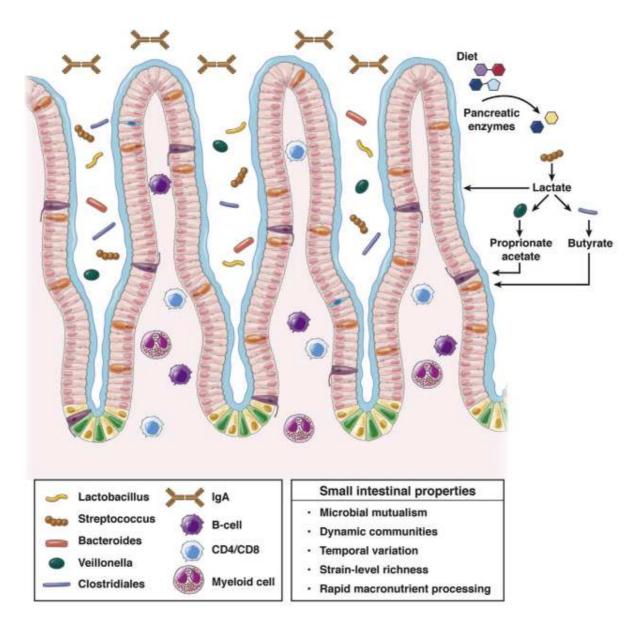


Figure I.4.4 - Dynamics of the upper small intestinal microbiome.

This figure shows the common bacteria genera in the small intestine and a brief overview of the properties of this ecosystem. *Streptococci* are lactate producers and cohabit with *Veillonella* and *Clostridium*, which are lactate secondary fermenters. A specific metabolic interaction between *Streptococcus*, *Veillonella*, and *Clostridiales* is highlighted to show an example of host–microbe and bacterial interactions. Figure reused from Kastl et al., (2019).

As introduced earlier, lipid digestion and absorption are complex physiologic processes central to the duodenum and jejunum (Volk and Lacy, 2017). The microbiome modulates lipid digestive physiology, and FM differs between mice on a low-fat and high-fat diet. HFD increase Clostridia abundance while decreasing Bifidobacteria and Bacteroides in faeces (Martinez-Guryn et al., 2018). Future investigations should consider the small

intestinal microbiome's response to fat saturation status and varying fatty acid composition (Kastl et al., 2019).

BAs are crucial for lipid and fat-soluble vitamin absorption and influence microbial growth and community structure (Begley et al., 2005; Carr and Reid, 2015; Friedman et al., 2018). They play many physiologic effects through activation of the nuclear hormone receptor farnesoid X receptor (FXR) and the G-protein–coupled receptor Takeda G-protein coupled Receptor-5 (Carr and Reid, 2015). Suppression of BAs synthesis increases SI gram-positive bacteria in human faecal samples (Friedman et al., 2018).

Primary BAs are deconjugated by bacteria in the ileum, with secondary BAs mediating microbial-host communication and are through to regulate the FXR pathway (Sayin et al., 2013). GF mice have increased conjugated BAs compared to conventionalised mice (Sayin et al., 2013; Swann et al., 2011). Increased BAs are thought to contribute to diarrhoea and inflammation and alter the composition of FM (Devkota et al., 2012). A high milk-fat diet (Martinez-Guryn et al., 2018) increased taurine-conjugated BAs and the abundance of sulfite-reducing *Bilophila wadsworthia*, which in turn was correlated with colitis in genetically susceptible interleukin-10 knockout mice (Devkota et al., 2012). Sulphate-reducing organisms (i.e., *desulfovibrios*) are found in greater abundance in mucosal biopsy specimens from patients with ileal Crohn's disease compared to controls (Pitcher et al., 2000; Zinkevich and Beech, 2000).

Current Knowledge from Disease Models of the Upper Small Intestinal Microbiome

Most of the current knowledge on USIM structure and function was acquired from studies on patients with ileostomies rather than patients with metabolic diseases. In the next section, I will highlight the major research in USIM and the knowledge acquired from disease models.

Knowledge of the Upper Small Intestinal Microbiome from Small Intestinal Bacterial Overgrowth

Small Intestinal Bacterial Overgrowth (SIBO) occurs when there is an abnormal increase in the overall bacterial population of the SI. A wide variety of commensal bacteria are increased in patients with SIBO, which indicates that SIBO could be a heterogeneous disease unlikely to be caused by a single bacterial strain. These bacteria that have been identified through duodenal and proximal jejunal cultures in patients with SIBO-induced diarrhoea and malabsorption include oropharyngeal and colonic commensal bacteria, including *Streptococcus, Escherichia, Staphylococcus, Klebsiella, Proteus, Lactobacillus, Bacteroides, Clostridium, Veillonella, Fusobacterium,* and *Peptostreptococcus*, among others (Bouhnik et al., 1999; Erdogan et al., 2015; Kastl et al., 2019).

This disease may result from gut stasis and has been studied in relation to various anatomic abnormalities and dysmotility (Swan, 1974; Wegener et al., 1994). In these disorders, due to ineffective food clearance, enhanced bacterial contact with food substrates can lead to excessive fermentation in the SI and symptoms such as bloating, nausea, abdominal pain, distention and acidic stool. In rare cases, excessive fermentation by *Lactobacilli* species, *Enterococci*, and *Streptococci* can lead to encephalopathy due to D-lactic acidosis, but this has been primarily observed in short bowel syndrome (SBS) (Bulik-Sullivan et al., 2018; Vitetta et al., 2017). Steatorrhea (increase in fat excretion in the stools), fatty acids malabsorption and fat-soluble vitamin deficiency can also occur due to premature bacterial deconjugation of primary bile salts in SIBO (Rana et al., 2017; Stotzer et al., 2003).

Usually, methanogenic archaea such as *Methanobrevibacter smithii* in the gut contribute to hydrogen depletion and methane production, a process that can be disrupted in SIBO, leading to altered microbial composition and resulting in positive hydrogen breath tests (Krajmalnik-Brown et al., 2012; Rezaie et al., 2017).

SIBO is known to affect certain vitamin biosynthesis. The small intestinal microbiome contributes to the synthesis and assimilation of several essential micronutrients (Blaner et al., 2016; Goncalves et al., 2015; Shearer and Newman, 2014). Vitamin K2 is mainly generated by intestinal bacterial biosynthesis (Shearer and Newman, 2014), and small intestinal bacterial overgrowth (SIBO) is associated with impaired vitamin K metabolism (Giuliano et al., 2010;

<u>Scarpellini et al., 2009</u>). Cobalamin (Vitamin B12) absorption is impacted by bacterial biology, with cobalamin deficiency being a complication of SIBO (Rowley and Kendall, 2019; Welkos et al., 1981; Wexler et al., 2018). Folate levels may be increased in SIBO due to bacterial biosynthesis (Rowley and Kendall, 2019). Iron, thiamine, and nicotinamide deficiencies have also been described in SIBO, though the mechanisms are not fully elucidated (Sachdev and Pimentel, 2013).

SIBO is associated with obesity and may alter intestinal barrier function and permeability, leading to increased circulating levels of endotoxins and chronic low-grade inflammation (Losurdo et al., 2020; Yao et al., 2023). This can lead to the onset of altered organ cross-talk and metabolic perturbations. However, the causal relationship between SIBO and obesity and the specific mechanisms have not been well elucidated. Further research is required to highlight the role of the USIM in metabolic diseases, such as the role of SIBO in obesity (Kastl et al., 2019; Yao et al., 2023).

Knowledge of the Upper Small Intestinal Microbiome from Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterised, notably, by changes in stool characteristics and associated with abdominal discomfort. IBS has been linked to gut microbiome alterations (Enck et al., 2016). Meta-analyses have shown that over one-third of IBS patients have small intestinal bacterial overgrowth (SIBO) (Chen et al., 2018). The efficacy of antibiotic treatment, such as Rifaximin (Pimentel et al., 2011), and dietary interventions like low fermentable oligo-, di-, monosaccharides, and polyols, further supports a microbial basis for IBS (Kerckhoffs et al., 2009).

Changes in populations of *Bifidobacteria*, *Prevotellaceae*, *Escherichia*, *Shigella*, *Aeromonas*, *Acinetobacter*, *Citrobacter*, and *Microvirgula* (Chung et al., 2016; Giamarellos-Bourboulis et al., 2015; Kerckhoffs et al., 2009) in the small intestine are associated with IBS, along with a decrease in diversity and an increase in the Firmicutes to Bacteroidetes ratio (Bhattarai et al., 2017). Germ-free mice colonised with stool from diarrhoea-predominant IBS patients showed faster intestinal transit, increased colonic permeability, and increased CD3+ T lymphocytes compared to mice colonised by stool from healthy controls (De Palma et al., 2017).

Microbially mediated effects on small and large intestinal secretion in IBS may be linked with BAs metabolism through deconjugation in the small bowel (Shin et al., 2013). Deoxycholic acid, a secondary BA, has been shown to induce intestinal peristalsis and contractions mediated by Takeda G-protein coupled Receptor-5 on enteric neurons and enteroendocrine cells and stimulate chloride and water secretion (Alemi et al., 2013; Alrefai et al., 2007; Ao et al., 2013). Additional evidence suggests that microbial metabolites, such as hydrogen sulfide, tryptamine, and hydrogen gas, contribute to intestinal motility regulation through their effects on smooth muscle (Jahng et al., 2012; Jimenez et al., 2017; Takaki et al., 1985).

Knowledge of the Upper Small Intestinal Microbiome from Environmental Enteric Dysfunction

USI is interesting in metabolic diseases such as malnutrition. Interestingly, the role of the USIM has been found to play an important role in environmental enteric dysfunction.

Childhood malnutrition is a worldwide health challenge, with new research indicating the role of microbiome maturation and enteropathogen burden as factors perpetuating malnutrition. Malnourished children exhibit impaired microbiome maturation, and FMT from these children into germ-free mice transmits impaired growth phenotype (Blanton et al., 2016). The addition of antibiotics to nutritional interventions has decreased mortality in children with uncomplicated severe acute malnutrition, showing the importance of studying microbial-based interventions (Trehan et al., 2013).

Environmental enteric dysfunction (EED) is a significant contributor to global malnutrition in children, characterised by the intersection of dietary macronutrient insufficiency and small intestinal dysfunction (Denno et al., 2017; Keusch et al., 2014; Syed et al., 2016). Increased inflammatory markers mark EED (Guerrant et al., 2016; Syed et al., 2018), such as small intestinal permeability markers and bacterial fragment translocation (Campbell et al., 2003; Kosek et al., 2017; Weisz et al., 2012; Welsh et al., 1998). Dietary interventions are insufficient for treating malnutrition in EED patients, and a disrupted resident small intestinal microbiome is hypothesised to play a critical role in EED pathogenesis (Bartelt et al., 2017; Kastl et al., 2019; Petri et al., 2014).

Enteropathogens commonly isolated in faecal samples from stunted children, such as Campylobacter species, Cryptosporidium, E. coli pathotypes, and Giardia, are typically found in the small intestine and have been associated with EED (Bolick et al., 2018, 2014; Costa et al., 2012). SIBO, diagnosed via a breath test, is also linked to malnutrition and poor sanitation (Kastl et al., 2019). Malnutrition can significantly alter the duodenal microbiome, causing a shift towards Bacteroidetes and Proteobacteria and changes in BAs and vitamin pools (Brown et al., 2015). Infection combined with malnutrition is required to replicate the small intestinal villus blunting and inflammation characteristic of EED in murine model. Several EED models with dietary restrictions and concomitant enteric pathogen exposure have been detailed, including Giardia, Cryptosporidium, Enteroaggregative E. coli, and a mixture of E. coli and Bacteroidiales. The dysbiosis induced by malnutrition, coupled with exposure to specific microbes, increases host susceptibility to adherent bacteria and enables pathogens to trigger ongoing immune responses even after pathogen clearance (Brown et al., 2015). In protein malnutrition, even a small Cryptosporidium inoculum triggered increased chemokine ligand 5, interferon γ , and B- and T-cell infiltration into the lamina propria, an effect not observed in fully fed mice (Bartelt et al., 2013). This evidence suggests that protein malnutrition in EED provides a platform for disrupted resident microbiome, intestinal pathogen colonisation, and small intestine injury. If the microbiome play such effects in malnutrition, it might also play a role in obesity. This will be discussed in the following section.

Upper Small Intestinal Microbiome in Metabolic Health

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Upper small intestine microbiome in obesity and related metabolic disorders: A new field of investigation

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ABSTRACT

The study of the gut microbiome holds great promise for understanding and treating metabolic diseases, as its functions and derived metabolites can influence the metabolic status of the host. While research on the fecal microbiome has provided valuable insights, it tells us only part of the story. This limitation arises from the substantial variations in microorganism distribution throughout the gastrointestinal tract due to changes in physicochemical conditions. Thus, relying solely on the fecal microbiome may not be sufficient to draw comprehensive conclusions about metabolic diseases. The proximal part of the small intestine, particularly the jejunum, indeed, serves as the crucial site for digestion and absorption of nutrients, suggesting a potential role of its microbiome in metabolic regulation. Unfortunately, it remains relatively underexplored due to limited accessibility.

This review presents current evidence regarding the relationships between the microbiome in the upper small intestine and various phenotypes, focusing on obesity and type 2 diabetes, in both humans and rodents. Research on humans is still limited with variability in the population and methods used. Accordingly, to better understand the role of the whole gut microbiome in metabolic diseases, studies exploring the human microbiome in different niches are needed.

1. Introduction

Obesity, characterized by the chronic and progressive accumulation of excess fat mass, has now reached pandemic proportions, posing a major public health concern worldwide [1,2]. The pathophysiology of obesity is complex, driven by the interplay between lifestyle/environment factors and a multitude of biological components including genetic/epigenetic factors [3]. In this challenging context, there has been a growing interest in elucidating the role of the gut microbiome in the development of obesity and its associated complications, including type 2 diabetes (T2D), cardiovascular diseases, and cancers [4–7].

The gut microbiome encompasses all the microorganisms inhabiting the gastrointestinal tract (GIT) and plays a critical role in various physiological processes, including digestion, immunity, and metabolism [8]. Recent research underscores that changes in the composition and function of the gut microbiome can lead to metabolic dysregulation or inflammation which in turn may contribute to obesity development and progression [9]. Therefore, targeting the fecal microbiome has emerged as a potential strategy for preventing and treating obesity and/or related comorbidities [10,11]. However, the distribution of bacteria along the GIT varies significantly [12-14]. Bacterial density gradually increases from the mouth to the colon, with a range of 10^{1} – 10^{3} colony-forming units (CFU)/mL in the stomach and duodenum, 10^4 – 10^7 CFU/mL in the jejunum and ileum, and 10^{11} – 10^{13} CFU/mL in the colon [15]. These changes are influenced by various physical and chemical factors that differentiate the small intestine (SI) from the colon. For instance, the SI presents a lower number of goblet cells and a thinner mucus barrier organized as a single layer compared to the colon, but it produces more antimicrobial peptides through Paneth cells, which help prevent bacterial colonization [15]. Additional factors such as increased luminal flow rate, intermittent food substrate delivery, downward peristalsis flow, and the release of bile acids (BAs) in the SI also contribute to reduced bacterial colonization. The pH of the intestine progressively

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Abbrevi	iations	HOMA-I	R homeostatic Model Assessment for Insulin Resistance
		KO	knockout
ACSL3	long-chain acyl-CoA synthetase-3	LFD	low-fat diet
BAs	bile acids	LFLD	low-fat, low-calorie diet
BMI	body mass index	LPS	lipopolysaccharides
BS	bariatric surgery	LS	longitudinal study
CD	control diet	NS↑	non-significant increase
CD36	cluster of differentiation 36	NS↓	non-significant decrease
CFU	colony-forming units	OB	group with obesity
CKK	cholecystokinin	ObD	group with obesity and diabetes
CLDN3	claudin 3	OB-1	group with moderate obesity
CSS	cross-sectional study	OB-2	group with severe obesity
CTRL	control group	REG3g	regenerating islet-derived protein 3 gamma
DJF	duodeno-jejunal fluid	rRNA	ribosomal ribonucleic acid
DW	drinking water	SGLT1	sodium-glucose cotransporter-1
EECs	enteroendocrine cells	SHAM	placebo surgery
GF	germ-free	SI	small intestine
GIT	gastrointestinal tract	T2D	type 2 diabetes
GLP-1	glucagon-like peptide-1	T2D-met	f group with type 2 diabetes treated with metformin
HFCD	high-fat, high-calorie diet	USI	upper small intestine
HFD	high-fat diet	WSD	western-style diet
HFHS	high-fat, high-sucrose	WT	wild type
HIF2a	hypoxia-inducible transcription factor 2 alpha	VSG	vertical sleeve gastrectomy

increases from 6.6 in the proximal tract to 7.5 in the terminal ileum, and stabilizes at 7.0 in the distal colon, affecting the distribution of microorganisms and influencing the microbiome's composition in each different GIT segment [16].

Furthermore, the upper small intestine (USI), which includes the duodenum and the jejunum, serves as the main site for the process of digestion and absorption of nutrients and minerals. This biological process involves a complex interplay of multiple factors including dietary signals, the hormone-secreting enteroendocrine cells (EECs), and the controlled release of bile [17]. As a result, microbial communities residing in the USI exhibit lower diversity compared to the colon in humans [18], displaying a remarkable level of dynamism that surpasses other regions of the intestine [19].

Several studies have investigated the mechanisms of lipid and glucose sensing that occur in the USI and the emerging role played by the USI microbiota in the absorption of macronutrients and in vitamin and micronutrient synthesis [20]. Upon food consumption, the intestinal uptake of lipids, which partly occurs through the CD36 transporter, stimulates the EECs to release intestinal peptides such as cholecystokinin (CKK) [20]. Similarly, the uptake of glucose in the intestine via the sodium-glucose cotransporter-1 (SGLT1) promotes the production of glucagon-like peptide-1 (GLP-1) by the L-cells located in the ileum and its subsequent release into the bloodstream. GLP-1, in turn, plays a critical role in glucose homeostasis by enhancing insulin secretion from pancreatic beta cell [17]. Additionally, similar to CKK, GLP-1 binds receptors in the brain thereby regulating food intake and decreasing endogenous nutrient production.

Recent findings suggest that the gut microbiome may play a role in regulating the secretion and transcriptome of EECs [21]. Surprisingly, germ-free (GF) mice exhibited higher colonic proglucagon expression compared to conventionally raised mice, leading to elevated circulating GLP-1 levels [22].

In light of these aspects, USI is an important site for neuroendocrine signaling, nutrient sensing, production of entero-hormones, and activation of different pathways that may induce energy expenditure and thermogenesis, promote satiety and lower food intake, or regulate glucose homeostasis by promoting insulin secretion or by lowering hepatic glucose production [23–25].

could represent a site of potential importance in the study of obesity and associated cardiometabolic diseases. However, most studies examining the gut microbiome's interplay with host metabolism have focused on fecal or colonic luminal samples partly due to their easier accessibility [26]. This leaves major gaps in our understanding of the complex relationship between the USI microbiome and host health and disease.

The purpose of this work is to present a comprehensive overview of the current evidence regarding the impact of the USI microbiome on different metabolic phenotypes including obesity and T2D.

2. Material and methods

The current literature was systematically reviewed up to September 10, 2023. To ensure a comprehensive and rigorous analysis, the research was conducted separately by two different researchers on multiple databases including PubMed, Cochrane, Web of Science, Scopus, and Embase. This method ensured to avoid mistakes and that no pertinent studies were overlooked during the initial screening process. The research was conducted by using a carefully selected set of keywords and their synonyms. The search string was the following one: "Microbiome" or "Microbiota" or "Microflora" or "Microbes" AND "Upper Small Intestine" or "Jejunum" or "Duodenum" AND "Obesity" or "Overweight" or "Metabolic Syndrome" or "Diabetes" or "High fat Diet" and all their synonyms. The search string was limited to "[Title/Abstract]" and publication date did not serve as a search criterion. Additionally, a thorough literature search was carried out across the references cited in the articles that were incorporated into this review. We included studies with the following criteria: 1) studies conducted on humans or rodents; 2) observational prospective and retrospective studies, cohort studies, and randomized clinical trials (RCTs); 3) studies written in English. Exclusion criteria were as follows 1) studies that did not include upper small intestinal microbiome analysis; 2) for clinical studies, cohorts that did not consist of at least one group of participants with metabolic dysregulations including overweight, obesity, insulin resistance, hyperglycemia or T2D.

3. Results

Given these considerations, it can be speculated the USI microbiome

We identified a total of 2266 studies through database searches and

by reviewing the reference lists of retrieved articles. After excluding 134 duplicates and 2041 studies based on their titles and abstracts, we carefully reviewed 91 full-text articles for eligibility. According to our inclusion and exclusion criteria, 17 articles were eligible for this review. Finally, we included 9 studies on rodents (Cf. Table 1) and 8 studies on humans (Cf. Table 2). Fig. 1 provides the flowchart with paper selection.

3.1. Upper small intestine microbiome in rodents: Effect of dietary manipulation

Studies first conducted in mice have shown that, at the phylum level, the microbiome of the duodenal mucosa comprises mainly Firmicutes, Proteobacteria, and Bacteroidetes [27]. At the genus level, *Lactobacillus* has been shown to be the most prevalent one in the USI microbiome of Wistar rats [28]. However, the USI microbial composition is altered by a wide range of external factors, with dietary changes being the most influential ones [29]. We summarize in Table 1 the results of animal studies analyzing the impact of high-fat diets (HFD) on the USI microbiome in rodents.

Martinez-Guryn and colleagues reported that HFD-induced obesity compared to a low-fat diet can increase the abundance of *Clostridiaceae* in the jejunum of C57BL/6 mice [30]. This finding aligns with the research conducted by Lacroix and colleagues who studied the impact of a high-fat, high-sucrose diet (HFHS) on the jejunum, ileum, and cecum microbiomes [31]. The analysis revealed that changes in the composition of the microbiome are dependent on the time elapsed since the start of the diet. Specifically, the relative abundances of Barnesiella, Defluviitalea, and Eubacterium were found to be reduced in the jejunum, ileum, and cecum as early as 10 days after the initiation of the HFHS diet. Akkermansia, Lactococcus, Coprobacillus, and Parasutterella decreased at later times in SI segments. Simultaneously, HFHS diet induced increases of two endocannabinoids, anandamide, in both ileum and plasma, and 2-arachidonoylglycerol, in plasma. These endocannabinoids are lipid derivatives that can activate cannabinoid receptors, primarily located in specific regions of the brain that are responsible for regulating food intake and rewards, namely the hypothalamus and the mesolimbic system [32]. This work highlights the involvement of the gut microbiome and the endocannabinoid system in obesity and related metabolic impairments.

Similarly, in a study by Bauer et al. involving eight-week-old adult male Sprague-Dawley rats, a HFD administered for three days led to a reduction in the relative abundance of *Lactobacillaceae* and an increase in the abundance of *Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Pseudomonadaceae,* and *Methylobacteriaceae* [33]. Importantly, they showed that the decreased relative abundance of *Lactobacillus* was associated with altered glucose sensing through SGLT1 expression, thus resulting in the alteration of overall glucose homeostasis. Interestingly, the authors demonstrated the causal impact of *Lactobacillus* on SGLT1 expression via the administration of metformin which restored *Lactobacillus* amount in USI. In addition, the transplantation of the USI microbiome from rats treated with metformin and fed with a HFD to untreated animals resulted in an increase in the abundance of *Lactobacillus* together with upregulation of SGLT1 expression, which in turn enhanced glucose sensing.

On the same line, HFD enriched with lard oil can cause a decrease in the abundance of *Lactobacillus gasseri*. The administration of this bacterial species is associated with improved metabolism in mice [34]. This change in the microbiome composition of the USI appeared to be related to a decrease in the long-chain acyl-CoA synthetase-3 (ACSL-3) fatty acid sensing pathway, which under physiological conditions, contributes to reduced hepatic glucose production. Thus, ACSL-3 reduction, as outlined by Bauer and colleagues, results in impaired glucose tolerance and hyperphagia in the animals. Moreover, the authors demonstrated that both USI microbiome transplantation from chow-fed animals and probiotic administration of *Lactobacillus gasseri* improved ACSL3 expression of USI and ACSL3-dependent glucoregulatory liposensing in HFD-fed rats [35].

Similar findings on HFD-dependent modulation of USI microbiome composition have also been observed in an unconventional animal model, p48-Cre (KC) mice, a model to study pancreatic inflammation and tumor progression promoted by obesogenic diets. Dong and colleagues observed a significant reduction in the presence of several genera, including *Dialister, Rikenellaceae*, and *Acetatifactor*, within the lumen of the duodenum. Conversely, the diet induced an increase in *Clostridium* sensu stricto. As far as microbial richness in the luminal duodenum is concerned, all measures of α -diversity, which is defined as the diversity of species within a particular habitat or community, (e.g., Chao1 and Shannon index) exhibited a significant decrease (p < 0.05) in response to the HFD when compared to the control diet [36].

Moreover, in a mouse model of HFD-induced obesity, the addition of inulin was found to have a significant prebiotic impact on USI microbial composition. Inulin is a fermentable fiber composed of a chain of fructose monomers linked together by β -(2-1)-D-fructosyl fructose bonds [37]. The β -configuration of its anomeric carbon renders it indigestible in the small intestine; however, it becomes a substrate for fermentation by the gut microbiome, primarily in the large intestine. Inulin serves important functions, including enhancing calcium absorption through passive diffusion, reinforcing calcium absorption via ion exchange mechanisms, and promoting the expansion of the colon's absorption surface by stimulating cell growth [38]. Shin and colleagues observed a decrease in the abundance of Firmicutes and an increase in the abundance of Bacteroidetes, Proteobacteria, and Actinobacteria both in the duodenal contents and feces of mice fed an inulin-enriched high-fat purified diet. At the family level, the duodenal microbiome of mice receiving inulin showed enrichment in Bifidobacteriaceae, Bacteroidaceae, and Sutterellaceae [39]. Noteworthy, mice fed an inulin diet supplemented with fermentable fibers showed an increase in the gene expression of the antimicrobial peptide, regenerating islet-derived protein 3 gamma (Reg3g), in all GIT segments compared with mice fed a cellulose diet. Therefore, a potential regulatory function of Reg3g in modulating glucose homeostasis and intestinal composition was hypothesized. In particular, the authors reported improvements in glucose tolerance in wild-type (WT) mice fed the inulin diet compared to those fed a cellulose diet, whereas Reg3g knock-out (KO) mice were refractory to this benefit of the inulin diet. Moreover, in mice gavaged with probiotics, including multiple strains of Lactobacillus and Bifidobacterium spp., the authors found increased levels of Reg3g both in the intestine and in the blood. Subsequently, the administration of lipopolysaccharides (LPS, 0.1 mg/kg) was found to increase intestinal permeability more in WT mice than in those without Reg3g, thus suggesting a contribution of Reg3g in regulating intestinal permeability in USI. Previous studies focusing on fecal exploration, have suggested that fermentable fibers can protect against metabolic disorders by acting on distal intestine microbiome composition [40,41]. As reported in a recent systematic review, the most consistent changes in fecal microbiome after inulin-type fructans supplementation include an increase in Bifidobacterium, and in the relative abundance of Anaerostipes, Faecalibacterium, and Lactobacillus, as well as a decrease in the relative abundance of Bacteroides [42].

Another factor that is capable of modulating the USI microbiome composition is mucus, which not only serves as a crucial immune barrier supporting colonization resistance, but also provides an energy source for selected mucus-degrading microorganisms including *Clostridium*, *Lactobacillus* and *Enterococcus* species.

The intestinal mucus barrier is sensitive to dietary changes in the lumen and research conducted in mice has demonstrated that dietary emulsifiers, detergent-like agents, commonly present in processed foods, may promote flagellated bacteria encroachment of the normally sterile inner mucus layer contributing to the establishment, promotion and chronicity of inflammatory diseases and to metabolic deregulations [43,44]. These studies were exclusively performed in the lower part of the intestine, where the mucus is made up of the gel-forming mucin

Table 1

Preclinical studies investigating the impact of HFD on the upper-small intestinal microbiome in mice.

First author	Year	Study design	Species	Duration	Type of diet/ Intervention	Control diet/ Intervention	Type of sequencing	Results
Birchenough et al. [47]	2023	LS	C57BL/6 WT male mice	8 weeks	high fat, high sugar, low fiber Western-style diet (WSD)	Normal chow diet (CD)	16S rRNA gene sequencing (Illumina MiSeq DNA platform)	 Exposure to a WSD drives region specific small intestinal mucus barrier dysfunction ↑ bacterial colonization and overgrowth in the small intestine; No significant effect of WSD exposure on mucus thickness, but ↑ penetrability; ↓↓ Muribaculaceae and Bifidobacterium and ↑↑ Faecalibaculum in the WSD-exposed microbiome.
Shao et al. [56]	2022	LS	Thirty 6-week- old C57BL6/J male mice	12 weeks	High-fat, iron-deficient diet	VSG (20) or SHAM (10) depending on weight	16S rRNA gene sequencing (Illumina MiSeq DNA platform)	 VSG results in metabolic improvements, † Lactobacillus spp. richness and activated HIF2a signaling specifically in the duodenum; Duodenal chyme samples: VSG microbiome showed †† Lactobacillus, Bifdobacterium and Akkermansia genus ††Verrucomicrobia phylum and Bacteroidales order; SHAM microbiota showed † Proteobacteri phylum and Actinomycetales order Duodenal epithelial scrape samples: VSG microbiome showed for only Bifdobacterium and Lactobacillus
hin et al. [39]	2022	LS	C57BL6/J and Reg3g KO or WT male mice	8 weeks	Inulin- or cellulose- enriched HFD	VSG or SHAM	16S rRNA gene sequencing (Illumina MiSeq DNA platform)	 Inulin-enriched diet was associated to ↓ Firmicutes and ↑ Bacteroidetes, Proteobacteria, and Actinobacteria, ↓ gut permeability in WT as compared to KO mice and expression of cell integrity (Occludin, Cldn.3) and antioxidant (Sod2, Cyba, Gpx2)-associated gen ↓ Firmicutes and ↑ Bacteroidetes i the duodenal content in WT-VSG mice relative to WT-sham controls but this was not observed in Reg3, KO-VSG mice
oteres et al. [53]	2020	LS	Thirty seven- week old C57BL6/J male mice	4 weeks	18 % anhydrous milkfat (HFD) + 200 μL of either a water vehicle control, rifaximin (30 mg/kg), or an antibiotic cocktail	4 % fat (LFD) + 200 μL of either a water vehicle control, rifaximin (30 mg/ kg), or an antibiotic cocktail	16S rRNA gene sequencing (Illumina MiSeq DNA platform)	 In the duodenum and jejunum, HFD ↓ Shannon diversity compare to the LFD In the jejunum HFD ↓↓ the abundance of all taxa except for Streptococcacee Antibiotic cocktail ↓↓ Shannon diversity in the duodenum and cecum of mice under LFD compare to HFD
oong et al. [36]	2019	LS	Offspring of LSL-KrasG12D/ + and p48- Cre+/- (KC) mice	3 months	High fat-high calorie diet (HFCD) with or without 5 mg/mL metformin in dw	Control diet (CD)	16S rRNA gene sequencing (Illumina MiSeq DNA platform)	 The mucosa-associated microbiome of the duodenum had abundance of Bacteroidetes and a of Firmicutes than the luminal microbiome of the duodenum. In the lumen of the duodenum, the HFCD ↓ the genera Dialister, Rikenellaceae, and Acetatifactor while ↑ Clostridium sensu stricto. Metformin with HFCD ↓ Clostridium sensu stricto to levels lower than mice fed on a CD, and the abundance of Akkermansia compared with both the HFCD alon and CD.
.acroix et al. [31]	2019	LS	Sixty 6-week- old C57BL6/J male mice	56 days	High-fat high-sucrose (HFHS)	low-fat, low-sucrose (LFLS)	16S rRNA gene sequencing (Illumina MiSeq DNA platform)	 HFHS ↓↓ Barnesiella, Defluviitalea, Eubacterium Akkermanisa and ↑ Sphingomona HFHS ↑↑anandamide, in both ileum and plasma, and 2-arachide noyl-glycerol, in plasma (continued on next page)

First author	Year	Study design	Species	Duration	Type of diet/ Intervention	Control diet/ Intervention	Type of sequencing	Results
Martinez- Guryn et al. [30]	2018	LS	C57BL/6 mice	4 weeks	Purified high saturated milk fat diet (HFD)	Purified low fat diet (LFD)	16S rRNA gene sequencing (Illumina MiSeq DNA platform)	 HFHS ↓↓ expression of <i>Ppara</i>, <i>Pparg</i>, and <i>Cnr2</i> in the ileum HFD-induced jejunal microbiome directly ↑ mucosal lipid absorption; HFD ↑↑ relative abundance of <i>Clostridiaceae</i> compared with LFD in the jejunum and ileum; HFD ↓↓ abundance of <i>Bifidobacteriaceae</i> and <i>Bacterioidaceae</i>.
Bauer et al. [33]	2018	LS	Eight-week old adult male Sprague- Dawley rats	3 days	Lard-oil enriched diet (HFD)/metformin ad.	Normal chow diet (CD)	16S rRNA gene sequencing (Illumina MiSeq DNA platform)	 Bacteroidaceae. HFD Disrupts USI Glucose Sensing, ↓↓ SGLT1 Expression and Glucose- Stimulated GLP-1 Release; Metformin Restores USI Glucose Sensing by Normalizing SGLT1 Expression; HFD ↓↓ the abundance of Lactobacillaceae and ↑↑ the abundance of Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Pseudomonadaceae, and
Bauer et al. [35]	2018	LS	Eight-week old adult male Sprague- Dawley rats	3 days	Lard-oil enriched diet (HFD)	Normal chow diet (CD)	16S rRNA gene sequencing (Illumina MiSeq DNA platform)	Methylobacteriaceae • HFD \1 Lactobacillus gasseri and \1 ACSL3-fatty acid sensing; • Transplantation of healthy USI microbiome to HFD-fed rodents restores LG levels and fatty acid sensing via \ACSL3 expression; • LG probiotic administration to HFD rodents is sufficient to restore intestinal ACSL3 expression and fatty acid sensing.

Abbreviations: LS, longitudinal study; HFD, high-fat diet; LFD; low-fat diet; WST, western style diet; HFCD, high-fat high-calorie diet; rRNA, Ribosomal ribonucleic acid; WT, wild type; KO, knock out; VSG, vertical sleeve gastrectomy; SHAM, placebo surgery; Reg3g, Regenerating islet-derived protein III-gamma; HIF2a, Hypoxia inducible factor 2; Cldn3, Claudin 3; DW, drinking water; ACSL3, Long-chain acyl-CoA synthetase-3,LFD, low-fat diet; LFLS, low-fat, low-sucrose; $\uparrow\uparrow$, significant increase; $\downarrow\downarrow$, significant decrease.

polymers (Muc2) that limit microbial access to the epithelium. Nevertheless, the mucus layer in the jejunum, which is looser and contains antimicrobial proteins secreted by epithelial enterocytes and Paneth cells, may serve as a habitat for those bacteria that can degrade Muc2 glycans [45,46].

In this context, Birchenough et al. have shown that exposure to a Western-style diet (WSD) can lead to region-specific small intestinal mucus barrier dysfunction, promoting bacterial colonization by the enteric pathogen *Citrobacter rodentium* and overgrowth in the SI [47]. The authors also observed a significant decrease in *Muribaculaceae* and *Bifidobacterium* and an increase in *Faecalibaculum* in the WSD-exposed USI microbiome. Although no significant effect of WSD exposure on mucus thickness was observed, there was an increase in permeability in the mid-jejunal segment, but not in the terminal ileal regions. Thus, diet-induced mucus alterations can profoundly affect Muc-2 dependent microbial colonization and related health outcomes.

Findings have also shed light on the complex role of the gut microbiome in regulating essential metabolic processes in the host, such as the digestion and the absorption of dietary lipids. These functions, which are of utmost importance in preventing both over- and undernutrition, involve a multitude of physiological mechanisms. To advance the understanding of the contribution of the small bowel microbiome in regulating host metabolic functions, Martinez-Guryn and colleagues utilized GF male C57B1/6 mice to which they transplanted the HFDinduced jejunal microbiome of other mice. After transplantation, mice exhibited increased lipid absorption, even when fed a low-fat diet, suggesting that the SI microbiome contributes to regulating host digestive and metabolic functions [30].

Thus, these findings highlight the importance of understanding the interplay between diet, mucus layer, and USI microbiome.

3.2. Upper small intestine microbiome in rodents and metformin administration

The USI (particularly the duodenum and the proximal jejunum) is the first site to be exposed to nutrients, as well as orally administered drugs, such as metformin. Metformin (1,1-dimethylbiguanide hydrochloride) is the first-line treatment in patients with T2D, yet its exact mechanism of action is still a source of investigation. In mice, after oral and even intravenous (IV) administration, metformin accumulates preferentially in the USI mucosa compared to other tissues [48]. Several studies indicate that the antidiabetic effects of metformin may be partly due to gut microbiome changes. Nonetheless, most of the evidence regarding this hypothesis still pertains to the fecal microbiome [49,50].

To delve deeper into the effects of metformin on the composition of the USI microbiome, Dong et al. conducted a study comparing the duodenal microbiome of mice randomly assigned to three groups: a control diet, a high-fat high-calorie diet (HFCD) and HFCD supplemented with metformin in drinking water. Metformin intake in the HFCD led to a non-significant rise in α -diversity indexes, while also reducing the presence of *Clostridium* compared to the control diet. The abundance of *Akkermansia muciniphila* was found higher in the group receiving the metformin-supplemented diet than in both the HFCD-only and control diet groups [36]. The authors also demonstrated a significant reduction in bacterial load in the cecum with the addition of metformin, compared to either a CD or HFCD alone.

As previously introduced, Bauer et al. showed that the transplantation of microbiome from metformin-treated HFD rats to the duodenum of untreated HFD rats resulted in the increased abundance of *Lactobacillus* with enhanced glucose sensing via upregulation of SGLT1 expression and consequently GLP-1 release [33].

Table 2

Clinical studies investigating the upper-small intestinal microbiome in humans with different metabolic phenotypes.

Author	Year	Country	Study design	Number and clinical characteristics of study subjects	Site of sampling	DNA (or RNA) extraction	Sequencing techniques	Results USI composition and diversity	Comments
Darra et al. [66]	2023	India	CSS	Hyperglycemic subjects ($n = 33$, median BMI 26.5 kg/m ² , 42,4 % female) Normoglycemic subjects ($n = 21$, median BMI 26.2 kg/m ² , 19 % female	Duodenal biopsies from the D2 region	DSP DNA Mini kit, Qiagen	16SrRNA sequencing (V3-V4 regions 16S rRNA gene; Illumina sequencing).	 70 bacterial families in duodenal microbiome including Burkholderiaceae, Prevotellaceae, Pasteurellaceae, Pseudomonadaceae, Neisseriaceae, Veillonellaceae, Carnobacteriaceae, Succinivibrionaceae, Lachnospiraceae, and Streptococcaceae; The variability in duodenal bacterial profile was associated with glycemic status; —In patients with hyperglycemia ↑ duodenal bacterial count, ↑ pathobionts and ↓in beneficial flora. 	Two distinct clusters representing fecal microbiome and duodenal microbiome; In feces ↑↑Proteobacteria while in duodenum ↓↓ Firmicutes
Villmones et al. [62]	2022	Norway	CSS	Ob (<i>n</i> = 60, median BMI = 41 kg/m ² , 10 with DM2)	 upper- jejunum samples (60 cm distal from Treitz), lower- jejunum samples (180 cm distal from the Treitz) 	DSP DNA Mini kit, Qiagen	16SrRNA sequencing (V3-V4 regions 16S rRNA gene; Illumina sequencing).	 and an beneficial fiola. No significant differences at the species level between the upper and middle part of jejunum; The most abundant species were associated with the oral cavity (Streptococcus sanguinis, Granulicatella adiacens/ para-adiacens); Actinobacteria, especially Corynebacterium spp., was ↑↑ in samples with low bacterial loads whereas the presence of Firmicutes, in particular Streptococcus sp., Gemella spp. and Granulicatella app. were 	All patients were prescribed a low- calorie diet (< 1000 kcal/day) 3 weeks before surgery and received perioperative antibiotic prophylaxis treatment
Gutierrez- Repiso et al. [77]	2020	Spain	CSS	Low HOMA-IR: $(n = 23; \text{ mean BMI} = 48.29 \text{ kg/m}^2, 75 \%$ females); High HOMA-IR: $(n = 10; \text{ mean BMI} = 54.76 \text{ kg/m}^2, 58 \%$ females); T2D-metf: $(n = 12; \text{ mean BMI} = 46.62 \text{ kg/m}^2, 82 \%$ females)	Jejunal biopsies sampled during gastric bypass, 40 cm distal from the Treitz.	PowerFecal DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA	16SrRNA sequencing (V2-4-8 and V3-6-7-9 regions) with an Ion 520 chip using the Ion Torrent S5 (Thermo- Fisher Scientific	the instantiation of the provided and the provided an	All patients were prescribed a very low-calorie diet (600–800 kcal/day) 15 days before surgery. All patients had perioperative antibiotic prophylaxis treatment

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tyrosine, and tryptophan

Author Year Study Number and clinical Site of DNA (or RNA) Sequencing Results USI composition Country Comments sampling techniques design characteristics of extraction and diversity study subjects biosynthesis in high HOMA-IR group vs low HOMA-IR. ↑pathways including glycosyltransferas in T2D-metf vs low HOMA-IR: 1 methane metabolism and linoleic acid metabolism in T2D-metf vs low HOMA-IR group; ↑enzymes involved in TMA. TMAO in T2D vs low HOMA-IR Sroka-Ctrl: (n = 27; BMI)MiniGenomic 16SrRNA ns↓ Chao1 index in Ob Exclusion criteria 2020 Poland CSS Biopsies from 23.2 kg/m², mean Oleksiak vs Ctrl and ObD; were: antibiotic the Kit. sequencing ns↑ Shannon index in Ob age = 42): descending (V3-V4 regions therapy and use of et al. A&A Ob: (*n* = 17, BMI [68] part of Biotechnology 16S rRNA gene; vs Ctrl and ObD groups; probiotic therapy =45 kg/m2; mean the duodenum Illumina ↓↓ Actinobacteria in Ob within the 30 days age = 40);(specific site sequencing). (13 %) and ObD (16 %) before duodenal ObD: (n = 22; BMI)groups vs Ctrl (21 %): not specified) biopsy sampling, =44 kg/m²; mean ↑↑ Bacteroidetes in ObD confirmed age = 45.5; gastrointestinal vs Ctrl group: administration of ↑↑ Gammaproteobacteria infections, chronic oral drugs for at in ObD vs Ctrl and Ob inflammatory bowel least 2 years after groups; disease, congenital diagnosis), ↑↑ Enterobacteriales in and acquired ObD group vs Ctrl and immune deficiencies, alcohol Ob groups; ns↑ Staphylococcus in Ob or drug addiction, (12,61 %) vs Ctrl (0.87 conditions requiring %) and ObD (0.99 %) psychiatric groups: treatment, pregnant ns↑ Lactobacillus in ObD women, patients (8.62 %) vs. control with LADA or MODY (0.58 %) and Ob (2.61 %): ns↑ Escherichia in ObD (18.42 %) vs Ctrl (4.84 %) and Ob (2.83 %) groups: ↑↑ *Bifidobacterium* in Ctrl (5.47 %) vs Ob (0.28 %) and ObD (0.69 %); Four species were identified in the Ctrl group: Bifidobacterium animalis (5.20 %), Bifidobacterium adolescentis (0.15%). Bifidobacterium longum (0.10 %), and Bifidobacterium bifidum (0.02 %) Granata 2020 Ctrl (n = 11, BMI = One duodenal mirVanaTM TruSeq RNA Exclusion criteria Italy CSS ↑↑ Firmicutes in Ob vs et al. 20.0-24.9 Kg/m²); biopsy miRNA Sample Ctrl; were: diabetes, Ob (n = 12, BMI =(specific site isolation kit Preparation kit ns↓ Proteobacteria in Ob [<mark>60</mark>] tumors, (Ambion, inflammatory bowel 40 kg/m²) not specified) v2 (Illumina vs Ctrl: Austin, TX, ↓↓ Actinobacteria in Ob diseases, Crohn's sequencing) USA) vs Ctrl; disease, viral ns† Streptococcus/ hepatitis, Prevotella ratio in Ob vs pharmacological treatment (i.e., Ctrl: ns†Firmicutes/ antibiotics, pro- and Bacteroidetes ratio in Ob prebiotics, antiviral vs Ctrl; or corticosteroid 45/55 overexpressed medications or genes in Ob vs Ctrl, of proton-pump these 16 genes (35 %) inhibitors) in the last involved in nucleotide 2 months before metabolism, 9 (20 %) in sample collection. carbohydrate metabolism, 9 (20 %) in

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the membrane transport

Author	Year	Country	Study design	Number and clinical characteristics of study subjects	Site of sampling	DNA (or RNA) extraction	Sequencing techniques	Results USI composition and diversity	Comments
								system, 4 (8.9 %) in amino acid metabolism, and 7 (15.6 %) in bacterial defense mechanisms.	
Nardelli et al. [59]	2020	Italy	CSS	Ctrl ($n = 16$, healthy volunteers; BMI 20.0–24.9 kg/m ² , 38 % females) suffering from gastroesophageal reflux disease symptoms Ob-1: group with moderate obesity (n = 13, mean BMI = 36.0 \pm 0.8; 54 % female); Ob-2: group with severe obesity (n = 6, mean BMI = 46.5 \pm 2.0 kg/m ² , 66 % females)	One duodenal biopsy (specific site not specified)	QIAamp DNA Stool Mini Kit, Qiagen	16SrRNA sequencing (V4-V6 regions 16S rRNA gene; Illumina sequencing).	no significant difference between OB-1 and OB-2; a significant difference in weighted UniFrac analysis between Ctrl, Ob-1and Ob-2; ns↓ richness and diversity in Ob vs Ctrls (Chao1, Shannon, Simpson); ns↓ Fusobacteria in Ob1 and Ob2 vs Ctrl; ns↑ Bacteroidetes in Ob1 and Ob2 vs Ctrl; ↑↑Proteobacteria in Ob1 and Ob2 vs Ctrl; ↓↓ Firmicutes in Ob1 and Ob2 vs Ctrls; ↓↓ Acidobacteria andActinobacteria in Ob1 and Ob2 vs Ctrls;	Exclusion criteria were: diabetes, tumors, inflammatory bowel diseases, Crohn's disease, viral hepatitis, any pharmacological treatment (i.e. antibiotics, pro- and pre-biotics, antiviral or corticosteroid medications for at least 2 months before the collection of samples).
Angelakis et al. [58]	2015	France	CSS	Ctrls (n = 5, healthy volunteers; BMI 20.72 \pm 2.3; mean age = 29.6; 40% females); Ob (n = 5; BMI = 36.8 \pm 8.4 kg/m2; mean age = 39, 40 % females)	Duodenal samples (1 mL) were aspirated at the ligament of Treitz (-10 cm with H2O).	QIAamp DNA Stool Mini Kit, Qiagen	16SrRNA sequencing (V6 regions 16S rRNA gene; Illumina sequencing).	ns† richness in the Ob vs Ctrl ns† Firmicutes in Ob (67 %) vs Ctrl (62 %) ns↓ Proteobacteria in Ob (4 %) vs Ctrl (9.5 %) ↑↑ anaerobic genera in Ob vs Ctrl	Aspiration was performed simultaneously to a test meal (with PEG4000) introduced into the stomach through the gastric tube using a 50 mL syringe over a period of 5 min
Corrodi et al. [57]	1978	USA	LS	Ob (n = 11, 45 % females)	Jejunal fluid was aspirated from the proximal jejunum (30 cm distal to the ligament of Treitz), and from the terminal ileum, (15 cm proximal to the ileocecal valve). For some patients, jejunal fluid was aspirated during follow- up endoscopy visits from the proximal end of the bypassed loop (proximal jejunum) and from the proximal bowel.	п.а	n.a After aspiration, the specimens were promptly injected into an anaerobic transport tube and brought to the laboratory where they were cultured within 2 h after collection from the bowel.	- Jejunal samples (8/11): Successful culture was obtained from 3/8 participants. - Ileal samples: The colony counts of the ileal contents were higher than those of the jejunal contents. 2/8 were considered sterile by bacterial culture; 6/8 showed wide variation in the total counts and species encountered (20 to 5.6×10^7 cft/mL). 3 samples showed equal counts of aerobes and anaerobes, 1 sample showed a clear predominance of aerobic organisms, 2 samples contained low counts of only one species each, <i>E.</i> <i>coli</i> and <i>B.fragilis.</i> - Follow-up endoscopy after bypass operation: Counts were higher than baseline (10^5-10^7 cft/ mL). Aerobes and anaerobes were present in equal numbers in 2 samples, whereas in the remaining specimen aerobes by	All patients did not have perioperative antibiotic prophylaxis treatment

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(continued on next page)

Table 2 (continued)

Author	Year	Country	Study design	Number and clinical characteristics of study subjects	Site of sampling	DNA (or RNA) extraction	Sequencing techniques	Results USI composition and diversity	Comments
								approximately 100-fold. <i>E. coli</i> and the <i>B. fragilis</i> group were present in high numbers.	

Abbreviations: BMI, Body mass index; LS, longitudinal study; CSS, cross-sectional study; Ob, Group with obesity; OB-1, group with moderate obesity; OB-2, group with severe obesity; ObD, group with obesity and diabetes; T2D-metf, group with type 2 diabetes treated with metformin; Ctrl, Ctrl group; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; ns↑, non-significant increase, ns↓, non-significant decrease; ↑↑, significant increase; ↓↓, significant decrease.

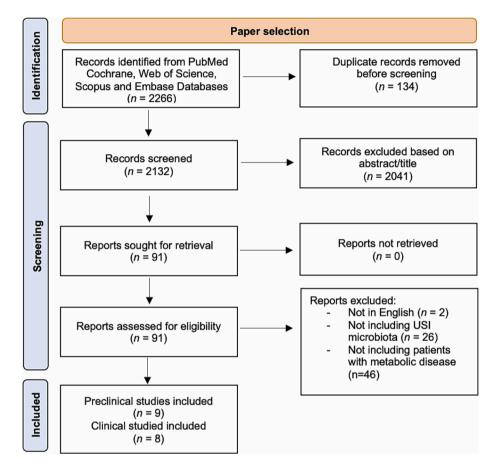


Fig. 1. Flowchart related to the selection of articles for this review.

3.3. Upper small intestine microbiome in rodents and antibiotic administration

Within the intricate interplay between drugs and the microbiome, antibiotics undoubtedly emerge as the agents exerting a profound influence on gut microbial communities. The discovery of the first antibiotic represented a real revolution in the therapeutic approach to infectious diseases, however, most of the antibiotics currently accessible in the market exhibit broad-spectrum activity, thereby impacting not only pathogenic bacteria but also commensal ones [51].

Antibiotic-induced gut microbial impairment, including reduced diversity and altered metabolic activity, has been extensively documented, highlighting multiple health consequences both in the short and long term [52].

Interestingly, a recent study has shown that antibiotic administration can cause regionally selective changes in the microbiome along the gut [53]. Poteres and colleagues explored C57Bl/6 mice fed either a low-fat diet (LFD) or HFD and treated with either a control vehicle, rifaximin or an antibiotic cocktail consisting of cefoperazone, vancomycin, metronidazole, and neomycin for four weeks. A main effect of the HFD on Shannon diversity reduction was observed in the duodenum and jejunum, but intriguingly not in the lower intestine, although previous articles have pointed to a detrimental role of WSD on colic microbiota richness and diversity [54]. The drug cocktail was the most powerful in increasing cecum size, as well as reducing fat mass and plasma lipids. In addition, the bacterial load was significantly reduced by the antibiotic cocktail under LFD in the cecum compared with the control vehicle. The only taxa that did not decrease after antibiotic administration was *Streptoccoccaceae*. According to the authors' findings, the HFD had the greatest influence on the USI microbiome, while the administration of antibiotics impacted all GIT regions, with a more notable effect on the duodenum and cecum, where a reduction was observed mainly in the abundance of Bacteroidetes and *Bifidobacteriaceae*.

3.4. Upper small intestine microbiome in rodents and metabolic surgery

Bariatric surgery (BS) stands as a highly effective treatment option for the management of severe obesity. Studies reported that not only BS leads to significant and sustained weight loss, improvements in comorbidities like T2D, and reduced mortality, but it also has profound effects on gut microbiome composition [11,15,55]. Despite its growing popularity and success, as well as the results of some studies conducted on mouse models, our understanding of the complex interplay between BS and gut microbiome remains limited.

Shin and colleagues have shed some lights on this fascinating topic. In particular, using vertical sleeve gastrectomy (VSG), the researchers investigated the changes in USI microbiome composition and function following BS in rats and mice [39]. Surprisingly, they found that VSG can increase the expression and circulating levels of Reg3g, involved in proper gut homeostasis.

Furthermore, to better understand whether the beneficial effects of bariatric surgery were Reg3g-dependent, the authors compared the outcomes after VSG in Reg3g KO and WT mice. While in the first 2 weeks after VSG, both WT and KO mice lost a comparable amount of weight to their respective sham controls, Reg3g-KO mice began to gain more weight after 2 weeks and recovered body weight up to the level of shamoperated mice. The authors also investigated changes in microbiota composition in the duodenal content of mice following VSG and there was a lower abundance of Firmicutes and a higher abundance of Bacteroidetes in the postoperative period compared to sham controls.

Using a Random Forest analysis, the researchers ranked microbial families based on their discriminatory significance. It revealed *Lactobacillus* in the duodenum of Reg3g KO mice as the primary taxonomic group distinguishing VSG-operated mice from sham controls. These observational findings suggest that Reg3g is fundamental in the metabolic benefits observed following BS or dietary interventions, while paving the way to the investigation of mechanistic aspects underpinnings of this association.

Shao and colleagues conducted a preclinical study to evaluate the effect of VSG on the composition of the microbiome isolated from duodenal epithelium in high-fat-fed mice [56]. Four weeks following bariatric surgery, mice that underwent VSG showed a noteworthy increase in the population of oxygen-tolerant anaerobes, including Lactobacillus, as well as strictly anaerobic bacteria like Bifidobacterium and Akkermansia in comparison to sham-operated mice. The diversity and richness indices of the microbiome in the duodenum and ileum were lower than those in the caecum, but no statistically significant differences were found between the VSG and SHAM groups within each intestinal segment. Furthermore, the authors demonstrated that the VSGmediated increase in Lactobacillus spp. is responsible, through increased lactate production, for activating Hypoxia-Inducible Factor 2α (HIF2a) signaling and its target genes Dmt1, Dcytb and Neu3, which are implicated in iron absorption and can play an integral role in intestinal physiology.

Abbreviations

GLP1	glucagon-like peptide 1
Reg3g	Regenerating islet-derived protein III-gamma
HIF2a	Hypoxia-inducible factor 2

3.5. Upper small intestine microbiome in human obesity and related disorders

There have been limited studies evaluating the composition of the USI microbiome in patients with metabolic disorders (Fig. 3). The first study that evaluated the USI microbiome dates back to the late 1970s. Corrodi and colleagues cultured material collected from eight patients with obesity undergoing jejunoileal bypass. Out of the eight jejunal samples, five were found to be sterile by bacterial culture, while three contained low counts of predominantly aerobic microorganisms. In the ileal content, the bacterial count was higher than in the jejunum, though the two sites were similar in terms of anaerobes and aerobes proportion. However, in three subjects the USI contents showed higher counts of

cultivable bacteria $(10^{5}-10^{7}$ CFU/mL) than the terminal ileum. Furthermore, in those patients for whom samples were obtained from a second surgery performed for bypass-related complications, the jejunal microbiome qualitatively resembled that of the feces, with a predominance of the facultative aerobic *Escherichia coli* and the anaerobic *Bacteroides fragilis* [57]. This observation can be related to the jejunoileal bypass intervention with colonic flora colonizing both the excluded loop and the remaining SI.

From the culture-based approaches of traditional microbiology, microbiome analysis has evolved, and bacterial genome sequencing now uses metagenomic, metatranscriptomic, and metabolomic analysis techniques. As such, Angelakis et al. conducted a small study using 16S rDNA V6 pyrosequencing and Illumina MiSeq deep sequencing to analyze the microbiome of duodeno-jejunal fluid (DJF) in 10 participants, 5 healthy volunteers and 5 subjects with obesity [58]. The results showed that both groups had similar duodenal microbiome at the phylum level, with Firmicutes and Actinobacteria being the most represented phyla. Compared to knowledge on fecal microbiome composition, the authors found lower levels of Bacteroidetes and increased Actinomyces and Streptococcus in the DJF. People with obesity had an increased duodenal microbiome richness compared to lean ones, but this difference did not reach statistical significance. At the genera level, low diversity, expressed as Simpson index, with mainly Streptococcus, Actinomyces, Propionibacterium and Granulicatella were reported in the DJF of patients with obesity.

Nardelli and colleagues confirmed that the taxonomic composition of the gut microbiome is altered in 6 patients with severe obesity when compared to individuals with normal weight [59]. Specifically using 16SrRNA sequencing, they found that the relative abundance of Proteobacteria was increased while Firmicutes were significantly less abundant in the gut microbiome of patients with obesity compared to controls. The authors reported specific anaerobic bacterial species, including *Oribacterium asaccharolyticum, Atopobium parvulum,* and *Fusobacterium nucleatum*, that were significantly reduced in the group with obesity compared to lean one. There was no statistically significant difference in taxa abundance between patients with moderate and severe obesity, but alpha diversity metrics indicated a trend of decreased microbial richness in patients with moderate and severe obesity when compared to controls.

The same group explored the functional activity of the duodenal microbiome in 12 subjects with severe obesity compared to lean controls, providing the first human duodenal metatranscriptome [60]. The authors discovered that the bacterial functional profile in the duodenum differed between the two groups. The most prevalent and active functionalities in the duodenal gut microbiome of individuals with obesity were related to the metabolism of carbohydrates. Moreover, the bacterial gene abundances showed a significant difference between patients with obesity and controls, as well as considerable intra-group variability. Notably, 45 genes were overexpressed in the group with obesity, mainly related to nucleotide metabolism (35.5 %), carbohydrate metabolism (20 %), the membrane transport system (20 %), and amino acid metabolism (8.9 %). In addition, the study revealed the upregulation of several enzymes associated with the glycolytic pathway, including aldolase, enolase, pyruvate kinase, phosphoglycerate kinase, pyruvateformate lyase, and pyruvate-ferredoxin oxidoreductase, in patients with obesity compared to lean controls. Many of these enzyme activities are shared by both Prevotella and Bacteroides. Notably, when coexisting in the same environment, one of these genera typically dominates and acts as an antagonist. The authors reported that Prevotella spp. exhibited greater activity than Bacteroides in the duodenum of patients with obesity versus lean subjects. However, unlike Bacteroides, Prevotella does not produce propionate, which, as previously described, can potentially affect the abundance of specific short-chain fatty acids available for host colonocytes and thus influence human host physiology [61]. Additionally, the authors found a non-significant increase in the Streptococcus/Prevotella ratio in the group with obesity. However, mRNA

expression abundance showed an opposite trend in the abundance of Firmicutes and Proteobacteria, which were more and less abundant in the group with obesity than in controls, respectively, than what was previously reported with 16S rRNA sequencing [59].

The bacterial microbiome was also investigated in two separate segments of the jejunum, collected at 60 and 180 cm from the Treitz angle in patients with obesity [62]. The composition of these two segments showed no significant differences, and the samples had a limited number of microorganisms, mainly belonging to the genera Corynebacterium, Streptococcus and Granulicatella, limiting the establishment of a core microbiome. Importantly, the most common and abundant species identified through the 16S rRNA sequencing were associated with the oral cavity. These species included the Streptococcus mitis group, the Streptococcus sanguinis group, Granulicatella adiacens/para-adiacens, the Schaalia odontolytica complex, and Gemella haemolysans/taiwanensis. Although it is acknowledged that there may have been oral microbiome contamination during the collection of jejunal fluid, these findings suggest that the microbiome in the USI is likely more similar to the oral microbiome than to the fecal microbiome in patients with obesity. In addition, direct contamination of the USI microbiome with the oral microbiome can be assumed since we continuously produce and swallow saliva throughout the day. Nevertheless, to date, there have been no studies comparing the microbiome of these three biological niches in patients with obesity.

3.6. Upper small intestine microbiome, pre-diabetes, type 2 diabetes and metformin administration in humans

Obesity is a major risk factor for T2D, which is now the most common endocrine disorder affecting between 5 and 15 % of the adult population worldwide and which is set to grow in incidence and prevalence [63]. A recent study found that patients who underwent total or partial colectomy had a higher risk of developing T2D than those who underwent other types of surgery not involving the GIT [64]. Therefore, there is a major interest in understanding the role played by the gut microbiome in the context of T2D and its precursor states.

USI microbiome may have a significant relevance since incretin hormones that contribute to glucose homeostasis are mainly produced at that site [65]. In a recent cross-sectional study, Darra and colleagues examined the duodenal mucosa-associated microbiota in individuals with pre-diabetes and normoglycemic subjects [66]. Their findings revealed an association between hyperglycemia and duodenal microenvironment. Specifically, when compared to normoglycemic individuals, those with fasting hyperglycemia displayed a significantly higher prevalence of duodenal bacterial load ($>10^3$ bacteria/ng DNA). They also exhibited an increased presence of pathogenic species such as Acinetobacter haemolyticus, Dolosigranulum pigrum, and Streptococcus cristatus, alongside a remarkable 23-fold decrease in beneficial bacteria like Akkermansia muciniphila. Given the elevated presence of pathogenic organisms in the duodenal microbiome of hyperglycemic individuals, the authors assessed the subjects' inflammatory status. Notably, they observed a correlation between bacterial overgrowth and levels of serum zonulin and TNF- α , indicating increased gut permeability and an inflammatory state, respectively. Moreover, zonulin expression also positively correlated to anthropometric parameters such as BMI and waist circumference. Furthermore, the duodenal environment in hyperglycemic individuals was characterized by reduced oxygen saturation, an increased total leukocyte count, and decreased levels of interleukin 10.

Interestingly, Gutierrez-Repiso et al. evaluated the USI microbiome in patients with morbid obesity, comparing subjects with insulin resistance or without and subjects on metformin treatment or not. To note, the authors found that the metagenomic richness of the USI microbiome inversely correlated with insulin resistance, with a statistically significant reduction in the number of genes observed in the group with insulin resistance compared with the group with low HOMA-IR. However, this reduction did not appear statistically significant between the metformintreated diabetic group and the high HOMA-IR group. Furthermore, regarding the composition of the USI microbiome, the relative abundance of the Phyla *Proteobacteria, Fusobacteria* and *Bacteroidetes* was higher in patients with high HOMA-IR than in subjects with low HOMA-IR as well as the abundance of *Verrumicrobia* in the metformin-treated group [67].

These results are in line with those of Sroka Oleksiak and colleagues, who showed that patients with T2D and patients with obesity have a common core microbiome in the duodenal mucosa at the phylum level, consisting mainly of Firmicutes, Actinobacteria and Proteobacteria, with significantly fewer *Bifidobacterium* than healthy subjects. Specifically, the authors report that in a group of 27 patients with T2D, the percentage of bacteria belonging to the phylum Bacteroidetes was statistically significantly higher than in the control group of 22 healthy subjects and the Gammaproteobacteria class was significantly more represented than both controls and patients with obesity [68].

4. Discussion

Factors, such as physico-chemical properties, the mucus layer, diet (and more broadly lifestyle), drug intake, and the host's immune system, among others, influence microbial distribution in different portions of the digestive tract [69,70]. While most studies exploring the relationship between the gut microbiome and host metabolism have relied on fecal or colonic luminal samples, research on the potential link between the USI microbiome and metabolic diseases is warranted. In this review, we provide an overview of the current knowledge on this topic which has gained growing interest in recent years.

As far as microbiome composition is concerned, at the phylum level, the USI is dominated by Actinobacteria, Proteobacteria, and Firmicutes while the colon shows higher proportions of Bacteroidetes. Consequently, the USI microbiome predominantly consists of facultative anaerobic and aerobic bacteria such as *Lactobacillus, Streptococcus, and Veillonella*. Conversely, in the colon, obligate anaerobes dominate, resulting in increased taxonomic diversity and the predominance of saccharolytic anaerobic bacterial groups like *Bacteroidales* and *Clostridiales* [15].

However, to appropriately compare the USI and fecal microbiome, it is essential to collect samples from the same individuals across the full length of the small and large intestines. This approach ensures that results are directly comparable within each patient. To this aim, two complementary studies were conducted by Shalon et al. [71] and Folz et al. [72] and provided valuable insights into the metagenome and metabolome of the entire GIT in fifteen healthy volunteers. These studies used pH-sensitive capsules which enable sampling from multiple regions of the human intestinal tract during digestion. Specifically, their research confirmed distinct variations in microbiomes throughout the GIT, such as an increase in the relative abundance of Proteobacteria in the USI compared to feces. Moreover, the presence of Bilophila wadsworthia, a bile-resistant bacteria was particularly encountered in the USI [71]. The viability of a proportion of USI bacteria was then confirmed through bacterial culture. To note, the metabolome also varied along the GIT. The authors reported a shift in proteome and metabolome from the proximal to the distal gut and 50 % of the molecules identified along the GIT were more abundant in the USI and with variations across individuals that were mainly attributed to dietary differences among participants. The USI showed increased concentrations of peptides while the colon had increased concentrations of SCFAs, Bas, and phospholipids.

Primary host-derived conjugates BAs decreased along the GIT, probably under bacterial action through microbial enzyme bile salt hydrolase, and microbially produced secondary BAs were increased in feces as already well reported in the literature [73]. The authors reported an increased abundance of the newly discovered microbially conjugated BAs in the gut compared to stools, although their action on

the host physiology is still unknown.

Interestingly, another recent study by Meier et al. compared the metabolome between conventional and GF mice, revealing the causal role of the local microbiome on the variation of the metabolome along the GIT [74]. Indeed, they were able to identify different niches determined by microbial activities along the GIT.

Whether such different metagenomic and metabolic profiles along the GIT are also observed in patients with obesity and type 2 diabetes remains to be clarified.

Within the articles reviewed here, only one study delved into the comparison of microbial compositions between the duodenum and stool. Interestingly, Darra et al. reported that the bacterial composition of the duodenal mucosa exhibited more variation among individuals than that of stool samples [66]. Moreover, by employing DESeq2, the authors identified taxonomic distinctions at the species level between stool and duodenal biopsy samples, revealing 153 taxa with differential abundance between the two sample types. Specifically, 97 genera contributed to the unique composition of the duodenal community, with notable features including *Delftia* spp., *Granulicatella* spp., *Pseudomonas* spp., *Carnobacterium maltaromaticum, Serratia* spp., *Prevotella nanceiensis*, and *Sphingomonas* spp.

In contrast, significant taxa distinguishing stool samples included Megasphaera spp., Bacteroides uniformis, Faecalibacterium prausnitzii, Ruminococcus bromii, Bifidobacterium longum, Dorea longicatena, Bacteroides thetaiotaomicron, Alistipes putredinis, and Lactobacillus delbrueckii.

Insights into the role of the USI microbiome in regulating host

metabolism and responding to dietary signals have been mostly provided by rodent models. Nine studies, listed in Table 1, demonstrated that HFD alters the duodeno-jejunal microbiome, while bariatric surgery or fiber intake partly contribute to its functional recovery. Still, differences in sample collection methods and variations in analytical methodologies (e.g., database for taxonomic identification) across studies limit a current and extended vision on proper taxonomic classification of the USI microbiome. More studies are also needed to decipher the mechanistic aspects connecting gut microbiome composition, mucus, intestinal layer, host metabolism, and inflammatory status.

Animal studies were principally conducted on the murine C57BL/6 strain undergoing HFD and USI microbiome exploration was mostly performed using 16S rRNA gene sequencing. However, none of the studies reviewed in this paper reported methods to prevent coprophagy in mice, and, therefore, the possibility that the fecal microflora affected that of the USI cannot be denied. When conducting research on USI using rodents, coprophagy should be avoided as it alters the microbiome of the USI and shifts its composition closer to that of the colon, such as altering the BA profile in the small intestine of mice [75]. Several precautions can be taken (e.g., mice wearing the tail cup) to avoid coprophagy-induced changes. Such precautions showed an increased relative abundance of genera such as Lactobacilli in the USI microbiome [76].

Different studies also suggest an USI dysbiosis in diet-induced obesity mice, reporting a reduction in *Bifidobacteriaceae* and *Lactobacillaceae* and an increase in *Clostridiaceae* and *Enterobacteriaceae* (Fig. 2). Multiple additional factors may nevertheless contribute to USI microbiome

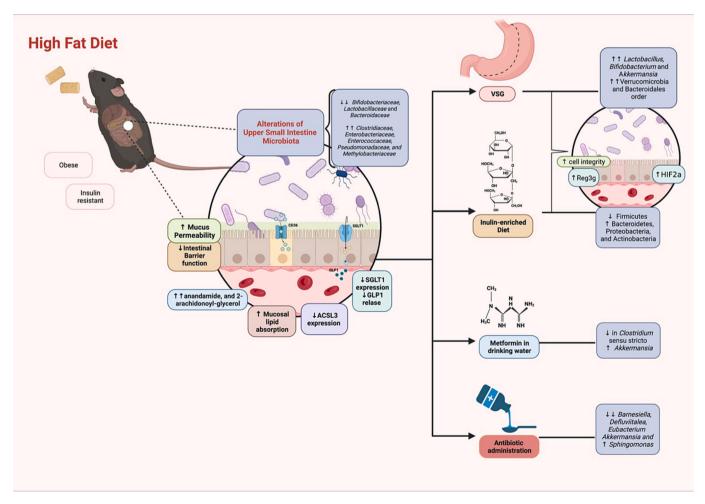


Fig. 2. Alterations of the upper small intestine microbiome in mice under high-fat diet and related modifications due to bariatric surgery, inulin, metformin or antibiotic administration. (created with Biorender.com).

modulation including medication, diet modulation or surgical modification of the GIT. VSG and inulin supplementation can contribute to the simultaneous increase in the expression of genes associated with cellular integrity in the intestinal epithelium and antioxidant activity. Conversely, metformin administration promotes an increase in *Akkermansia* abundance, ultimately leading to improved bacterial composition with supposed effects on host metabolism improvement.

As far as research on humans is concerned, we retrieved eight articles examining the USI microbiome obesity and/or T2D as summarized in Table 2.

Most clinical studies in this field have primarily relied on fecal or colonic luminal samples and research investigating the potential link between the human USI microbiome and metabolic diseases is still scarce. Therefore, it is important to acknowledge the limitations of using stool as a surrogate for the entire GIT.

Fecal sampling offers advantages such as accessibility, minimal invasiveness, high bacterial biomass, making DNA extraction easier, and cost-effectiveness. In contrast, USI represents the site of convergence between food, antimicrobial peptides Bas, and pancreatic secretions, as well as the principal absorbent surface of the GIT. USI sampling with endoscopy or during surgical procedures may provide more precise location targeting, potentially yielding more accurate data, particularly relevant to upper digestive tract and metabolic conditions. However, it requires specialized equipment, carries higher costs, and may raise ethical concerns. In this regard, a technical (and ethical) reason for the current low information is the access to the USI microbiome which can be mainly done by fibroscopy. Bariatric or metabolic surgery approach nevertheless facilitates this access. The resection of an intestinal fragment during a gastric bypass may indeed provide the opportunity to collect the luminal contents of USI. Nevertheless, endoscopy and surgery remain invasive and still provide restricted access to specific intestinal regions and are dedicated to patients with severe obesity. In addition, apart from emergencies, the gut is always "cleaned" with antibiotic therapy and laxatives before surgery. Indeed, these preparatory

measures may have an impact on the microbial composition and metabolic status of the gut. Among the clinical studies included in this review, two analyzed the composition of the USI microbiota in a cohort of patients with severe obesity undergoing bariatric surgery [62,67]. As reported by the authors, these patients received perioperative antibiotic prophylaxis, and it is critical to consider this limiting factor when interpreting data from these cohorts, as they may not fully represent the natural, pre-surgical state of the intestine.

Whereas the number of studies regarding the human USI microbiome is scarce, methods and results are already inconsistent across studies, making it difficult to integrate the results for generalization, as is also the case often for fecal microbiota studies. This inconsistency is due to various factors.

Cohort sizes ranged from 10 patients [58] to 66 participants [68], with each study including at least one group of subjects with overweight or obesity. Except for the study by Angelakis et al. including overweight participants with a body mass index (BMI) > 28 kg/m², other studies involved individuals with severe obesity BMI \geq 35 kg/m²). One study featured participants from a cancer-screening program [68], while all other studies included candidates for bariatric surgery. It is key to note that several studies before or during bariatric surgery procedure follow a low-calorie diet or modify their diet, which can strongly impact the microbial composition in the USI region.

Regarding study design, out of the seven included papers, two did not have a comparison group and only described the microbial composition in the group with obesity [57,62]. Three studies compared the group with obesity to lean participants without metabolic disorders [58–60], while one study compared the group with obesity to both a control group and a group with obesity and T2D. [68] Finally, in one study the group with obesity was stratified into Low Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) and high HOMA-IR, and the T2D group was stratified into T2D without metformin and T2D with metformin [77]. All human studies are observational and based on a one-time point analysis, except for one that compared the USI

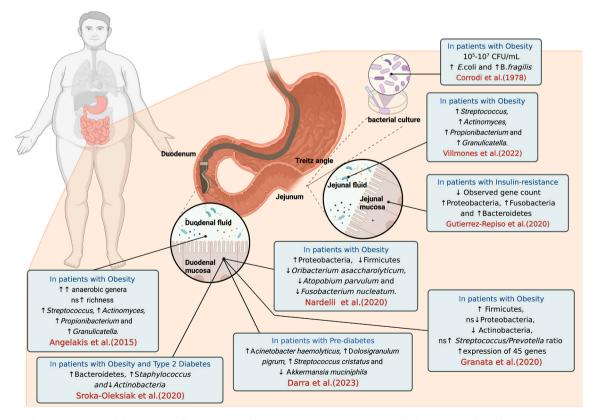


Fig. 3. Alterations of the upper small intestine microbiome composition in patients with obesity (created with Biorender.com).

microbiome before and after bariatric surgery [57]. Sampling methods varied among the selected studies, being performed during bariatric surgery [62,77], or through the endoscope during fibroscopy that can provide result variability [57–60,68]. In this regard, it is important to note that endoscopic sampling is a potential source of contamination from segments proximal to the sampling site. As the USI microbiome has a low bacterial load, it is important to prevent contamination from microbe-rich areas and, in this case, oral bacteria. As such, the mouth and teeth must be thoroughly cleaned and the endoscope closed [26]. No study reported such precautions in their methods.

In addition, we found inconsistencies in sampling sites among studies. In particular, two studies did not specify the exact portion of the duodenum in which samples were collected [59,60], while others took samples from the descending part of the duodenum [68]; the ligament of Treitz [58], or at different distances from the angle of Treitz: 30 cm [57], 40 cm [67], or either 60 cm or 180 cm [62]. In Corrodi et al. report, the jejunal fluid was aspirated during follow-up endoscopy visits from the proximal end of the bypassed loop (proximal jejunum) and from the proximal functioning small bowel [57].

The sample types were different among the studies and allowed either luminal or mucosa-associated microbiome to be investigated. In two trials luminal fluid was collected thus enabling the study of luminal microbiome [57,58], while others analyzed mucosal-associated microbiome which was collected with swab-rubbed biopsies [62] or conventional biopsies [59,60,67,68].

In addition, microbiome analysis techniques were different with either bacterial culture [57,62], 16SrRNA sequencing [58,59,62,67,68] or metatranscriptomics [60]. One study shared gene function analysis based on metagenomic in addition to microbial composition results based on 16SrRNA gene analysis [58].

Importantly, none of the studies reported an association between obesity-related metabolic or clinical phenotypes and microbiome in the USI and the systematic comparison of the digestive tract region is scarce.

These differences in study design, sampling methods, eligibility criteria and consequently in the definition of the groups analyzed among the various studies make it difficult to compare results and to draw firm conclusions. Despite these limitations, our review provides a thorough overview of the current knowledge regarding the relationship between the USI microbiome and metabolic diseases, identifies gaps in current research, and calls for further studies to standardize sampling methods and analytic pipelines.

5. Conclusions

New studies shed light on the intricate interplay between diet, USI microbiome, and host metabolism, and their potential implications in metabolic disorders. Emerging evidence from preclinical studies suggests the role of the USI microbiota as a specific ecosystem and as an important regulator of intestinal physiology. This is an area that merits further study in the context of human disease, considering the methodological shortcomings.

CRediT authorship contribution statement

Conceptualization: E.S, K·C; data curation: E.S. and D.M.; funding acquisition: K.C., T.L.R., A.R. and P.S.; investigation: E.S. and D.M.; methodology: E.S. and D.M; project administration: T.L.R., K.C.; supervision: K.C. and T.L.R.; validation: K.C., T.L.R., A.R., and P.S.; visualization: D.M.; writing—original draft, E.S. and D.M.; writing—review and editing, K.C., T.L.R., A.R., P.S., All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors have nothing to disclose regarding this review.

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HYPOTHESIS

The gut microbiome plays a significant role in the development of obesity, which has been extensively studied through non-invasive methods, primarily focusing on faecal microbiome analysis. However, it is important to note that the intestine consists of distinct ecological niches, each influenced by various factors, including physicochemical, biochemical, mechanical and physiological parameters and nutrient availability.

Research on the upper small intestinal microbiome in humans is challenging due to limited accessibility and the invasive nature of sampling. Nevertheless, studying the small intestine and its ecology has become an appealing area of interest in metabolism research. The small intestine is a critical interface where intricate interactions occur between the host, microbes, and environmental factors such as diet.

Given that the small intestine is responsible for nutrient digestion, sensing, and absorption, enterohomone secretion and whole-body glucose regulation such as food intake regulation, it is crucial to comprehensively understand the complex interplay between gut physiology, dietary factors, and the small intestinal microbiome to gain insights into its impact on host metabolic health.

My PhD hypothesis is that the duodenojejunal microbiome and its metabolome are strong indicators and actors of the host's metabolic health and environment (particularly its nutrition). It might also be an indicator of the host's intestinal integrity, local epithelial transcriptome activity or transit time.

I expected some of these associations to be stronger regarding effect size with the USI compared to the associations found between the same variables and the faecal microbiome. For example, I hypothesise that corpulence and body composition variables such as fat mass accumulation will associate more strongly with the USIM than the faecal microbiome.

This research could lead to finding specific microbiome members from the duodenojejunal compartment that could either play a role in the promotion or protection of intestinal inflammation, intestinal barrier integrity and chronic inflammation; that could play a role in nutrient digestion, sensing and absorption, and ultimately to associate with metabolic health status such as obesity.

Findings from this study may lead to mechanistical studies in animal models, which could help to understand the physiopathology of obesity better and lead to the development of innovative treatments such as the use of specific probiotics or personalised lifestyle advice to promote an optimal upper small intestinal microbiome and host symbiosis.

Finally, the literature proposes that upper-small intestinal microbiome residents include species known to colonise oral cavities. I thus also hypothesise that the USIM is more similar to the oral microbiome compared to the faecal microbiome and that it would be interesting to look if the OM is a proxy for the USIM and associates with metabolic health.

OBJECTIVES

The main objective of my PhD project was to evaluate the changes of duodenojejunal microbiome in humans with or without severe obesity.

To answer my hypothesis and objectives, I settled up and ran a clinical research as described in detail in the following section (Cfr : <u>Materials and Methods</u>). Briefly, the cohort (n = 30) is composed of two groups: a group of non-obese participants (n = 15) and a group of participants with severe obesity involved in a bariatric surgery programme (n = 15). The subjects were characterised in depth by lifestyle and clinical variables, and phenotyping included metagenomic and metabolomic analyses.

We required technical developments before launching the "omics" analyses as our laboratory had not previously studied oral and DJF microbiomes. The aim was to establish our study's sample collection, DNA extraction and sequencing methods.

Using these developed methods, I aimed to investigate the changes in the USIM, its inferred functions and the associated metabolome between the two groups of participants. The objective was to decipher the associations between this ecosystem and the participant's phenotype and lifestyle and to observe if we could identify promising candidates in this association study that could be further investigated in mechanistic research, such as specific bacterial strains or metabolites.

MATERIALS AND METHODS

The materials and methods section has been organised as follow:

- 1) The Je-MiMe study: description of the clinical protocol;
- 2) The materials and methods section related to the results section of my Ph.D. project.

1. Je-MiMe Study: Clinical Protocol

To answer my PhD hypothesis and meet my objectives, I designed a clinical study protocol under the supervision of my thesis supervisor, Prof. Karine Clement, during the first year of my PhD (<u>Annex I: Clinical protocol</u>). In this clinical investigation work, I was in charge of all administrative processes to receive all authorisations to launch this clinical study. We received a favourable response from the local ethics committee in July 2021 and the authorisation to launch the inclusions in February 2022.

To carry out the study I undertook the 'Bonnes Pratiques Cliniques' training (Groupement Inter-régional de Recherche clinique et d'Innovation d'Ile-de-France, GIRCI). The 'Good Clinical Practice' consists of standards that include the protection of human rights for the subjects and volunteers in clinical trials and guidelines on how clinical trials should be conducted.

Overall this experience has taught me a lot about the ins and out of clinical research and how fastidious it is to carry out such research and accumulate human data.

The Je-MiMe Protocol: Design and Population

The Je-MiMe study is a monocentric, observational study carried in France (Hôpital Privé des Peupliers- Ramsay Santé, 8 Pl. de l'Abbé Georges Hénocque, 75013 Paris). Subjects were recruited between March 2022 and July 2022.

Informed written consent was obtained from all individuals prior to study inclusion. The study was conducted following Helsinki Declaration and received approval from the local ethics committee, CPP Ile de France 8 (CPP: 21 06 48, CNRIPH: 21.04.29.67842, Protocol: C20-86 / Étude JE/COL-MIME, ID RCB: 2021-A01074-37) and was registered on the ClinicalTrials.gov website (clinicaltrials.gov/study/NCT05186389).

The present studied cohort explored in this work is composed of two groups of 15 participants (n=30): 1) a "Control Group" (Ctrl group), composed of non-obese participants without known metabolic disorders (BMI: 18.2 - 24.9 kg/m²); 2) and an "Obese group" (Ob

group), composed of participants with severe obesity and candidates for bariatric surgery (BMI: $\geq 40 \text{ kg/m}^2$ or BMI $\geq 35 \text{ kg/m}^2$ with at least one obesity-related comorbidity). Initially, the clinical protocol aimed to recruit a third group: an "Obese and Type 2 Diabetes group" (ObD). However, during my PhD project, no patient with T2D met our inclusion criteria at the Hôpital Privé des Peupliers.

All participants had a gastroscopy planned as part of the patient's required medical examinations (<u>Annex I: Clinical protocol</u>). Lean participants had a gastroscopy planned for minor epigastralgia that did not require medication, and gastroscopy is a prerequisite for bariatric surgery for patients from the Ob group.

General inclusion criteria were, amongst others, age between 18 and 60 years old and weight stable for the previous three months. Specific inclusion criteria for the Ctrl group were: BMI between 18.5 and under 25 kg/m². Inclusion criteria for the participants from Ob and ObD groups are: being a candidate for bariatric surgery and meeting the HAS criteria (Haute Autorité de Santé, 2009; BMI \geq 40 kg/m² without comorbidities or BMI \geq 35 kg/m² with at least one obesity-related comorbidity such as hypertension, dyslipidemia, obstructive sleep apnea, joints disease, non-alcoholic steatohepatitis).

General exclusion criteria were, amongst others, the use of treatments for the previous 12 weeks that could alter gastrointestinal motor function, acidity (i.e., proton pump inhibitors, H2 receptor antagonist), gut microbiome (i.e., antibiotics, prebiotics, probiotics), diagnostic of acute or chronic inflammatory bowel disease or, to cite another, abdominal colorectal cancer.

All participants underwent extensive multidimensional clinical phenotyping, and a biobank was constituted as described in the section '<u>Materials and Methods of the Je-MiMe study</u>'.

2. Extensive Phenotyping

Participants underwent multidimensional and extensive phenotyping (Figure M.1.1). Clinical and lifestyle data were collected through an online electronic-Case Report Form (e-CRF; REDCap, Vanderbilt University) that I designed for the study.

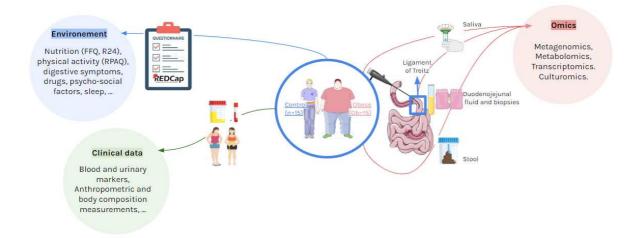


Figure M.1.1 - Patients' circuit and clinical phenotyping.

I met the participants for two visits during which clinical phenotyping was performed. Saliva, duodenojejunal fluid and biopsies, stools, urines, and blood were sampled and aliquoted for subsequent analysis. Environmental data were collected directly by our participants on our electronic Case Report Form (eCRF; REDCap) through online questionnaires. I encoded the rest of the clinical data manually on our eCRF during the visits and phone calls with the participants or when results were available.

Lifestyle, Psychosocial and Socio-Demographic Phenotyping through Online Questionnaires

To establish the list of questions and standardised questionnaires for the phenotyping of the Je-MiMe cohort, I followed the OBEsity Diverse Interventions Sharing (OBEDIS) guidelines (Alligier et al., 2020). This European project provided guidelines to homogenise patient phenotyping with minimal variables to include in clinical trials on obesity. The end goal of such guidelines is to enable project harmonisation and to merge data from multiple trials.

I transformed the paper versions of these questionnaires into .csv files and uploaded them on the projects' eCRF. Participants could complete the questionnaire from their homes, at their own pace, using a unique link to an online survey. I followed the participant's completion of those questionnaires and corrected all aberrant or missing values, as detailed later.

It is important to note that all these questionnaires do not provide a clinical diagnosis and are not intended to replace clinicians' practice. These self-administered questionnaires report the participants' subjective evaluation of their lifestyle and personal situation. Bias can thus be associated with such questionnaires (Choi and Pak, 2004; Cook, 2010).

<u>Alcohol consumption – Alcohol Use Disorders Identification test:</u> The Alcohol Use Disorders Identification test (AUDIT) is a 10-items alcohol screening instrument. **Scores range from 0 to 40**. A score of 0 indicates an abstainer who never had alcohol consumption-related problems, scores ranging from 1-7 indicate low-risk consumption according to WHO guidelines, from 1-14 suggest hazardous or harmful alcohol consumption, a score above 15 may indicate alcohol dependence (moderate to severe alcohol use disorder) (Bohn et al., 1995; Saunders et al., 1993; Saunders and Aasland, 1987).

<u>Nicotine Dependence – Fagerström Questionnaire:</u> The Fagerström Test for Nicotine Dependence is a 6-items instrument measuring physiological dependence on nicotine. **Scores range from 0 to 10**. The items assess the quantity of cigarette consumption, the compulsion to use, and dependence. A score below or equal to 2 indicates low nicotine dependence; Scores ranging from 3 to 4 correspond to low to moderate dependence; From 5 to 7 indicates moderate nicotine dependence; 8 and over indicates a high nicotine dependence (Heatherton et al., 1991; Pomerleau et al., 1989).

<u>Quality of life and depression - Patient Health Questionnaire:</u> The Patient Health Questionnaire (PHQ-9) is a nine questions screening tool to rate depression and quality of life for adult patients. **Scores range from 1 to 27** and respectively from 5, 10, 15, and 20 represent mild, moderate, moderately severe, and severe depression (Kroenke et al., 2010, 2001; Spitzer et al., 1994).

<u>Perceived Stress</u> – The Perceived Stress Scale: The Perceived Stress Scale (PSS) is an instrument to assess individual personal stress perception. **PSS scores range from 0 to 40.** Participants took the PSS-14, from which I calculated the PSS-10 score as proposed in the literature. Ranging from 0-13, 14-26 and 27-40, scores indicate respectively low, moderate, and high perceived stress (Cohen et al., 1983; Cohen and Williamson, 1988; Taylor, 2015).

<u>Anxiety – Hospital Anxiety Depression Scale</u>: The Hospital Anxiety and Depression Scale (HAD) comprises 14 items. Seven items are related to anxiety and seven items to depression. The depression and anxiety scores are **calculated and interpreted separately** as the sum of their 7-related items. **Calculated scores range from 0 to 21 for anxiety** or depression. I only assessed anxiety with this scale as depression was evaluated through the BDI questionnaire, which is more sensitive and specific to depression (Brenner et al., 2020). Scores below 7 correspond to 'absence of symptoms', scores from 8-10 indicate 'doubts of symptoms', and a score of 11 or above indicates 'symptoms of anxiety' (Bjelland et al., 2002; <u>Snaith and Zigmond, 1986)</u>.

<u>Depression – The Beck Depression Inventory Questionnaire</u>: The Beck Depression Inventory (BDI) is a 21-item instrument assessing the severity of depressive symptoms during the past weeks. **Scores range from 0 to 63.** Scores ranging from 1 to 10 are considered normal, with no mood disturbance; from 11-16 indicate mild mood disturbance; from 17-20 borderline clinical depression; from 21-30 moderate depression; from 31-40 severe depression; and scores from 41 to 63 indicates extreme depression symptoms (Beck et al., <u>1988, 1961</u>).

<u>Sleep Apnea – The Stop-Bang Questionnaire</u>: The STOP-BANG questionnaire is an 8-item tool to identify Obstructive Sleep Apnea (OSA). **Scores range from 0 to 8.** A score ranging from 0 to 2 indicates a low risk for OSA, from 3 to 4 indicates an intermediate risk, and from 5 to 8, there is a high risk for OSA (<u>Chung et al., 2014, 2012, 2008</u>).

<u>Sleep Quality - The Pittsburgh Sleep Quality Index</u>: The Pittsburgh Sleep Quality Index (PSQI) is an 11-item questionnaire assessing sleep quality and disturbances over the previous month. **Scores range from 0 to 21**. Answers to the 11 items and sub-questions lead to the calculation of 7 sub-scores (subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction) and one global score (Buysse et al., 1989).

<u>Circadian rhythm - The Horne and Ostberg Questionnaire</u>: The circadian rhythm of participants was assessed by the 19-item morningness-eveningness questionnaire (MEQ). Scores ranging from 16 to 30 indicate that the participant is a 'definite evening type', from 31 to 41 a 'moderate evening type', from 42 to 58 an 'intermediate type', from 59 to 69 'moderate morning type' from 70 to 86 'definite morning type' (Horne and Ostberg, 1976). Scores range from 16 to 86.

Eating Behaviour - Dutch Eating Behabiour Questionnaire: Eating behaviour was assessed with the Dutch Eating Behaviour Questionnaire (DEBQ). This questionnaire comprises 33 items grouped into three independent dimensions: cognitive restraint, externality and emotionality (Brunault et al., 2015; Lluch et al., 1996). Scores are calculated from their deviation to the median score of the group.

<u>Nutrition - The Food Frequency Questionnaire</u>: The food frequency questionnaire (FFQ) used in our laboratory was developed for the MetaCardis cohort (Verger et al., 2017). This questionnaire, designed to measure usual dietary intake during the previous year, is a 158-items, semiquantitative questionnaire with generic portion sizes. The food items are selected from the major food groups and adapted to represent local dietary habits. Additional questions concern dietary supplement intake or additional food items that would not appear in the 158-item list (i.e., type of fat used for cooking, sugar or milk consumption with hot beverages). The SU.VI.MAX food composition database (Economica, Paris, France) was used to calculate consumption of various food groups and nutrients.

<u>Nutrition - 24-hours dietary recalls</u>: We utilised self-administered 24-hour dietary recalls (R24) to record all foods and beverages consumed over a 24-hour period. Participants were instructed to complete three days of recording, including two weekdays and one weekend day following a meal-based approach, recording all foods and beverages consumed during a midnight to midnight-period. Portion sizes were estimated using standard measurements or validated photographs. Food intakes were calculated using a comprehensive food composition database following a method developed by Dr S. Adriouch, a postdoctoral fellow in our laboratory.

Physical Activity - Recent Physical Activity Questionnaire:

This questionnaire (RPAQ) assesses physical activity (PA) in 4 domains of life: work, travel, recreation, and domestic life during the previous month in order to calculate energy expenditure (Besson et al., 2010).

<u>General medical questionnaire</u>: A general questionnaire with a set of questions regarding the participant's personal history of CVD, T2D and obesity, the participant personal's history of weight, medication, socio-demographic background (including marital status, number of children, ethnicity, household composition or income) and other variables known to impact the gut microbiome (i.e., delivery mode, breastfeeding) was built on the e-CRF. The rationale for constructing the general questionnaire is detailed in the study protocol (<u>Annex I: Clinical Protocol</u>).

I designed all questionnaires through REDCap platform. These were then validated by my supervisors and members of the laboratory. Each participant answered more than 500 questions, taking approximately 1 to 3 hours. Few participants took the survey on a printed-paper version, and were then recoded through Redcap.

Anthropometric Measures and Body Composition:

Neck, waist, and hip circumferences were each measured with a medical flexible measuring tape, and data was collected in our eCRF. Participant height was measured, and body composition was analysed through bioelectric impedance balance (MC-780MA P, Tanita, Amsterdam, The Netherlands). These analyses were carried out three times for data quality purposes. An example of impedancemetry results is shown below (Figure M.1.2). These variables were integrated to omics data (i.e., fat mass %, visceral fat rating, etc.).

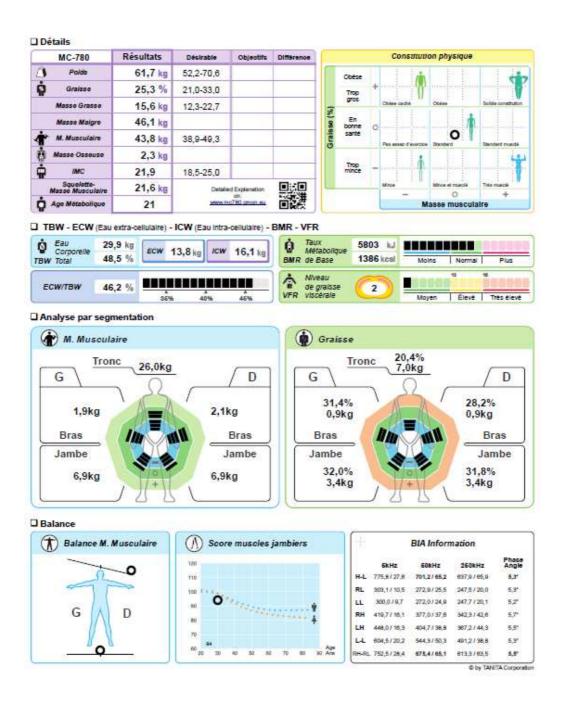


Figure M.1.2.: <u>Body composition variables acquired through a bioelectric impedance</u>. This figure displays all body composition results acquired with our multi-frequency segmental body composition bioelectric impedance.

Biological Samples

All samples were collected fasting (except for stools, which were collected at the participant's home) at the Hôpital Privé des Peupliers before the gastroscopy, early in the morning. Participants were fasting for at least 8 hours.

Fasting blood samples

Blood samples were collected fasting.

Variables related to glucose metabolism (fasting glycemia, insulin, Hba1c %), lipid profile (total cholesterol, LDL, HDL, triglycerides), liver function (aspartate transaminase - ASAT, alanine transaminase - ALAT, gamma-glutamyl transferase - GGT and alkaline phosphatase) thyroid function (ultra sensible measurement of thyroid-stimulating hormone - TSHus) and inflammation (C-reactive protein - CRP) were measured by Cerballiance (Paris).

Additional tubes of serum and plasma were sampled for biobanking. After sampling, one tube of serum was directly mixed with dipeptidyl peptidase-4 (DPPIV) inhibitors (DPPIV-i) and Protease Cocktail Inhibitors (cOmpleteTM, Mini Protease Inhibitor Cocktail, Roche, Basel, Switzerland) to quantify circulating levels of GLP-1 and Amylin.

Inflammatory and Metabolic Markers Measurements

I performed the quantification of circulating levels of Amylin Active, C-Peptide, Ghrelin Active, GIP, GLP-1 Active, Glucagon, IL-6, Insulin, Leptin, MCP-1/CCL2, PP, PYY, Secretin and TNFα using a human metabolic hormone immunoassay panel using a Luminex on the serum mixed with DPPIV-i and protease inhibitors (MILLIPLEX® Human Metabolic Hormone Panel V3, Millipore).

Some inflammatory markers were undetected in some or most participants (TNF α , IL-6). This is explained by the reduced sensibility using multiplex assay. We thus performed a second ultra-sensitive dosage using MSD's platform.

Low-Grade Inflammatory Markers and Score Measurements

MSD's ultra-sensitive assay platform was utilised to evaluate inflammatory cytokines levels in serum. In detail, Interleukin-6 (IL-6) was assayed with the S-Plex assay, while Interleukin-8 (IL-8), C-reactive protein (CRP), serum amyloid A (SAA), Monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor-alpha (TNF α) were measured using the U-Plex assay.

Subsequently, a cumulative score of low-grade inflammation, known as the Maastricht-z score, was calculated in our cohort following the methodology from the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study (van der Kolk et al., 2019; van Greevenbroek et al., 2011). The calculation of the cumulative inflammation z-score, which includes IL-6, IL-8, CRP, SAA, and TNF α , involved the following steps: 1) Transform all cytokines concentrations to logarithm to have a normal distribution for each; 2) Calculate the mean and standard deviation for each logarithm of the inflammatory marker within the reference population; 3) Calculate the z-scores of each inflammatory marker using the formula: z-score = (logarithm of individual value - mean) / standard deviation; 4) Combine the z-scores: Sum the z-scores of each inflammatory marker to obtain the overall inflammation z-score for the obese patient.

Adiponectin, Growth Differentiation Factor-15 and Fibroblast Growth Factor-21 Measurements

Quantitative determination of human High Molecular Weight Adiponectin (HMW Adiponectin) was performed through QuantikineTM ELISA Human HMW Adiponectin/Acrp30 Immunoassay, of human Growth Differentiation Factor-15 (GDF-15), also known as macrophage inhibitory cytokine-1 (MIC-1) through QuantikineTM ELISA Human GDF-15 Immunoassay, and of human Fibroblast growth factor-21 (FGF-21) through QuantikineTM ELISA Human FGF-21 Immunoassay (Catalogue Number DF2100).

Tryptophan Metabolites Measurements

Tryptophan metabolites were quantified by Prof. H. Sokol's laboratory through liquid chromatography coupled with high-resolution mass spectrometry from human serum, as described in their previous work (Lefèvre et al., 2019).

Urine sample

Urine samples were collected fasting, transported on ice, aliquoted within two hours and stored at -80°C for subsequent analysis.

Duodenojejunal Biopsies

Two duodenojejunal biopsies were sampled (fasting) between the second segment of the duodenum and 10cm distal to the angle of Treitz. One biopsy was immediately placed in dry ice, transported and stored for further RNAseq analysis at -80°C. The second biopsy was placed in Dulbecco's Modified Eagle Medium (DMEM +/+ with 1% penicillin-streptomycin and 10% fetal bovine serum) at 4° C and transported to the laboratory. Within two hours, the milieu was replaced with acidified formal alcohol (AFA) for fixation overnight. Biopsies were then paraffin-embedded for subsequent histological analysis.

Saliva

Approximately 5 mL of saliva were collected, transported, aliquoted on ice, and stored within two hours at -80°C. Participants were asked not to wash their teeth in the morning before saliva sampling, and they were fasting (not allowed to eat and drink anything) for at least 8 hours. Participants followed the protocol developed based on the results presented later (Table M.2.1). I was always next to the patient to ensure proper collection and avoid contaminations as much as possible.

La salive sera collectée à jeun, à votre arrivée à l'hôpital le jour de votre fibroscopie.

Collecter la salive si les critères suivants sont respectés:

Pour la fibroscopie, il vous est demandé de vous rendre à l'Hôpital Privé des Peupliers à jeun: il ne faut rien manger et ne rien boire depuis la veille au soir, il ne faut pas fumer le matin qui précède la fibroscopie. Nous insistons sur ces points et vous demandons de ne pas vous brosser les dents le matin avant la collecte de la salive. Vous pouvez prendre votre brosse à dents avec vous afin de vous brosser les dents après la collecte de salive.

La collecte de salive doit se faire **sans substances ou matériel dans la bouche** (ex: appareil dentaire non-permanent type gouttières, dentier, élastiques,...).

Si les conditions ci-dessus sont toutes respectées, procéder à la collecte de la manière suivante:

- Avant de collecter la salive, lavez et séchez vous les mains et/ou portez les gants stériles.
- Réalisez la collecte de la salive sur une surface propre, sans courants d'air, dans un endroit calme en étant assis. Ceci permettra d'éviter de faire tomber le tube ou de contaminer l'échantillon.
- Ouvrez le tube en faisant attention de ne pas toucher l'intérieur du tube ou du bouchon avec vos doigts
- Laisser couler la salive, ne pas cracher pour éviter la formation de bulles
- Si la collecte de salive est difficile, nous vous demandons de récolter l'échantillons sur un période de plusieurs minutes. Merci de ne pas boire, même une seule gorgée d'eau.
- Collecter 5mL de salive
- Si vous remarquez des bulles dans l'échantillon, taper délicatement le tube sur une surface dure afin d'éliminer les bulles. Si besoin, continuez à collecter la salive jusqu'à atteindre 5mL.
- Bien refermer le tube
- *Remettre immédiatement le tube à Emilie pour garder l'échantillon sur glace.*

Table M.2.1 - Saliva collection procedure developed for the Je-MiMe study

Duodenojejunal Fluid

After saliva collection, participants were still fasting and were asked to thoroughly wash their teeth as proposed by the previous literature in order to prevent (as much as we could) DJF contamination from oral cavities that have a higher bacterial load (Sundin et al., 2017). Afterwards, participants underwent anaesthesia and an endoscopy was performed. We could not proceed to the aspiration of DJF with a clean catheter inserted in the endoscope when it was inserted in the USI. I asked the surgeon to thoroughly wash the endoscope with water within the stomach to avoid contamination as much as possible. The surgeon continued the aspiration for a few seconds. After taking these precautions, the surgeon entered the duodenum with the endoscope. 1-3 mL of DJF was aspirated through the endoscope and collected in a sterile tube. Aspiration was performed between the second segment of the duodenum and 10 cm distal to the angle of Treitz. DJF was immediately aliquoted in the surgery unit for subsequent metagenomic and metabolomic analysis and placed within five minutes after sampling on dry ice. In addition, 400 µL of DJF were injected in a vial under anaerobic conditions in a rich non-selective growth medium (i.e., containing yeast extract, peptones, glucose, raffinose, vitamins, antioxidants, trace elements, carbonate, hemin, SCFAs).

Stool

Participants were provided with a sampling protocol regarding stool collection. I read and explained the protocol during the inclusion visit, and participants could ask any question they had to ensure proper sampling and reduce potential contaminations.

Total fresh stools were collected in a hermetic box. When the sample was collected, participants placed an anaerocult (bioMérieux, Paris, France) on the sample and closed the box. The sample was transported to Dr Benoit Chassaing laboratory on ice and aliquoted by his team in an anaerobic hood within two hours for different analyses.

Metabolomics from Duodenojejunal Fluid and Stool

Untargeted metabolomics was performed by Metabolon (Durham, North Carolina, United States) using Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS).

To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionisation (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation quality controls and analysis.

All methods utilised a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionisation (HESI-II) source and Orbitrap mass analyser operated at 35,000 mass resolution. The sample extract was dried and then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analysed using acidic positive ion conditions, chromatographically optimised for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analysed using acidic positive ion conditions. However, it was chromatographically optimised for more hydrophobic compounds. In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analysed using basic negative ion optimised conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however, with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analysed via negative ionisation following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSⁿ scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z.

DNA Extraction, Library Preparation and Sequencing from Saliva, Duodenojejunal fluid and Stool

Total DNA extraction was performed using 400 µL of saliva, 400 µL of DJF or approximately 150 mg of homogenised faeces. We used the "AutoPure 96" automatic extractor (Auto-Pure96, Nucleic Acid Purification System Hangzhou Allsheng Instruments CO., Ltd. Hangzhou, Zhejiang, China), marketed by Dutsher, whose principle is based on the use of a kit containing magnetic beads that retain the genomic DNA present in the sample (NucleoMag DNA Microbiome for DNA purification from soil, stool and biofilm, Macherey -Nagel Vertrieb Gmbh & Co. Kg). The samples were subjected to two cycles of chemical lysis followed by mechanical lysis. The aim of this treatment is that chemical lysis at 95°C weakens the bacterial wall and membrane, which in turn facilitates the action of mechanical Hz lysis (Bead-beating: 1000 6x 30 sec; Precellys[®], Bertin Technologies, Montigny-le-Bretonneux, France), leading to rupture of the bacterial wall and access to the DNA. After this step, we obtained a solution of approximately 1 to 1.5 mL of lysate, and 310 µL is used for the purification steps using Auto-Pure96. After the run with the robot, approximately 100 µL of lysate were retrieved.

The purity ratio was analysed with a spectrophotometer (NanoDrop, ThermoFisher) and DNA quantity was assessed with a fluorometer (Figure M.2.1; Qubit, ThermoFisher).

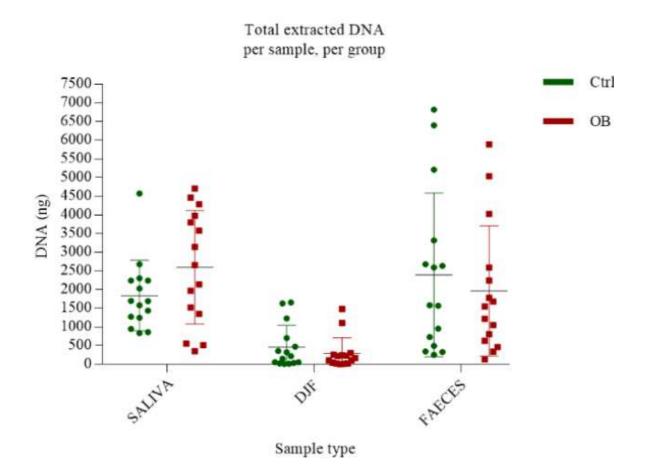


Figure M.2.1 - <u>DNA extraction results from saliva</u>, duodenojejunal fluid and faecal samples <u>of the Je-MiMe Cohort</u>.

This graph displays the total extracted DNA results from the Je-MiMe cohort. Total extracted DNA per sample type: saliva (2225,66 ng \pm 1301; min 352 - 4708 max), duodenojejunal fluid (DJF; 379,478 ng \pm 508.3; min 12.1 -1657 max), faeces (2181,52 ng \pm 1960, min 132 - 6820 max); Total extracted DNA per sample type, per group: saliva (Ctrl: 1846,82 ng \pm 943, min 840 - 4576 max vs Ob: 2604,50 ng \pm 1519, min 352 - max 4780), DJF (con 466,722 ng \pm 581, min 13 - max 1657; vs ob 292,23 ng \pm 426, min 12 - max 1485), stool (con 2397.85 ng \pm 2194, min262 - max 6820; vs ob 1965.18 ng \pm 1745, min 132 max 5896). Green: Control group; Red: Ob Group.

For some DJF samples, the total extracted DNA was very low (i.e., total extracted DNA was 12 ng for one sample in approximately 100 μ L). For these samples, a second extraction from a second aliquot of 400 μ L DJF was performed, and we also used the remaining lysate from the previous extraction (*) and ran the following purification step. All eluted DNA tubes were pooled (per patient, per sample type) when we repeated the extraction and purification steps and all aliquots were concentrated to obtain no more than 50 μ L with a SpeedVac Vacuum Concentrator (ThermoFisher Scientific). Even though at least 1 ng DNA

was required in a maximum 50 μ L for the following steps, we aimed to retrieve minimum 5 ng DNA in 50 μ L per sample.

For DNA library preparation, we used the Invitrogen Colibri[™] PS DNA Library Prep Kit for Illumina[™] (ThermoFisher Scientific, Waltham, Massachusetts, United States) and the QIAquick Purification Kit (Qiagen, Hilden, Germany). I performed these library preparations with Charlène Dauriat and Dr Rohia Alili at Dr Chassaing Laboratory (INSERM U1016, Institut Cochin, Paris). Sequencing was performed by the Illumina platform at Cochin.

Quantification of the library was performed after each cleanup step, using Quant-iTtm PicoGreen dsDNA Reagent and Kits (Invitrogen, P7589), an ultrasensitive fluorescent nucleic acid stain for quantitating double-stranded DNA in solution with the microplate reader from BMG and the CLARIOstar Plus.

First, DNA was physically sheared up to approximately 250-550 bp through sonication using the Bioruptor® Pico and Bioruptor® Pico (glass) Microtubes (Diagenode, Seraing, Belgium; 5 cycles). A first test was performed on six samples with the highest DNA yields to have enough DNA for a second test if needed (30 seconds ON, 30 seconds OFF). We selected two samples for each sample type (2 x saliva, 2 x DJF, 2 x faecal samples; 25μ L sample + 25μ L H₂O placed in Bioruptor microtubes). Fragment size was controlled with a 1% agarose gel, and fragment migration was performed at 110 V for 40 minutes. A trace view of the analysis showed adequate shearing for the ligation protocol, as the required fragment size should range from 150 bp to 1000 bp. However, traces showed low DNA quantity for saliva and DJF compared to stool (Figure M.2.2). Thus, all samples were sonicated following this first test, but while we sonicated 25 μ L extracted DNA elution for faecal samples, we sonicated total eluted DNA or 50 μ L for DJF and saliva samples.

GeneRuler 1 kb Plus DNA Ladder, ready-to-use

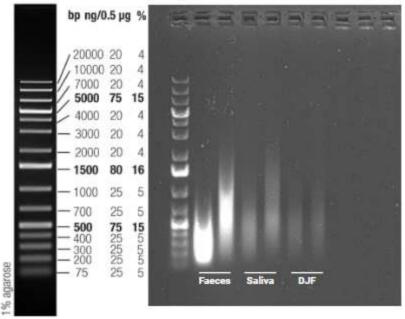


Figure M.2.2: <u>Agarose gel analysis of 6 DNA samples after physical shearing through sonication</u>.

Fragment size was controlled for 6 samples after sonication (2 saliva samples, 2 DJF samples, 2 faecal samples) with agarose gel analysis (1% agarose gel: 2g agarose (Biorad) in 200 mL TAE 1X + 2,5 μ l de SYBR safe), fragment migration was performed at 110 V during 40 minutes. Gel trace view of the analysis were acquired with the E-Box (Vilber, Marne la Vallée, France).

Following shearing, a first purification step was performed with QIAquick Purification Kit (Qiagen, Hilden, Germany) to avoid residual traces of contaminating proteins, organic solvents and salts that could degrade DNA or decrease the activity of enzymes that are necessary for efficient DNA library preparation.

End repair and conversion were performed, followed by unique dual indexed-adaptor ligation to uniquely label sequencing libraries that are generated from each individual and each sample type and to enable multiplexed sequencing. Each adaptor consists of 8 nucleotide indexes or barcodes.

A second, Post-ligation cleanup, using reagents included in the Colibri kit, was performed to remove any unligated adaptors and adaptor-dimer molecules from the library before amplification. The size selection of the ligated library was performed with paramagnetic beads following the protocol: DNA fragments can be size selected in a range no smaller than 150 bp and no larger than 800 bp.

After these steps, PCR amplification of the purified adaptor-ligated DNA library was performed, followed by a third purification of the amplified DNA library using reagents included in the Colibri kit.

Verification of size distribution and quality of prepared DNA library was by performing capillary electrophoresis analysis on AgilentTM 2100 Bioanalyser instrument using the AgilentTM High Sensitivity DNA kit (Agilent Technologies, Santa Clara, California, United States) and showed that both DNA quantity and fragment size were sufficient for sequencing (Figure M.2.3).

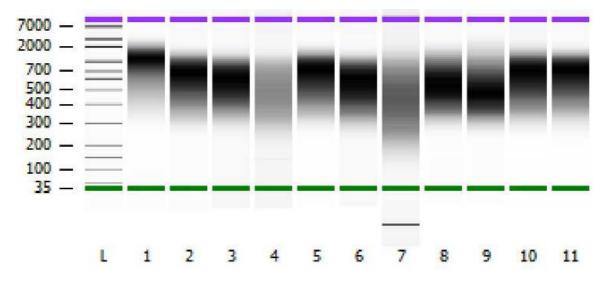


Figure M.2.3 - <u>Trace views of electrophoresis analysis.</u>

L: Ladder, 1: Saliva 'low' (sample with low DNA concentration compared to other saliva samples), 2: DJF 'high' (sample with high DNA concentration compared to other DJFsamples), 3: saliva 'low', 4: DJF 'very low', 5: saliva 'high', 6: Stool 'low', 7: DJF 'very low', 8: Stool 'high', 9: Saliva 'middle', 10: Jejunal 'middle', 11: Stool 'middle'.

Sequencing was performed with NextSeq 2000 (P2 300 cycles : 2x150 bp).

Data Analysis and Integration

Clinical and Lifestyle Data Analyses:

I continuously controlled and curated data during the study to ensure quality. The dietary recalls were controlled by Véronique Pelloux through phone calls with the participants. After completion of the study, the eCRF was duplicated. The original eCRF has remained unchanged (kept as raw data). Data were corrected in case of any remaining missing values or aberrant values on the duplicate. I documented all the corrections. These were made with the participants before the gastroscopy or during phone calls. The data was then uploaded from Redcap as a .csv file to RStudio for data management and analysis (Annex 4: RStudio script). The data management steps on RStudio included, amongst many other processes, the verification of the outliers (i.e., verification of minimal and maximal values, visualisation through dot plots of each variable), the distributions, the missing values, the adaptation of the classes (i.e., date to numeric) or the creation of new variables (i.e., Homeostatic Model Assessment for Insulin Resistance or HOMA-IR, age, ...).

The scores from the standardised questionnaires were calculated based on the literature. I documented all processes. Calculating the scores required data management similar to that explained above and the transformation of the raw data. For example, the score attributed to each answer to each question of each questionnaire was reviewed and adapted for calculations of the questionnaire's score. Afterwards, the scores were computed on Rstudio following the unique procedure of each validated questionnaire.

Metagenomics Analyses

Metagenomics is the direct genetic analysis of genomes contained in an environmental sample (Thomas et al., 2012). A dual approach was followed and developed by Dr Eugeni Belda: 1) Quantitative profiling thanks to a catalogue of references, 2) De-novo metagenomic assembly.

In the first approach, the DNA sequences obtained from the sample are compared to a pre-established reference catalogue, which contains genomic sequences of various known microbial organisms. The advantages of this method include better accuracy in identifying the

microorganisms present and a reduction in computation time and resources required for analysis.

On the other hand, catalogues have limited diversity and may result in an underestimation of true microbial diversity if organisms not represented in the catalogue are present in the sample. In the second approach, metagenomic analysis by de novo assembly, the DNA sequences obtained from the sample are assembled *de novo* without a reference catalogue.

De novo assembly reconstructs the genomes of organisms in the sample by reassembling overlapping sequences. This method is particularly interesting for discovering new organisms or genetic variants not represented in reference catalogues (Li et al., 2023). However, this second approach can be complex, requiring more computational resources and analysis time, and it can also be less accurate for identifying certain already known microorganisms.

In summary, metagenomic analysis with a reference catalogue is generally faster and more accurate for identifying known microorganisms. At the same time, de novo assembly is more suitable for discovering new organisms and studying diversity.

In our case, we followed both methods as both met our specific objectives of the metagenomic study in question: we aimed to characterise the duodenojejunal microbiome in comparison to FM and OM, and this was more convenient with the catalogue approach; we also aimed to describe the duodenojejunal microbiome and to identify microorganisms that are not already described.

Reference-Based Metagenomic Profiling

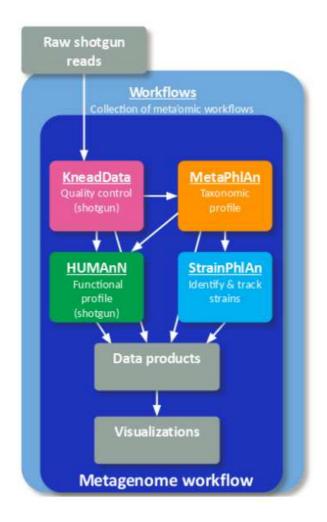


Figure M.2.4 - Reference-based metagenomic profiling.

Whole metagenome shotgun sequencing data can be profiled using a pipeline constituted of several tools. In our case, read-level quality control was performed using <u>KneadData</u>; taxonomic profiling was performed using <u>MetaPhlAn</u>; and quantification and functional profiling using <u>HUMAnN</u>. Figure adapted from the bioBakery workflows page of Huttenhower lab website (huttenhower.sph.harvard.edu/biobakery_workflows/).

For the first approach, Dr Eugeni Belda used the bioBakery workflows on Python (Figure M.2.4). The metagenomic data was cleaned and tested for quality control with the Kneaddata tool. Taxonomic profiling was performed using the Metaphlan catalogue, and quantification was performed using the HumaNN tool (Blanco-Míguez et al., 2023). These tools are described below.

Kneaddata is a bioBakery tool from Huttenhower lab. It performs quality control, especially from microbiome sequencing data. This tool separates bacterial reads from human reads. The tool also performs various filtering tasks that can be adapted (i.e., dropping the reads if below a specified length, trimming overrepresented sequences, cutting adapters or other Illumina-specific sequences, repetitive sequences, cutting bases off the start or the end of a read if below a given threshold quality, in our case, below PHRED 33 score,...) ("KneadData – The Huttenhower Lab," n.d.).

Metaphlan is a taxonomic profiling tool for shotgun metagenomic data based on clade-specific marker genes. These genes were identified from the reference genome sequences and are unique for bacterial species. The fourth version of the catalogue (Metaphlan4) comprises ~1M prokaryotic sequences organised in 5.1 million marker genes and further organised into 21 978 species-level genome bins (SGB) (Blanco-Míguez et al., 2023). SGB corresponds to a taxonomic level under the species level. In the results section, I may refer to this 'SGB' level, or 'marker gene', meaning that the granularity of the chosen taxonomic level is higher than the species level.

HUMAnN (the HMP Unified Metabolic Analysis Network) is a functional profiling tool. This pipeline is used to determine the presence or absence and abundance of molecular functions, such as microbial metabolic pathways in a community, from metagenomic or metatranscriptomic data (Beghini et al., 2021, p. 3).

These tools are available on the biobakery platform Curtis Huttentower lab (<u>https://huttenhower.sph.harvard.edu/biobakery_workflows/</u>).

In our case, we sequenced samples from three different ecological niches and differences in metagenomic richness could be potentially confounded by variations in sequencing depth, which was not homogenous across ecosystems. Indeed the number of clean reads used as a source for taxonomic profiling was the highest per faecal samples, with an average of 3.6 million read pairs, compared to the two other ecological niches. Each saliva sample obtained an average of 2.1 million reads. DJF samples have the lowest sequencing depth, with 1.2 million reads on average per sample. This was mainly explained by a much higher contamination with human reads in the two proximal niches, and particularly in DJF samples where bacterial reads were in the minority compared to human reads.

Whereas species genome bins (SGB) abundances in Metaphlan4 were corrected by sequencing depth by normalising the marker gene coverages by the metagenome size (number of reads), diversity estimates (especially gene and SGB richness) are still impacted by variations in sequencing depth. To avoid this, a rarefaction analysis over the cleaned sequence files was performed by random selection of a fixed number of read pairs across all samples from which Metaphlan profiling is performed. This procedure was carried out 15 times with sequencing depth from 0.35 million read pairs (all samples retained) to 2.4M reads at steps of 150 000 read pairs, from which SGB and marker gene richness and Shannon diversity were computed for samples reaching each downsizing level with the R package Phyloseq ("phyloseq." 2023).

With the obtained estimates for the samples retained at different downsizing thresholds, the process of "upsizing" was applied. This allows us to infer microbiome diversity (i.e., richness, Shannon) for all the samples of the cohort (including those not reaching a given downsizing level). This procedure consists of fitting linear models of diversity (i.e., Shannon, observed SGB or marker genes) at consecutive downsizing levels (diversity at downsizing 0.45 M ~ diversity at downsizing 0.35 M reads for example) and using these linear models to infer the diversity values of samples that did not reach a given downsizing level (i.e., 0.45M reads).

Finally, the output, or "data product" from this metagenomic pipeline, consists of abundance tables and gene count tables at different taxonomic levels (from the Phylum level to the marker gene level of the Metaphlan4 catalogue). With Eugeni Belda, I learned to analyse and visualise this data and the results are presented in the following section.

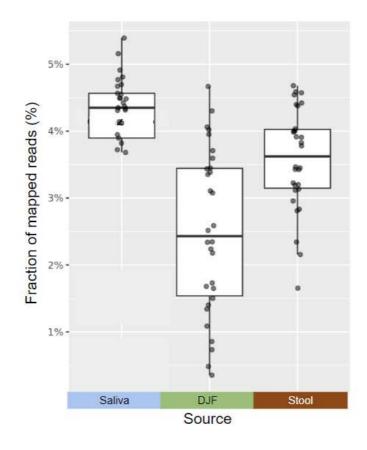
All analyses using metagenomic richness displayed in the section "<u>Part II: Results</u> <u>from the Je-MiMe cohort</u>" were performed using the metagenomic richness at the gene-marker level of the Metaphlan 4 catalogue (5.1 M genes) and upsized to 0.06 M reads.

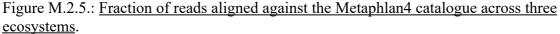
N.B.: the current version of the manuscript addressed to the rapporteurs is missing the sequencing data of 6 stool samples. The final version of the manuscript will include metagenomics from all 30 participants for the three microbiomes.

de novo Metagenome Assembly

During the first catalogue-based approach, we observed that the fraction of reads that were aligned over the 5.1 M gene of the MetaPhlan4 catalogue varied across ecological niches. While faecal samples had the highest fraction of annotated genes, the saliva samples had a lower fraction of mapped reads, and in particular, duodenojejunal fluid samples had the lowest fraction of aligned reads against the catalogue (Figure M.2.5). This can be explained by the fact that this ecosystem has not been extensively studied, in contradiction to the FM or the oral microbiome (OM) in public sequence databases.

Reads that are not aligned against the catalogue are rejected from the analyses. Thus, the Metaphlan4 catalogue underestimates the diversity of the DJF microbiome.





This boxplot shows the fraction of mapped reads (%) against the Metaphlan4 catalogue for each ecosystem: saliva (in blue), duodenojejunal fluid (DJF, in green) and stool (in brown).

RESULTS

Part I. Technical Development

Oral and duodenojejunal microbiome had never been studied in our laboratory and thus required protocol development. This work was performed during the first two years of my PhD, in parallel to the redaction and monitoring of administrative processes related to the clinical trial (carried out during the first year of my PhD) and to the clinical trial (carried out during the second year of my PhD). The challenge was to develop the protocols regarding the collection method, DNA extraction and library preparation protocols and sequencing method for saliva and particularly for the duodenojejunal fluid (DJF).

In our laboratory, we proceed to Nanopore sequencing using the MinION device (Oxford Nanopore Technologies (ONT), Oxford, United Kingdom), an in-house portable real-time and long-read sequencing technique whose associated protocols and analysis have been optimised in our lab by Dr Rohia Alili and Dr Eugeni Belda for faecal samples (Alili, Belda et al., 2021b).

The rationale for this choice is that 1) Nanopore sequencing supports longer fragment reads than shotgun sequencing (Alili, Belda et al., 2021b), making it easier, for example, to cover repeated sequences in a genome or to assemble a known genome. Moreover, with whole-genome long-read sequencing, metagenomics analysis or machine learning approaches can give us the identification of the functional role of identified genes (Shoaie et al., 2015); 2) This technique does not require amplification, thus removing amplification biases; 3) The MinION is a small size, portable device for sequencing based on nanopore technology as introduced earlier. We can thus perform in-house sequencing without the need to work with sequencing platforms. Finally, its cost is much lower than Illumina platforms.

However, the routine protocol for bacterial DNA preparation and ligation before sequencing used in our team requires a minimal amount of 1000 ng extracted DNA (in a maximum volume of 50 μ L). This was a challenge with duodenojejunal fluid samples (Cfr: <u>Conclusion for part I</u>). Finally, DJF has different properties compared to faeces. These properties (i.e., AMPs, BAs, mucus) could degrade DNA or inhibit DNA extraction or sequencing.

These aspects first needed to be controlled before working on samples from the Je-MiMe cohort, as we had low quantities of materials. Indeed, only 1 to 3 mL of DJF could be aspirated during the endoscopy, and we knew DJF had a lower bacterial DNA concentration compared to stool. It was thus impossible to run these technical developments on the samples of our cohort as it would compromise further analyses or even lack DNA material.

Protocol Development for Duodenojejunal Fluid Collection and DNA Extraction

First Experimentation Round

The first steps of this tuning phase were:

- 1. To observe how DJF could be sampled through endoscopy or during bariatric surgery and verify if the sampling procedure could impact DNA concentration.
- 2. To aliquot DJF in different manners to test:
 - a. the quantity of duodenojejunal fluid required to extract sufficient DNA amounts for Nanopore sequencing;
 - b. if washing the cells with PBS could ameliorate DNA extraction results in terms of quality (i.e., purity ratio).
- 3. To test different DNA extraction protocols. At this point, we used three methods to which I attributed a "name" to facilitate the following reading and comprehension:
 - a. The "Invitrogen" protocol (or silica membrane protocol): This DNA extraction kit and protocol was optimised by Dr R. Alili and is routinely used in the laboratory for faecal samples (PureLink[™] Microbiome DNA Purification Kit, ThermoFisher Scientific, Waltham, Massachusetts, United States);
 - b. An "Automated" protocol (or magnetic beads protocol):

The bacterial lysis and the first purification steps of the "automated" protocol are performed with a commercially available kit (NucleoMag DNA Microbiome for DNA purification, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The following steps are performed by a

robot (Auto-Pure96, Nucleic Acid Purification System Hangzhou Allsheng Instruments CO., Ltd. Hangzhou, Zhejiang, China). DNA is bound to magnetic beads during the purification steps. This method has been optimised and validated for DNA extraction from faeces in our laboratory by Dr Rohia Alili. The standard version of this protocol contains two lysis cycles (chemical and mechanical). Purified DNA retention is performed with magnetic beads.

c. A "Manual" protocol:

The 'manual' protocol is an adapted version of the protocol developed by Godon and colleagues (1997). Most reagents are prepared in the laboratory. As we know which reagents are used, each step can be adapted to the sample (Godon et al., 1997). This protocol uses precipitation methods (with ethanol and isopropanol) and does not use prepared kits or silica columns. Silica columns might reduce obtained DNA yield as they could retain DNA despite several washing steps.

Results from the First Experimentation Round

At first, I compared samples aspirated during endoscopy versus bariatric surgery (Figure R.1.1). Samples from bariatric surgery appeared contaminated (i.e., blood, visceral fat, epithelium debris). We thus decided to aspirate DJF from endoscopy and submitted the protocol as found in the annexe section.

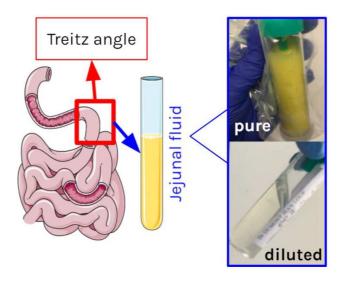


Figure R.1.1 - <u>Duodenojejunal fluid is aspirated at the Angle of Treitz during endoscopy</u>. On the left, the illustration shows the sampling site. The red square corresponds to the anatomical separation between the duodenum and the jejunum: the Angle of Treitz. On the right, the two tested sampling conditions are displayed: pure duodenojejunal fluid aspirate is shown on the top, and duodenojejunal fluid aspirated with water is shown on the bottom.

During the first year of my PhD, I had the opportunity to assist endoscopy examination weekly to develop the sampling protocol and to collect biological waste for the tuning experiments. I asked the endoscopist to always sample gastrointestinal luminal fluid between the Angle of Treitz to 10 cm distal to this region. This corresponds to the most distal region we can access through endoscopy. The endoscopist could either aspirate a minimum of 1 mL of pure DJF and, depending on the patient, this quantity could increase to approximately 3mL. The endoscopist could also wash the epithelium with 1-3 mL water, sent through the device into the duodenum and aspirate a slightly higher fluid quantity corresponding to a "mix" of water and DJF. This method could have facilitated the aspiration of bacteria close to the mucus.

Results from DNA extraction (all protocols and sampling conditions combined) showed that pure jejunal fluid aspiration gives a higher DNA yield (n=29; ~50 ng) compared to aspiration of jejunal fluid with water (n=35; ~4.1 ng) (Figure R.1.2). DNA quantification results displayed here come from spectrophotometry (NanoDrop spectrophotometer, Labtech, France) as DNA yields were too low to enable Qubit assays on most samples (QubitTM dsDNA assay, ThermoFisher Scientific, Waltham, Massachusetts, United States).

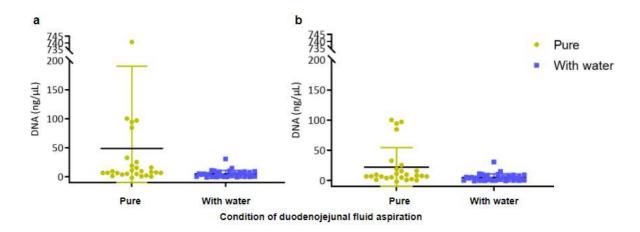


Figure R.1.2.: Effect of jejunal fluid sampling condition on DNA yield. These graphs display DNA concentration obtained from DJF aspirate for two sampling conditions: pure (in yellow) and with water (in blue). a) Data displayed for all participants: pure DJF (n=29; ~50 ng/ μ L ± 142), DJF aspirated with water (n=35; ~4.1 ng/ μ L ± 4.3). b) The same data is displayed for all participants except one outlier (the participant could have bacterial overgrowth or increased human DNA contamination).

After DJF sampling and transport to the laboratory (1-4 hours on ice), I aliquoted the jejunal fluid in three different manners to investigate if different factors could affect DNA extraction yield and purity ratios. This allowed us to compare the results from different amounts of DJF (Figure R.1.3; 400 μ L of DJF, which corresponds to the maximal quantity that can be added to the beads-containing tubes used for mechanical lysis of the bacterial cells) as well as to compare pellets obtained DJF to a "washed" pellet (pellet from 1mL of DJF vs pellet from 1mL DJF washed with 1 mL PBS). All aliquots were then directly frozen at -80°C for future DNA extraction.

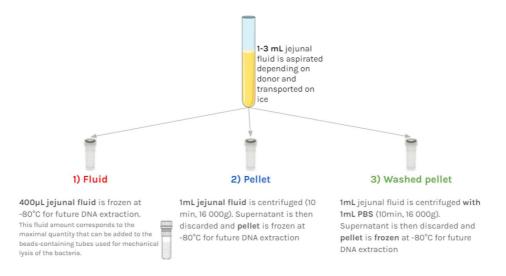


Figure R.1.3 - Plan of the duodenojejunal fluid aliquoting methods.

DJF was aliquoted in three different manners: 1) Fluid: 400 μ L DJF; 2) 1 mL DJF was centrifuged, and the pellet was retained for subsequent analyses; 3) 1 mL DJF was mixed with PBS, centrifuged, and the pellet was retained for subsequent analyses.

For these tuning experiments, we could extract bacterial DNA from different participants (Figure R.1.4). However, we could only use the three extraction protocols on only six aliquots from the same participants and with the same aliquot option. I will, thus, only share these results here (R.1.5). We used controls (mouse faeces) for each protocol to ensure low DNA yields were not due to experimental errors.

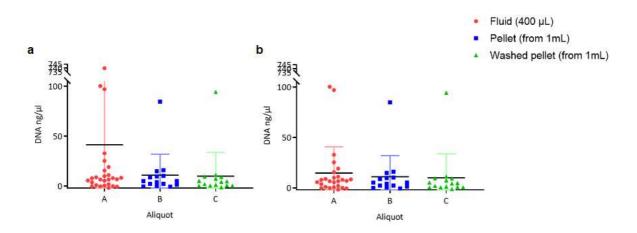


Figure R.1.4.: <u>DNA yields after extraction depending on the aliquoting technique</u>: a) Data displayed for all participants: Aliquots "A" are shown in red (n= 31; ~42,25 ng / μ L \pm 133,4), Aliquots "B" are shown in blue (n=15; ~10,96 ng / μ L \pm 21,08), aliquots "C" are shown in green (n=15; ~10,15 ng / μ L \pm 20,99). B) The same data is displayed for all participants except one outlier.

The adapted Godon protocol gave the higher DNA yield (n=6; ~24.47 \pm 37,04 ng / μ L), followed by the MN (n=6; ~7.83 \pm 0.87 ng / μ L) and the Invitrogen protocol (n=6; ~3.89 \pm 6,53 ng / μ L). The MN protocol combined with the robot gave a lower standard deviation (Figure R.1.5). However, low purity results may lead to an overestimation of DNA concentration using the Nanodrop.

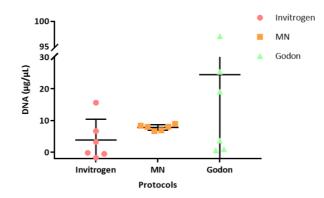


Figure R.1.5 - Impact of the DNA extraction protocol on DNA yield. Results from the Invitrogen protocol is shown in red (n=6; ~3.89 ng / μ L ± 6,53); from the MN protocol in orange (n=6; ~7.83 ng / μ L ± 0.87); from the Godon protocol in green (n=6; ~24.47 ng / μ L ± 37,04).

PCR-free Library construction of the whole genome of 12 samples was performed based on the optimised protocol developed in the laboratory (Alili et al., 2021b). Unfortunately, the maximum yield of bacterial DNA obtained from DJF was less than 1000 ng. We started the protocol with 750 ng when it was possible. However, we had to start the library preparation with lower quantities for most samples. Due to our low DNA yields, we adapted the protocol developed by Rohia Alili. Instead of filtering-out shorter fragments, we selected all fragments to optimise total DNA reads instead of optimising the DNA fragment's length.

Filtering fragments is performed with Agentcourt AMPure XP beads for DNA purification (Beckman Coulter, Villepinte, France). DNA binds to these paramagnetic beads allowing to remove contaminants though washing steps (i.e., salts, primers). In addition, the quantity of beads can be adapted to filter DNA fragment size. Library preparation was

performed with New England Biolabs (NEB) Kits (NEBNext® #E7595, #E7645 and #M0367S NEB, Evry, France). We used 1D Native barcoding genomic DNA kit (ONT) for DNA multiplexing.

Whole genome metagenomics of the 12 samples was simultaneously sequenced in one 48 hours-long run on a MinION flow cell (multiplexing).

Sequences were analysed with the bioinformatics pipeline developed in our laboratory by Eugeni Belda (<u>Alili, Belda et al., 2021b</u>) and available online (<u>https://git.ummisco.fr/pipelines/nanopore</u>).

The sequencing experiment from 12 samples generated 6.6 million reads, of an average length of 336.6 base pairs. Such fragment length is unusually small in comparison to previous results obtained with ONT where at least more than 2.5 kilobases on average were previously observed (Alili, Belda et al., 2021a; Debédat et al., 2022). However, this result is explained, in part, by the adaptation of the library preparation protocol.

Our short reads can be explained by the fact that 1) As explained above, in the protocol developed in our laboratory, the different stages of library preparation are accompanied by DNA fragments selection using paramagnetic beads (Agentcourt AMPure XP beads for DNA purification), which allows the retention of long fragments. Due to the low DNA yields that we obtained from this first experimentation round, we introduced some modifications in the sequencing protocol to keep the maximum number of DNA fragments and optimise the sequencing result; 2) Moreover, the DNA extraction protocols developed for faeces comprise two cycles of chemical and mechanical lysis which might increase DNA fragmentation in our case.

This is problematic because DNA fragment length is crucial for reads' classification during bioinformatics metagenomics analysis. Indeed it has been observed that unclassified reads (the reads that cannot be aligned against the reference genomes) are usually shorter, with an average length of 3 168 base pairs, compared with those that can be aligned, with an average read length of 8 064 bp (Alili, Belda et al., 2021b). This is also what we observed in this experiment: a high number of fragments were unclassified, and this was particularly the

case for shorter fragments (Figure R.1.6D). Thus, the second round of experiments was developed to optimise our fragment length and, thus, the number of classified reads.

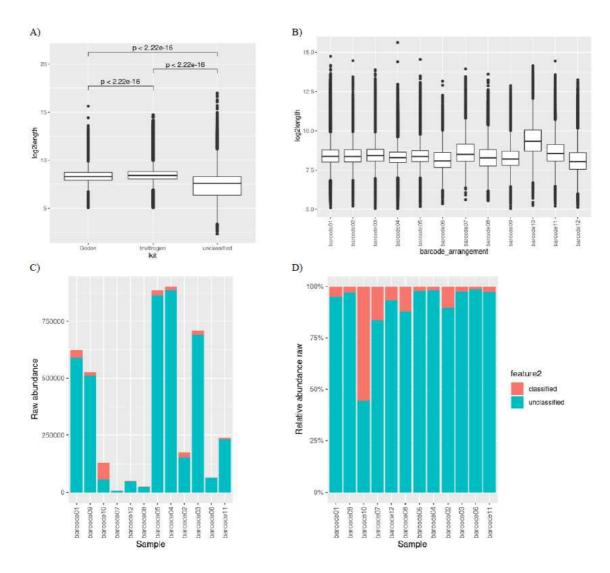


Figure R.1.6 - <u>DNA fragment read length and read classification against the reference catalogue</u>.

A) Graph A (top-left, boxplots) shows the distribution of fragments read length (log2-transformed) for classified reads (demultiplexed), per DNA extraction protocol (Godon or Invitrogen), and for unclassified fragments reads that could not be assigned to a barcode. B) Graph B (top-right distribution plot) shows the distribution of log2-transformed read lengths for the entire run. C) Graph C (bottom left, boxplot) shows read lengths for each sequenced sample. Barcode 10 shows longer reads on average. This is associated with an increased number of classified reads aligned against the reference genomes. D) Graph D (bottom right) shows the proportion of classified versus unclassified reads for each barcode. The unclassified fraction dominates all samples except for barcode 10 where the average read length is longer. Through observation, we can identify an association between read length and classification against the reference genomes (graphs B and D).

Rarefaction curves represent the diversity (i.e., metagenomic richness; y-axis in Figure R.1.7) detected as a function of sequencing depth (x-axis in Figure R.1.7). The ideal scenario is represented by a flattened curve, indicating that most of the sample's diversity has been sampled. Based on this analysis, we can simulate an even number of reads per sample, thereby avoiding sequencing depth biases between samples.

Rarefaction curves were thus performed to assess the necessary sequencing depth for optimal species richness and diversity index calculation. Curves reached a plateau at a sequencing depth of 36 1436 on average. For the analysis, a rarefaction threshold is usually chosen based on the samples having the lowest sequencing run. Some samples may be removed from the analysis. In this case, we could, for example, run the experiments a second time with the removed samples to improve their sequencing depth. In our case, we could have chosen to remove one sample whose threshold was much lower than the other samples (Figure R.1.7, Sample 4A, extracted with the Invitrogen protocol, barcode number 07). However, due to a lack of sample availability and as we were undertaking tuning experiments, we kept all samples for the analyses.

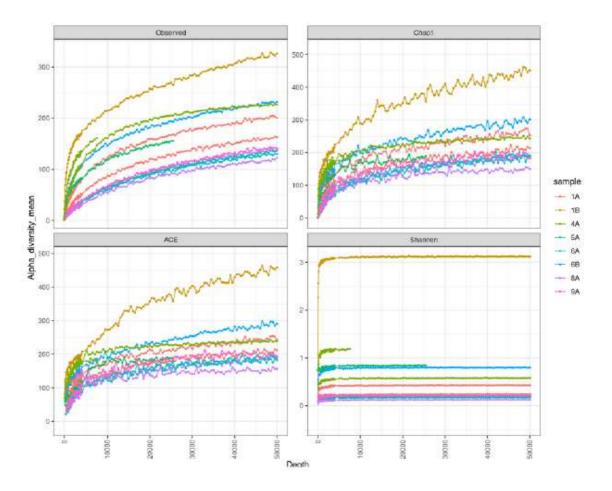


Figure R.1.7: Rarefaction curves from the first sequencing experiment.

This figure shows the rarefaction curves of the 12 samples based on four diversity indexes coloured by sample label. Rarefaction thresholds for each sample were estimated at 7751 (sample 4A, extracted with the Invitrogen protocol, barcode number 07); 25 959 (5A, Godon, barcode 08); 50 516 (4A, Godon, barcode 12); 65 644 (8A, Godon, barcode 06); 13 0224 (1B, Invitrogen, barcode10); 173 070 (6B, Invitrogen, barcode 02); 237 126 (9A, Godon, barcode11); 527 052 (1A, Godon, barcode09); 623 015 (1A, Invitrogen, barcode 01); 708 952 (8A, Invitrogen; barcode 03); 884 528 (6A Invitrogen, barcode 05); 903 395 (6A, Godon, barcode 04). No threshold was chosen for the subsequent analysis, and all samples were taken into account. Legend: ACE: (abundance-based coverage estimators).

Our results showed an influence of the extraction protocol on microbiome characteristics as visualised by 1) species richness and diversity graphs, 2) microbiome similarity as visualised by the distances between the samples on principal component analysis (Figure R.1.8), and 3) the graphs displaying relative abundances per sample and protocol (Figure R.1.9). Variations of microbial diversity and composition induced by different DNA extraction protocols have already been reported in the literature (Costea et al., 2017).

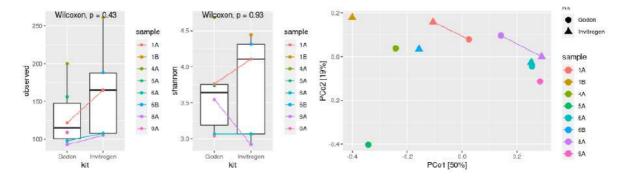


Figure R.1.8. <u>Variation of alpha diversity indexes with DNA extraction protocol</u>. From left to right. The two first boxplot graphs display the difference in microbial diversity for each DNA extraction protocol we used in this tuning experiment. The first graph displays microbial observed diversity, and the second graph displays the microbial diversity calculated with the Shannon index. The third graph is a PCOA map showing the distance between each sample.

These (and other parameters such as fragment length) were considered for the second round of experiments to confidently assess the contributions of the GM to human health and for reproducibility. Metagenomics results are known to be subject to variations induced by the sample collection, preservation, DNA extraction protocol, library preparation, and analytical processes. Batch effects also need to be minimised.

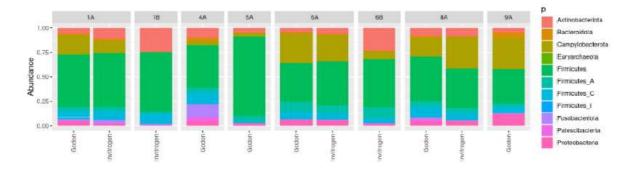


Figure R.1.9 - <u>Variation of the relative abundance at the phylum level with DNA extraction</u> protocol.

This graph shows the relative abundance at the phylum level for each sample and each DNA-extraction protocol of DJF. Replicates are side-by-side, and the influence of the extraction protocol on relative abundances can be observed. Names of Phyla are listed on the right (p).

Second Experimentation Round

The second round of experiments tested two different DNA extraction protocols: the 'automated' protocol and the 'manual' protocol, as detailed above. Each protocol was tested with or without the use of lysozyme (Auto+L and Manu+L). The four protocols were used on the same 12 samples to ensure optimal comparisons. The 12 samples were pure DJF (n=6) or saliva (n=6). Saliva samples were collected using the F2 kit (Falcon 2 kit, without conservatives), described in the following section.

For the adapted versions of the 'automated' or 'manual' protocol with lysozymes (named "Auto+L" and "Manu+L"), we replaced one of the two lysis cycles with the use of lysozymes (A49720001, PanReac AppliChem, Darmstadt, Germany). Lysozymes are enzymes that facilitate lysis and improve nucleic acid extraction by destabilising bacterial cell walls (Borrero-de Acuña et al., 2017). The objective of this modification was to have more delicate lysis to preserve DNA integrity as much as possible.

The strengths of the automated protocol are that it could be used for all samples along the GIT in one run. This could ensure a better comparison between these samples without batch effects or variability induced by different extraction protocols. Compared to the previous protocol version, we added a step using proteinase K to reduce protein contamination.

Regarding the manual protocol, for this second tuning experiment round, we added a purification step using phenol-chloroform to improve DNA purity (Phenol-Chloroform promotes the isolation of DNA from lipids, proteins, and cellular debris; 15593031, Invitrogen, Waltham, Massachusetts, US). As previously developed, we used an alcoholic precipitation method with isopropanol. The expected benefit of this protocol is that it could increase DNA yields (using silica beads/columns might reduce final DNA yields as it could retain DNA despite several washing steps). The limitation of this protocol is that there might be contamination of the DNA with reagents (i.e., phenol, guanidine, ...).

After extraction, DNA purity was assessed using a spectrophotometer (NanoDrop[™], ND-1000, ThermoFisher Scientific, Waltham, Massachusetts, United States). Concentration

was determined using a fluorometer with a High-Sensitivity kit (Qubit[™], Invitrogen[™], Waltham, Massachusetts, United States).

DNA purity was assessed with the absorbance ratio 260/280 nm and 260/230 nm (NanoDrop, Thermofisher). The 260/280 ratio, when calculated between 1.8 and 2, indicates a sample containing mainly nucleic acids. The 260/230 ratio, between 2 and 2.2, indicates pure and contaminant-free DNA. Lower 260/230 ratios indicate that the sample is contaminated (i.e., with guanidine, phenol, residual proteins, or other contaminants). This may affect sequencing experiments or other downstream analyses. In our case, the Automated version of the protocol without lysozymes had the best DNA purity as assessed by the spectrophotometer (Figure R.1.10).

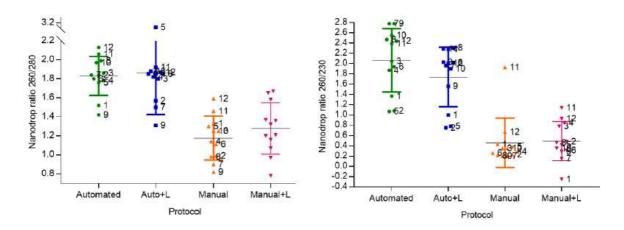


Figure R.1.10 - <u>Variation of DNA purity with DNA extraction protocols</u>. The left graph displays the nanodrop 260/280 purity ratios for the extracted DNA of each sample, extracted with the four DNA extraction protocols. The second graph on the right displays the nanodrop 260/230 purity ratios for the extracted DNA of each sample, extracted with the four DNA extraction protocols.

The quantity of total extracted DNA was higher for the versions of the protocols using lysozyme compared to the versions without lysozyme (Figure R.1.11; p < 0.05). The highest quantity of extracted DNA was obtained with the Automated+L protocol (p < 0.001 compared to the Automated without lysozymes protocol).

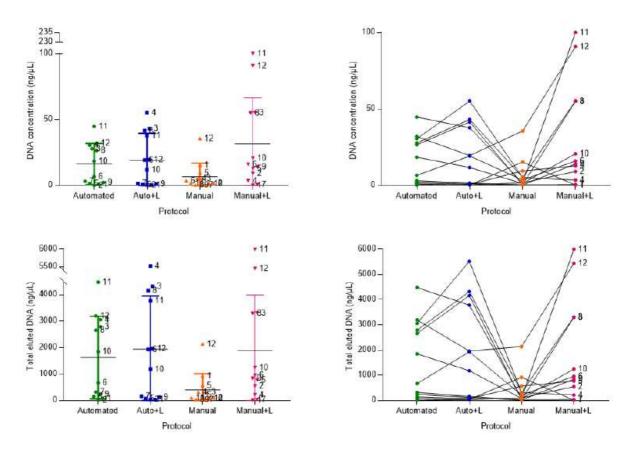


Figure R.1.11 - <u>Variation of DNA quantity with DNA extraction protocol</u>. The graphs on the top display the DNA concentration $(ng/\mu L)$ assessed with the fluorometer for all 12 samples and for each DNA extraction protocol we used: Automated, Automated + lysozymes (Auto+L), Manual, Manual + lysozymes (Manual+L). The graphs at the bottom display the total quantity of DNA assessed with the fluorometer extracted for all 12 samples and each DNA extraction protocol. The total extracted DNA was eluted in 100 μ L for the two automated protocols and in 60 μ L for the manual protocols.

Due to the low purity ratio and low DNA yields, I did not pursue the experiments on the DNA extracted with the manual protocol (with and without lysozymes).

The objectives of the sequencing were to compare the impact of lysozymes added to the extraction protocol. For the sequencing, I selected the samples whose DNA was extracted with the Automated protocol and that had the best purity ratio and for which I extracted more than 1 μ g DNA. Replicates were systematically used (to compare extracted DNA with the Auto vs Auto+L protocol). DNA libraries were prepared with the protocol developed by Rohia Alili. In contradiction with the first experimentation round, we filtered out the short DNA fragments, and the sequencing was run in September 2022. Results from sequencing showed that read length was increased to an average of 2 375 kb (versus 300 bp in the first experimentation round) and that classification of reads against the reference genome was increased with the Automated protocol, without lysozymes. This was explained, in part, by the increased read length with the protocol without lysozyme.

Based on these results, we decided to extract bacterial DNA from saliva, DJF, and stools of the cohort Je-MiMe, with the Automated protocol (without lysozymes) that gave the best sequencing results in terms of reads length, sequencing depth, and rarefaction curves. Depending on the total extracted DNA quantities on the samples of the Je-MiMe cohort, we could proceed to shotgun sequencing with Illumina instead of ONT as a minimal amount of 1 ng DNA is required instead of 1000 ng to start the library-preparation and amplification steps.

Protocol Development for Saliva Collection, DNA Extraction and Sequencing

Briefly, I ran a tuning experiment to test the collection, preservation, and DNA extraction methods of saliva samples. The objective was to assess the choice of these methods on DNA yields (Figure R.1.12).

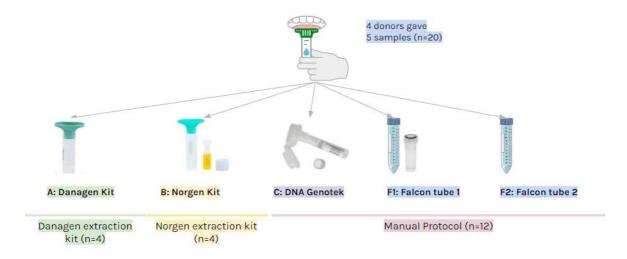


Figure R.1.12 - Experimental plan of saliva collection methods and DNA extraction protocol round of experiments.

Four donors sampled saliva on five consecutive days, using 5 different kits. All kits included conservatives except for the F2 kit. DNA extraction was done using the provided commercial kit and protocol or the manual protocol mentioned earlier (Godon, 1997).

I showed that sampling the saliva by spitting directly in an empty and sterile Falcon 50 mL tube gave the highest DNA yields. Thus, as it was convenient and, most importantly, less expensive, we chose this sampling method for the Je-MiMe study. As mentioned above, the second set of tuning experiments included saliva samples. Results showed that the DNA fragment, obtained using the developed method, had an average read of 3 507 kb, and no apparent issues were raised.

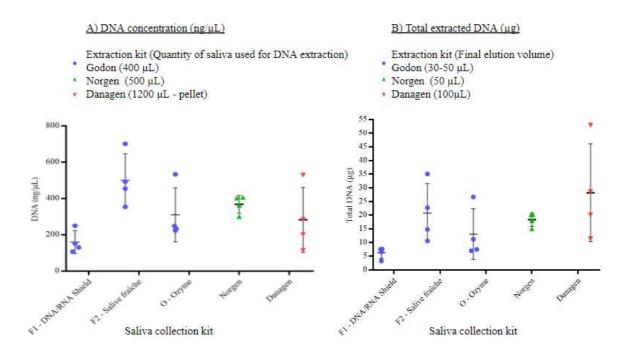


Figure R.1.13 - Results from DNA extraction from saliva samples.

Four donors sampled saliva on five consecutive days, using five different collection kits (x-axis) as described in Figure R.1.12. DNA extraction was performed using three different kits. Each kit required different quantities of saliva to start the extraction (Godon: 400 μ L; Norgen: 500 μ L; Danagen: 1 200 μ L). A) The left panel shows the DNA concentration (ng/ μ L). B) The right panel shows the total quantity of extracted DNA. The final elution volume varied with the extraction kits (Godon: 30-50 μ L; Norgen: 50 μ L; Danagen: 100 μ L).

As we selected the F2 kit for saliva collection (Figure R.1.12). All three samples for metagenomics and metabolomics (saliva, DJF and stools) were devoid of any conservatives mixed with the samples.

Conclusion: Rationale of Choice for Whole Genome, Next-Generation Sequencing using Illumina Platform

USI contents have low bacterial biomass, which comes with challenges: decreased DNA yields and increased difficulty of non-16S sequencing approaches (Morgan and Huttenhower, 2014).

Shotgun sequencing with the Illumina platform and Nanopore sequencing with the MinION device (ONT, Oxford, United Kingdom) were the two options considered for the work of my PhD project. Both methods involve sequencing the whole genome present in a sample. In the laboratory, we developed the Nanopore sequencing; thus we addressed the question of whether this platform is suitable to sequence the duodenojejunal luminal microbiome.

As introduced earlier, Illumina sequencing was developed in the early 2000s. The associated metagenomic catalogues have been developed over the years. They could allow for better identification of duodenojejunal microbiome species compared to the catalogues frequently used with nanopore sequencing. Moreover, as previously exposed, it is better suited for short DNA fragments, which was one of our concerns. Indeed, in the library preparation process, there is a random fragmentation step (350-550 bp fragments). Finally, it is convenient for materials with low bacterial load, such as duodenojejunal fluid, as Illumina sequencing is often combined with amplification steps. Up to 96 samples can be sampled in one run, reducing batch-experiment biases.

On the other hand, Nanopore sequencing can read very long intact DNA fragments (up to 2 million bp fragments), making classification processes easier against the reference genome. We can use Nanopore sequencing without amplification steps, reducing amplification biases. However, nanopore sequencing is still less widely used than the Illumina platform, meaning developments are still needed to optimise this technology. For example, accuracy is decreased compared to Illumina (i.e., the accuracy of MinION, per base, is much lower, 95% versus 99.9%; Stevens et al., 2023).

In my case, the most critical point of comparison was the quantity of extracted DNA required to initiate library preparation before sequencing. While library steps with Nanopore sequencing, using the developed protocol in our laboratory, require at least one µg DNA, on

the other hand, library preparation for Illumina sequencing can be started with one ng DNA (i.e., using the Colibri kit from ThermoFisher).

Based on experimental tests performed in the laboratory, we finally decided to sequence the DNA from the 90 samples of our cohort with Illumina sequencing (Cfr: DNA extraction, library preparation and sequencing from saliva, DJF and stools).

Part II: Results from the Je-MiMe Cohort

Description of the Je-MiMe Cohort

The studied cohort explored in this work is composed of two groups of 15 participants (n=30). A "control Group" (Ctrl group), composed of non-obese participants without known metabolic disorders (BMI: 22.3 kg/m², \pm 3.2; min: 18.6, max: 28.2) and a group with obesity (Ob group), composed of participants with severe obesity involved in a bariatric surgery program (BMI: 40.4 kg/m² \pm 4.8; min: 35.5 kg/m²; max: 50.6 kg/m²).

The cohort characteristics are described in the following tables (Table R.II.1, Table R.II.2. Table R.II.3).

There were no significant differences between the two groups regarding age (32,9 vs 34,4, p = 0.69) and sex ratio (male: n = 3, 20% vs n = 1, 6,6 %, p = 0.3). The Ob group had higher fasting glycemia (4.73 vs 5.32 mmol/L, p < 0.001), insulinemia (4.5 vs 17.8 mIU/L, p<0.001), glycated haemoglobin (5.1 % vs 5.6, p < 0.001), as expected. No participant had T2D. There were no significant differences between groups regarding total cholesterol levels. Compared to the control group, the Ob group had higher levels of triglycerides (0.7 vs 1.2 mmol/L, p < 0.001) and low-density lipoprotein cholesterol (LDL; 2.5 vs 3.1 mmol/L, p < 0.001) and low-density lipoprotein (HDL; 1.6 vs 1.1 mmol/L; p < 0.001). GGT, a liver health marker was higher in the "Ob group". The Ob group had higher C-Reactive Protein levels (CRP; 1.2 mg/L vs 5.4, p < 0.001), a common circulating marker of inflammation compared to the control group (Table R.II.1).

In addition to these circulating markers detailed in the table below, we quantified various other markers, including the circulating level of various entero-hormones, adipokines, cytokines and other inflammatory markers (from which the low-grade inflammation Z-score was calculated as detailed in the <u>Materials and Methods</u> section) or metabolites from the tryptophan pathway, amongst others. A total of 61 circulating variables were integrated to metagenomics and metabolomics data in the following section.

Several prescription drugs were exclusion criteria for this study (Annex 1: <u>Clinical</u> <u>protocol</u>). Analysis of prescription drug utilisation over the 3-months previous to the endoscopy, revealed that more than 10% of participants reported the following usage patterns: occasional use of analgesics (n = 14), occasional use of benzodiazepines (n = 4), antidepressants (n = 3), oral contraceptives (n = 3), and antihistamines (n = 3). The comparison of drug use patterns between groups showed no significant differences, with one exception: benzodiazepine use was reported exclusively by four participants within the control group, while none of the participants from the Ob group reported usage of this medication.

	Control Group	Obese Group	p-value
Sample size, n (%)	15 (50.0)	15 (50.0)	-
North European, n (%)	11 (73.3)	6 (40.0)	ns
Age (years)	32.9	34.4	ns
Sex (male), n (%)	3(20.0)	1(6.6)	ns
Height (cm)	169.0 (8.1)	165.9 (4.9)	ns
Adiposity markers			
Weight (kg)	63.8 (11.3)	111.1 (14.3)	****
BMI (kg/m2)	22.3 (3.2)	40.4 (4.8)	ગેર મેર મેર
Fat mass (%)	23.9 (7.2)	46.0 (4.1)	非非非非
Visceral fat mass (kg)	2.7 (1.5)	12.4 (2.8)	****
BMR (Kcal/day)	1447 (237.8)	1894 (290.9)	4: 4: 4: 4:
Plasma Glucose homeostasis			
Glycemia (mmol/L)	4.73 (0.35)	5.32 (0.42)	zije zije
Insulinemia (mIU/L)	4.5 (2.5)	17.9 (10.0)	4.4.4.4.
HOMA-IR	0.9 (0.6)	4.1 (2.5)	એર છેર છેર છેર
HbA1c (%)	5.1 (0.3)	5.6 (0.3)	****
Plasma lipid homeostasis			
Total cholesterol (mmol/L)	4.5 (0.9)	4.8 (0.7)	ns
Total triglycerides (mmol/L)	0.7 (0.3)	1.2 (0.3)	非非非非
HDL cholesterol (mmol/L)	1.6 (0.4)	1.1 (0.2)	nit nit nit
LDL cholesterol (mmol/L)	2.5 (0.7)	3.1 (0.7)	2 2
Liver Enzymes			
ASAT (IU/L)	27.7 (6.9)	23.9 (5.1)	ns
ALAT (IU/L)	28.3 (13.3)	34.3 (21.5)	ns
GGT (IU/L)	18.1 (7.7)	42.1 (23.3)	* * *
Inflammation marker			
C-Reactive Protein (mg/L)	1.2 (0.4)	5.4 (4.2)	라 라 라

Table R.II.1. - Description of the clinical parameters of the Je-MiMe cohort.

This descriptive table summarises the major clinical parameters of the Je-MiMe Cohort. Results are expressed as mean (SD) for continuous data and n (%) for categorical data. *P* values result from the Mann-Whitney-Wilcoxon non-parametric test (W test). Legend: BMI: Body Mass Index; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance. ASAT: aspartate transaminase; ALAT: alanine transaminase; GGT: gamma glutamyl-transpeptidase; ns *P*-value>0.05; * *P*-value ≤ 0.05 ; ** *P*-value ≤ 0.01 ; *** *P*-value ≤ 0.001 ; **** *P*-value ≤ 0.0001 Phenotyping also included various questionnaires to study patient's lifestyle, digestive symptoms, sleep, psycho-emotional health or eating behaviour amongst others (Table R.II.2). Importantly, there was a significant difference between groups regarding the Alcohol Use Disorder Test (AUDIT). While the control group had a significantly higher score compared to the obese group (p < 0,001), both groups had a mean score considered "low-risk consumption". While scores obtained for all other questionnaires were not different between groups (p > 0,05), the control group reported lower depressive symptoms ("mild" vs "moderate symptoms" of depression for the ob group) and higher anxiety ("doubts of symptoms" for the control group vs "no symptoms"). In addition, the control group reported lower eating behaviour stimulated by "external" and "emotional" stimuli compared to the Ob group. On the other side, the control group reported increased "restriction" eating behaviour (p > 0,05).

	Control Group	Obese Group	p-value
Alcohol consumption	6.3	1.8	***
	"Low risk co	onsumption"	
Nicotine dependence	0.7	0.11	ns
	"Low nicotine dependence"		
Quality of life	5.0	7.5	ns
	"Mild depression" vs "Moderate depression"		
Perceived stress	14.5	16.5	ns
	"Moderate per	ceived stress"	
Anxiety	8.2	6.1	ns
	"Doubts of symptom	s" vs "No symtoms"	
Depression	7.7	12.1	ns
	'No mood disturbance" vs	"Mild mood disturbance"	
Chronotype	55.8	51.1	ns
	"Morning type	" vs "Neutral"	
Eating behaviour (external)	3.1	3.2	ns
Eating behaviour (emotional)	2.9	3.3	ns
Eating behaviour (restriction)	3.0	2.6	ns

Table R.II.2 - Description of the Je-MiMe cohort: Psychosocial questionnaires scores. This table shows the scores obtained from various questionnaires. Alcohol consumption was evaluated by the Alcohol Use Disorders Identification Test (AUDIT); Nicotine dependence by the Fagerström Test; Quality of Life and Depression by the Patient Health Questionnaire (PHQ-9); Perceived stress by the Perceived Stress Scale (PSS-10); Anxiety by the Hospital Anxiety and Depression Scale (HAD, "A" items only); Depression with The Beck Depression Inventory (BDI); Chronotype or 'circadian rhythm' by the Horne and Ostberg Questionnaire; Eating Behaviour was assessed with the Dutch Eating Behaviour Questionnaire (DEBQ). Results are expressed as mean (SD). When available, the corresponding score interpretation is mentioned below the scores. *P* values result from the W test. Legend: ns *P*-value>0.05; * *P*-value ≤ 0.05 ; ** *P*-value ≤ 0.01 ; *** *P*-value ≤ 0.001 .

Regarding nutritional aspects, there were differences in the mean repartition of carbohydrates and alcohol consumption. The control group drinks more alcohol (p < 0.001) and consumes less carbohydrates (p = 0.029). However, the control group had a higher diet quality as assessed by two dietary scores (alternate Healthy Eating Index - aHEI, p < 0.001; Dietary Approaches to Stop Hypertension - DASH, p = 0.019). Regarding main food groups, the Ob group consumes less beer and wine (p < 0.001), coffee (p = 0.002), nuts, pulses, and vegetables (p < 0.05) and consumes more red meat, sweetened beverages and processed meat (p < 0.05).

There was no difference regarding oily fish consumption between groups (p = 0.090). No other significant difference regarding food groups' consumption between the Ctrl and Ob groups than those reported in Table R.II.3.

Repartition of macronutrients and alcohol (%)Alcohol $4.46 [2.53;5.38]$ $0.11 [0.01;0.98]$ Carbohydrates $39.9 [36.1;43.0]$ $44.3 [41.6;46.8]$ Proteins $17.2 [16.5;19.3]$ $17.6 [15.0;19.8]$ Lipids $37.2 [34.9;41.4]$ $38.1 [35.0;41.5]$ Diet quality scoresaHEI % $52.4 [43.5;62.8]$ $31.0 [20.9;37.4]$ DASH % $58.5 [51.0;64.8]$ $48.4 [41.8;56.3]$ Food groupsCoffee $180 [90.0;225]$ $0.00 [0.00;38.7]$ Beer $35.0 [8.75;108]$ $0.00 [0.00;0.00]$ Wine $51.6 [34.2;73.2]$ $0.00 [0.00;8.40]$	*** * NS NS
Carbohydrates $39.9 [36.1;43.0]$ $44.3 [41.6;46.8]$ Proteins $17.2 [16.5;19.3]$ $17.6 [15.0;19.8]$ Lipids $37.2 [34.9;41.4]$ $38.1 [35.0;41.5]$ Diet quality scores aHEI % $52.4 [43.5;62.8]$ $31.0 [20.9;37.4]$ DASH % $58.5 [51.0;64.8]$ $48.4 [41.8;56.3]$ Food groupsCoffee $180 [90.0;225]$ $0.00 [0.00;38.7]$ Beer $35.0 [8.75;108]$ $0.00 [0.00;0.00]$ Wine $51.6 [34.2;73.2]$ $0.00 [0.00;8.40]$	* NS
Proteins17.2 [16.5;19.3]17.6 [15.0;19.8]Lipids37.2 [34.9;41.4]38.1 [35.0;41.5]Diet quality scoresaHEI %52.4 [43.5;62.8]31.0 [20.9;37.4]DASH %58.5 [51.0;64.8]48.4 [41.8;56.3]Food groupsCoffee180 [90.0;225]0.00 [0.00;38.7]Beer35.0 [8.75;108]0.00 [0.00;0.00]Wine51.6 [34.2;73.2]0.00 [0.00;8.40]	ns
Lipids 37.2 [34.9;41.4] 38.1 [35.0;41.5] Diet quality scores aHEI % 52.4 [43.5;62.8] 31.0 [20.9;37.4] DASH % 58.5 [51.0;64.8] 48.4 [41.8;56.3] Food groups Coffee 180 [90.0;225] 0.00 [0.00;38.7] Beer 35.0 [8.75;108] 0.00 [0.00;0.00] Wine 51.6 [34.2;73.2] 0.00 [0.00;8.40]	
Diet quality scores aHEI % 52.4 [43.5;62.8] 31.0 [20.9;37.4] DASH % 58.5 [51.0;64.8] 48.4 [41.8;56.3] Food groups Coffee 180 [90.0;225] 0.00 [0.00;38.7] Beer 35.0 [8.75;108] 0.00 [0.00;0.00] Wine 51.6 [34.2;73.2] 0.00 [0.00;8.40]	ns
aHEI % 52.4 [43.5;62.8] 31.0 [20.9;37.4] DASH % 58.5 [51.0;64.8] 48.4 [41.8;56.3] Food groups Coffee 180 [90.0;225] 0.00 [0.00;38.7] Beer 35.0 [8.75;108] 0.00 [0.00;0.00] Wine 51.6 [34.2;73.2] 0.00 [0.00;8.40]	
DASH % 58.5 [51.0;64.8] 48.4 [41.8;56.3] Food groups Coffee 180 [90.0;225] 0.00 [0.00;38.7] Beer 35.0 [8.75;108] 0.00 [0.00;0.00] Wine 51.6 [34.2;73.2] 0.00 [0.00;8.40]	
Food groups Coffee 180 [90.0;225] 0.00 [0.00;38.7] Beer 35.0 [8.75;108] 0.00 [0.00;0.00] Wine 51.6 [34.2;73.2] 0.00 [0.00;8.40]	***
Coffee180 [90.0;225]0.00 [0.00;38.7]Beer35.0 [8.75;108]0.00 [0.00;0.00]Wine51.6 [34.2;73.2]0.00 [0.00;8.40]	**
Beer 35.0 [8.75;108] 0.00 [0.00;0.00] Wine 51.6 [34.2;73.2] 0.00 [0.00;8.40]	
Wine 51.6 [34.2;73.2] 0.00 [0.00;8.40]	***

Red meat 17.0 [3.69;27.6] 48.9 [39.2;56.0]	**
Nuts 3.24 [0.81;9.94] 0.00 [0.00;1.62]	**
Pulses 21.4 [16.1;65.9] 10.7 [0.00;21.4]	**
Sweetened beverages 0.00 [0.00;21.0] 71.0 [0.00;176]	**
Processed meat 3.89 [0.00;6.29] 19.1 [7.13;41.6]	*
Vegetables 271 [243;424] 202 [135;276]	*
Oily fish8.83 [4.41;18.0]4.41 [3.31;5.52]	ns

Table R.II.3 - <u>Description of the Je-MiMe cohort: nutrition</u>.

This table shows the mean repartition of macronutrients and alcohol intake in terms of percentage; two diet quality scores expressed as the percentage of the maximal score: the aHEI (Alternate Healthy Eating Index) and the DASH (Dietary Approaches to Stop Hypertension) scores; and the consumption of different food groups. Results were computed from the Food Frequency Questionnaire. Results are expressed as median [min; max]. *P* values result from W test. Legend: ns *P*-value > 0.05; * *P*-value ≤ 0.05 ; ** *P*-value ≤ 0.01 ; *** *P*-value ≤ 0.001 .

Integration of Metagenomics and Metabolomics to Lifestyle and Clinical Data

We first aimed to characterise the microbiome across three ecosystems in the digestive tract in humans. To reach that goal, we analysed the microbiome from three samples: saliva (oral microbiome, OM), the duodenojejunal luminal fluid obtained during endoscopy (upper small intestinal microbiome, USIM), and stool (faecal microbiome, FM). We also compared the USIM and -associated metabolome to the faecal metabolome.

<u>Compositional characteristics of luminal duodenojejunal fluid microbiome compared</u> to oral and faecal microbiome

We first calculated the metagenomic richness based on the MetaPhlAn4 catalogue. Metagenomic richness corresponds to the count of different features (i.e., bacterial genes at a given taxonomic level) identified in a sample. We observed a significant difference in metagenomic richness between the three ecosystems (p < 0.001; Figure R.II.1a). Metagenomic richness was highest in the faecal microbiome and the lowest in the duodenojejunal luminal microbiome, while the oral microbiome was in between. Interestingly, there was a high interindividual variability of the marker gene count in USIM (min ~ -1 100 to max ~15 500 marker genes in the USIM; vs. metagenomic richness in FM: min ~ 8000, max ~ 18 700 marker genes).

Afterwards, we quantified the compositional dissimilarity between samples from the table of relative abundance at the marker gene level. As shown after principal coordinate analysis (Figure R.II.1b), we observed a low distance between OM and USIM samples and a high dissimilarity between FM samples compared to samples from the two other ecosystems. An intra-individual connection was observed between the saliva and DJF samples (PCo2).

An effect size calculation between species and ecosystems (Figure R.II.1c, Vulcano plot showing Cliff's delta analysis) confirms the shared characteristics of OM and USIM, as most species were not significantly associated with one or the other ecosystem, while Cliff's delta analysis between OM or USIM with FM showed almost no shared species between ecosystems.

Strain tracking across ecosystems (Figure R.II.1d) confirmed that the duodenojejunal microbiome shares a higher count of species with saliva than FM. Indeed, while OM and

USIM shared 231 species (9% of all species found accross the GIT) in at least one individual of the whole group, USIM and FM shared 47 species (4%), and OM and FM shared only one species. This analysis showed a minimal set of shared strains across samples, as only 25 species were found in the three ecosystems. Strain tracking, with a prevalence of 50% in our cohort, showed that only one strain was shared across all three ecosystems: *Streptococcus salivarius*, which was indeed identified in all three samples in 25 participants.

We further examined the relative abundance of bacterial Phyla and families across ecosystems (Figure R.II.S1). At the phylum level, all three microbiomes from the ecological niches are dominated by Firmicutes (renamed 'Bacillota').

Specifically, the USIM is dominated by Firmicutes, Actinobacteria (renamed Actinomycetota) and Bacteroidetes (renamed Bacteroidota), whereas the relative abundance of Proteobacteria (renamed Pseudomonadota) varies between ~1% to ~19%. OM is dominated by Firmicutes, followed by Bacteroidetes and Actinobacteria, while the relative abundance of Proteobacteria varies between 4% to 20% in saliva. FM is largely dominated by Firmicutes, followed by Bacteroidetes (Figure R.II.S1).

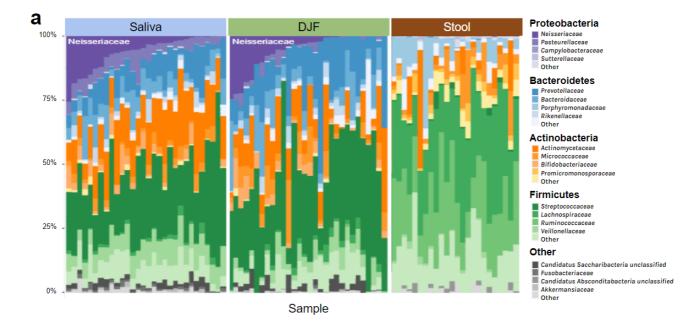


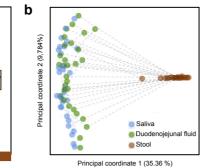
Figure R.II.S1 - Relative abundance of bacterial Phyla and family in USIM and the two other ecosystems. (Supplemental). These barplots display the relative abundance per ecosystem (OM in blue, USIM in green, FM in brown). The different Phyla are colour-coded (Purple: Proteobacteria; Blue: Bacteroidetes; Orange: Actinobacteria; Green: Firmicutes; Grey: Others) and within each Phyla, the bacterial families are coloured in different shades (From dark light purple: to Neisseriaceae, Pasteurellaceae *Campylobacteraceae*, Sutterellaceae: from dark to light blue: Prevotellaceae Bacteroidaceae, Porphyromonadaceae, Rikenellaceae, Other; From dark to light orange: Actinomycetaceae, Micrococcaceae, Bifidobacteriaceae, Promicromonosporaceae, other; From dark green to light green: Streptococcaceae, Lachnospiraceae, Ruminococcaceae, Veillonellaceae, other; From dark to light grey: Candidatus Saccharibacteria unclassified, Fusobacteriaceae Candidatus Absconditabacteria unclassified, Akkermansiaceae, other). The relative abundance is expressed in percentage (y-axis). Each column represents a participant. N.B.: as mentioned in Materials and Methods, the current version of the manuscript is missing metagenomics data from 6 faecal samples (OM: n = 30; USIM: n =30; FM: n = 24).

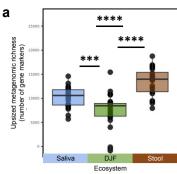
Figure R.II.1e reveals the composition of each ecosystem at the species level. While the OM and USIM are colonised by various *Prevotellaceae, Streptococcaceae, Veillonellaceae, Actinomycetaceae,* amongst others, the FM is colonised by species such as *F. Praustnitzii, Dysosmobacter welbionis, Eubacterium rectale, Roseburia faecis,* various *Clostridiales, Ruminococcaceae, Roseburiaceae* or *Blautia* species amongst others.

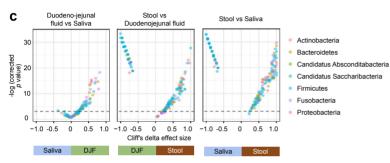
4	Saliva	Si luid	0
Anaerobutyricum hallii			
Roseburia faecis			
Faecalibacterium prausnitzii			
Anaerostipes hadrus Dorea longicatena	\vdash		
Romboutsia timonensis	HH		
Blautia faecis			
Clostridiaceae bacterium			
Ruminococcus torques Blautia wexlerae			
Clostridia bacterium	\vdash	=	t I
Fusicatenibacter saccharivorans			
Lachnospiraceae bacterium			
Dysosmobacter welbionis			
Coprococcus catus Roseburia inulinivorans	\square		
Coprococcus comes	\vdash		
Dorea formicigenerans	H		
Blautia massiliensis			
Eubacterium rectale			
Eubacteriaceae bacterium Blautia SGB4815	\square		
Clostridium sp AF34 10BH	\vdash	_	
Candidatus Cibiobacter qucibialis	H		
Ruminococcaceae bacterium			
Clostridiales bacterium KLE1615			
Blautia obeum			
Streptococcus thermophilus Oscillibacter sp ER4			
Coprococcus eutactus			
Ruminococcus bromii			
Roseburia hominis			
Lacrimispora amygdalina Lachnospiraceae bacterium WCA3 601			
Lachnospiraceae bacterium WCA3 601 Lachnospira eligens	\square		
Dorea sp AF36 15AT	\vdash		
Agathobaculum butyriciproducens	\vdash		
Gemminer formicilis	H		
Firmicutes bacterium AF16 15 Peptostreptococcus SGB749			
Gemella haemolysans		\square	
Mogibacterium diversum			
Eubacterium sulci			
Oribacterium sinus			
Streptococcus sanguinis Streptococcus sp A12			
Megasphaera micronuciformis			
Veillonella atypica		Н	1
Streptococcus mitis			
Streptococcus salivarius Streptococcus parasanguipis			
Streptococcus parasanguinis GGB4538 SGB6253			
Parvimonas SGB6649		Н	
Abiotrophia defectiva GGB3886 SGB5269			
Veillonella tobetsuensis			
Granulicatella elegans			
Selenomonas SGB5880		\square	
Panvimonas micra		Н	
Oribacterium parvum Peptostreptococcus stomatis			
Eubacterium brachy			
Eubacterium nodatum		\square	
Lachnospiraceae sp oral taxon 096 Bulleidia extructa		Н	
Bulleidia extructa		Н	
Mogibacterium timidum			
Filifactor alocis Oribacterium asaccharolyticum			
Lachnoanaerobaculum orale		\square	
Streptococcus anginosus		Н	
Stomatobaculum longum		Н	
Streptococcus pneumoniae			
Eubacterium saphenum Streptococcus constellatus			
GGB3394 SGB4498			
Granulicatella adiacens		Н	
Streptococcus gordonii		Η	
Veillonella rogosae			
Stomatobaculum SGB5266 Streptococcus cristatus			
Streptococcus cristatus Gemella morbillorum		Н	
Lachnoanaerobaculum sp ICM7		Н	
Streptococcus sp 263 SSPC		Η	
Streptococcus infantis			
Gemella sanguinis Veillonella dispar		\square	
Streptococcus oralis		Н	
Solobacterium SGB6833			
Veillonella parvula			

Firmicutes

e	Salles
Bifdobacterium iongum Collinsella aerofaciens Actinomyces sp S6 Spd3 Trueperella pyogenes Lancefieldella parvula Actinomyces oris Actinomyces sp ICMAT Rothia mucilaginosa Isoptericola variabilis Actinomyces graevenitzii Actinomyces neesiundii Actinomyces saevenitzii Actinomyces SGB17168 Actinomyces dentalis Olsenella sp oral taxon 807 Rothia aeria Corynebacterium durum Rothia dentocariosa	Actinobacteria
Parabacteroides distasonis Bacteroides uniformis Phocaetical vulgatus Barnesiella intestinhomnis Odorbacter splanchnicus Bacteroides thetistolare dinis Bacteroides thetistolare dinis Bacteroides vulatus Bacteroides vulatus Prevotella jeluni Allogrevotella innerae Porphyromonas endodontalis Bacteroidaceae unclassified SGB1468 Prevotella instincel Prevotella instincel Prevotella pieuritidis Tannerella sp oral taxon HOT 286 Prevotella anternae Prevotella denticola Prevotella splanti Alloprevotella splanti Alloprevotella spa 2879 Prevotella splanti GGB1403 SGB1453 Bacteroidaceae bacteroides denticola Prevotella splanti Alloprevotella rava Capnocytophaga laddeticola Prevotella rancella sis GGB1403 SGB1568 Prevotella mancella sis Prevotella concella son Prevotella palensi Prevotella pienettalis Prevotella pienettalis	Bacteroidetes
bsconditabacteria GGB4936 SGB6889	C.A.
Saccharibacteria SGB19850 TM7 phylum sp oral taxon 351 Saccharibacteria SGB19782 nosynsacchari sp TM7 ANC 38 39 G1 1	an.Sacc.









Saccharibacteria SGB 19850 TM7 phylum sp oral taxon 351 Saccharibacteria SGB 19782 Nanosynaacchari sp TM7 ANC 38 39 G1 1 Nanoperiomorbus periodonticus	
Leptotrichia wadei Leptotrichia sp oral taxon 215 Fusobacterium pseudoperiodonticum Fusobacterium nucleatum	
Bilophila wadsworthia Neisseria subflava Aggregatibacter sp oral taxon 458 Haemophilus sputorum Lautropia mirabilis Campylobacter concisus Neisseria sicca Haemophilus parinfluenza	
prev 0.00 0.25 0.50 0.75 1.00	D



Figure R.II.1 - Microbiome patterns across three ecosystems.

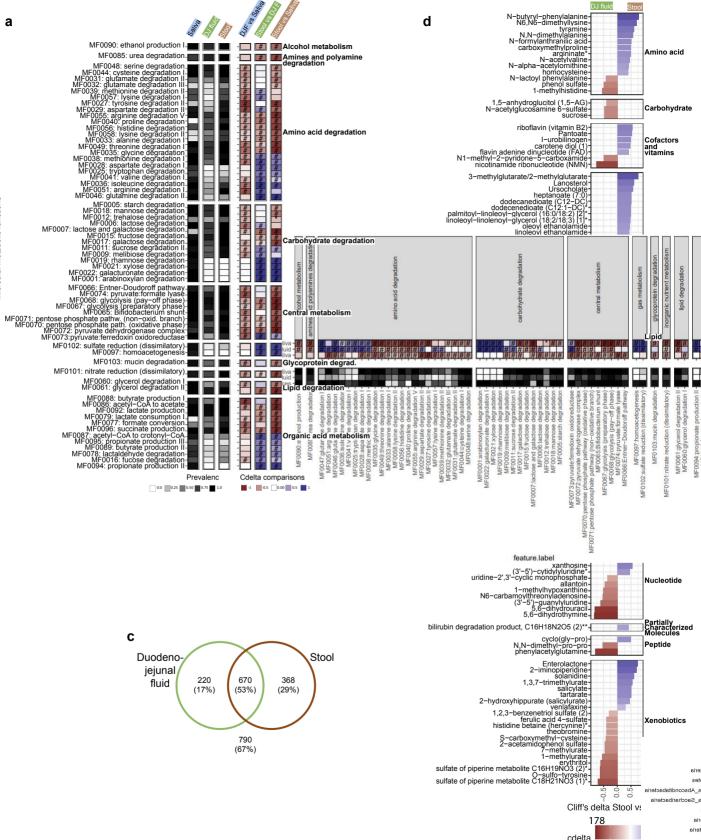
a.) Metagenomic richness across the three ecosystems. This boxplot shows the metagenomic richness (y-axis) of each ecosystem (x-axis): saliva (OM, in blue), duodenojejunal fluid (USIM, in green) and stool (FM, in brown). P-values result from W test. Legend: *** $p \le 0.001$; **** p < 0.0001). **b.)** Dissimilarity matrix between the three ecosystems. This principal component analysis shows the distance between each sample (represented by a dot coloured in their corresponding ecosystem) in each ecosystem: saliva (in blue), duodenojejunal fluid (in green) and stool (in brown). Samples from the same patient are connected with a dotted line (PCo2). c.) Vulcano plot of Cliff's delta association analysis between species and each ecosystem. Each dot represents a species, coloured in their corresponding Phylum (Red: Actinobacteria; Yellow: Bacteroidetes; Green: Candidatus Absconditabacteria; Turquoise: Candidatus Saccharibacteria; Blue: Firmicutes; Purple: Fusobacteria; Pink: Proteobacteria). Statistical significance is displayed on the y-axis, and the horizontal dotted line indicates the p < 0.05 threshold. Cliff's delta is displayed on the x-axis. d.) Venn Diagram showing the strain tracking of species across three ecosystems. The Venn Diagram represents the species repartition between the three ecosystems: saliva (in blue), duodenojejunal fluid (in green) and stool (in brown). Results are expressed as count (%). e) Species prevalence and abundance across the three ecosystems. These heatmaps show the presence-absence (grey-shaded heatmap) of species across ecosystems. Phyla are colour-coded using the same legend as panel 1c of this figure. This graph only displays species significantly altered between ecosystems (Kruskall-Wallis: *p* < 0.001). Legend: # FDR < 0.05.

Functional Characteristics of the Duodenojejunal Fluid Microbiome and Metabolome

We first examined functional profiling with the HUMAnN pipeline from metagenomic data to reveal the metabolic potential of each of the three ecosystems. In the proximal digestive tract (USIM and OM), we found enrichment of ethanol production, simple carbohydrate degradation, and most 'central metabolism' functions, such as half of all amino acid degradation, whereas the 'butyrate production I' functional module was predominant in OM. In FM, we found enrichment in complex carbohydrate degradation, half of all amino-acid functional modules and gas degradation functional potential, as expected (Figure R.II.2a).

We extended this exploration by examining the metabolomics of DJF and stools. In DJF samples, 890 metabolites were identified, and 1038 metabolites were identified in faecal samples. Comparative analyses showed that, while 220 metabolites (17%) were solely identified in DJF, 368 metabolites (29%) were unique to the faecal samples, and 670 metabolites (53%) were common to both ecosystems (Figure R.II.2b).

We found an enrichment of metabolites related to carbohydrate and lipid pathways in the USIM. Regarding lipids, there was, in particular, higher levels of sphingolipids, acylcarnitines and primary bile acids (BAs) in the USI, while secondary or conjugated fatty acids were higher in faeces.



-0.5 0.0

Figure R.II.2 – <u>Microbiome inferred functions and metabolome variation across</u> ecosystems.

a) Metabolic potential of each ecosystem. These heatmaps reveal the presence/absence of functional modules across ecosystems (shades of grey, from black, for presence, to white, for absence) and the Cliff's Delta association analysis (colour-coded in red for negative associations and blue for positive associations with stool). Functional modules are listed on the left and organised per group of functions listed on the right. Legend: * p < 0.05; # FDR p < 0.05. b) Relationship between the two metabolomes. This Venn Diagram shows the relation between the two metabolomes, the duodenojejunal metabolome (DJF, in green) and the faecal metabolome (stool, in brown). Results are expressed as counts of different identified metabolites (%). c) Association analysis between metabolites and ecosystem. This graph shows Cliff's delta effect size analysis between metabolites and stool, listed on the left, and organised per their corresponding pathway, listed on the right, with one of the two ecosystems: the duodenojejunal fluid (in red) and the faeces (in blue). Only the core metabolites shared between the two ecosystems and passing the FDR correction (FDR < 0.05) are shown in this figure (n=118).

Lifestyle and Clinical variables explaining Duodenojejunal Fluid Microbiome Compositional Variation

Based on our detailed phenotypic characterisation of our population of participants with or without obesity, we aimed to identify the variables explaining the interindividual variation in terms of composition within each microbiome and metabolome and in terms of metagenomic richness as shown in Figure R.II.3.

For USIM, we observed that microbiome variance was explained (p < 0.05, Figure R.II.3a) by a series of circulating markers (triglycerides, ghrelin, C-peptide HDL-cholesterol and ASAT / ALAT ratio), corpulence and body composition variables (BMI, percentage of fat mass and visceral fat score) and dietary-related factors (wine, processed food, processed meat, potatoes, pastries and animal fat consumption). The score obtained from the AUDIT questionnaire assessing the alcohol consumption risk was also an explicative factor of USIM variance. Only HDL-cholesterol and body composition-related factors remained significant after multiple testing corrections (False Discovery Rate, FDR; BH method). The explanation of variance reported by the sum of squares showed HDL-cholesterol to have the highest effect size and to impact the variance of USIM significantly ($R^2 = \sim 9\%$; FDR < 0.05). Equally, all body composition variables, including fat mass accumulation, significantly explained the variance of USIM ($R^2 = \sim 7\%$ FDR < 0.05).

Corpulence-related factors (BMI and visceral fat rating) also explained FM variance but revealed non-significant after FDR correction. Similarly, FM variance was explained by various circulating markers (leptin, ASAT/ALAT ratio, circulating tryptophan levels) and dietary variables (both diet-quality scores: DASH and aHEI, food items as well as plant fat, animal fat and coffee consumption) such as delivery mode (being born by C-section or not). All of these variables, except birth by C-section, did not pass the FDR correction (p < 0.05FDR > 0.05). Finally, only a few variables were found to explain OM variation (HDL-cholesterol circulating levels, consumption of hot beverages and processed meat and age). However, none met the FDR correction (p < 0.05, FDR > 0.05).

Regarding the DJF metabolome, the same trend was observed. Indeed, all corpulence and body composition variables (BMI, visceral fat mass score and percentage of fat mass) and circulating HDL-cholesterol significantly explained DJF metabolome variation after FDR correction. In addition, beer consumption significantly explained metabolomic variance and resisted statistical significance after FDR correction (p < 0.05, FDR < 0.05).

Other variables explained DJF metabolomic variation but did not pass multiple corrections. This was the case for dietary variables (the aHEI score, yoghourt, wine, egg, coffee and beer consumption).

The variables explaining the most DJF metabolome variance after FDR correction were: beer consumption ($R^2 \sim 15\%$; FDR < 0.05), BMI ($R^2 \sim 10\%$; FDR < 0.05), followed by fat mass accumulation variables ($R^2 \sim 8\%$; FDR < 0.01).

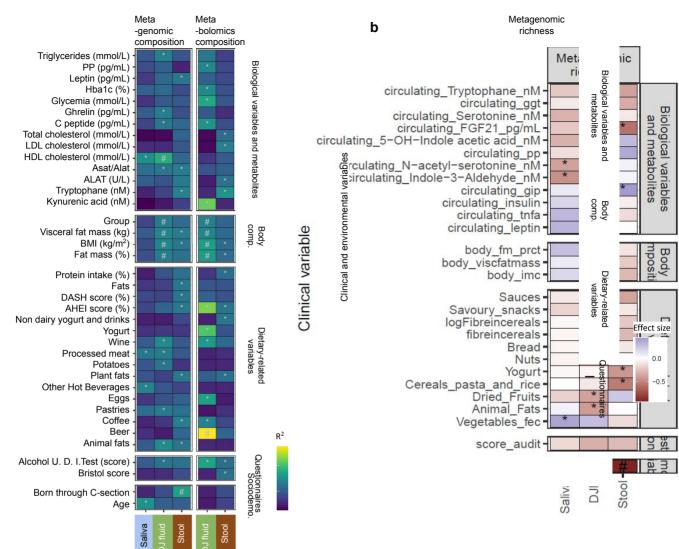
The faecal metabolome was not significantly explained by any variable after FDR corrections. However, certain circulating variables (total cholesterol, LDL cholesterol, ALAT and circulating tryptophan levels), corpulence and body composition variables (BMI and percentage of fat mass) such as dietary variables (percentage of consumed proteins, aHEI score, non-dairy alternatives such as soja drinks and yoghourts and plant fats consumption) explained the variance of the metabolome (p < 0.05, FDR > 0.05). In addition, the scores obtained from the AUDIT and Bristol questionnaires also explained metabolomic variance in faeces (p < 0.05, FDR > 0.05).

Furthermore, because metagenomic richness is an important feature of microbiome composition, we then examined the association between microbiome richness and the series of environmental and clinical variables (Figure R.II.3b). The consumption of several food items (fibres in cereals, nuts, dried fruits, savoury snack and animal fat) was negatively associated with USIM richness (p < 0.05), with savoury snacks remaining linked with USIM richness after FDR correction (FDR < 0.1). On the other hand, we observed a positive association between the percentage of fat mass and USIM gene richness (p < 0.05, FDR < 0.1).

There was an opposite trend regarding the association between FM richness and body composition variables, which were associated negatively. However, this association did not reach statistical significance (p > 0.05). Moreover, while negative associations were found between food items and FM richness (yoghurt and cereals, pasta and rice consumption), these associations did not remain after the FDR correction (p < 0.05, FDR > 0.1).

Relationships between OM richness and the tested variables showed N-acetyl-Serotonine and Indole-3-Aldehyde were associated negatively with metagenomic richness and, in opposition, the consumption of plant-based carbohydrates was negatively associated with gene richness in saliva. No association pass the FDR correction (p < 0.05, FDR > 0.1).

Interestingly, birth by C-section was associated with lower microbial richness in both USIM and FM after FDR correction (p < 0.05, FDR < 0.1).



Clinical and environmental variables

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Figure R.II.3 – <u>Contribution of lifestyle and clinical variables on microbiome and</u> metabolome composition and metagenomic richness across the ecosystems.

a) Permutational multivariate analysis of variance of metagenome and metabolome composition explained by lifestyle and clinical variables. This figure shows the proportion of compositional variance explained by different clinical or environmental variables. The Permutational Multivariate Analysis of Variance using the Adonis function was computed from the Bray-Curtis dissimilarity matrix for metagenomic and the Euclidian distances for metabolomics data. The effect size of the determination is colour-coded from blue ($R^2 = 0\%$) to yellow ($R^2 = 15\%$). Legend: * p < 0.5; # : FDR < 0.05. b) Association analysis between metagenomic richness and lifestyle and clinical variables. This heatmap shows the association between metagenomic richness and lifestyle and clinical variables (listed on the left) in each ecosystem (listed at the bottom) using either Spearman's rank correlation coefficient, W test or Kruskal–Wallis test. Positive associations are coloured in blue, while negative values are coloured in red. This panel 3b, only displays the associations that reached at least p < 0.1. Legend: * p < 0.05; # FDR < 0.1.

Microbiome Signatures of the Upper Small Intestinal Microbiome in Obesity

As we observed that corpulence and body composition variables explained the compositional variance of the USIM and were associated with microbiome richness, we examined the compositional differences of each ecosystem in subjects with or without obesity (Figure R.II.4a).

We observed USIM relative abundance of Proteobacteria was lower in the group with obesity (p < 0.001). On the other hand, FM relative abundance of Firmicutes was lower in the group with obesity compared to the control group (p < 0.01; Figure R.II.4a).

Metagenomic richness was not significantly altered between groups in any ecosystems, as shown in Figure R.II.4b. There was a non-significant trend towards higher metagenomic richness in the obese group in the USIM (p = 0.06)

We further investigated the compositional differences at the species level between groups with or without obesity (Figure R.II.4e). This analysis revealed that, in the USIM, 18 bacteria showed higher abundance with obesity (p < 0.05). In contrast, only three bacterial species were higher in the control group (p < 0.05). In opposition to USIM, in FM, more bacteria showed higher abundance in the control group: 14 bacteria were significantly higher in the group of non-obese participants, while eight bacteria were higher in the Ob group (p < 0.05). In the OM, there were equal numbers of species associated with each group (7 bacterial species were higher in the control group and seven others in the obese group; p < 0.05).

However, across the three ecosystems, only two species were significantly altered between groups after FDR correction (Figure R.II.4c): *Actinomyces sp* S6 Spd3 an Actinomycetota (previously named Actinobacteria) was associated with obesity in the USIM (Cliff's Delta $> \pm 0.65$; FDR < 0.001), and the most important effect size was observed for *Neisseria subflava*, a Proteobacteria associated with the control group in the USIM (Cliff's Delta $> \pm 0.7$; FDR < 0.001). No significant changes in the relative abundance of any species pass the FDR correction between the control group and the obese group in OM and FM.

We then looked at the relationships between the abundance of these species and obesity-related phenotypes in the whole cohort. The USIM abundance of *Actinomyces sp* S6 Spd3 was positively associated with the circulating levels of Leptin, GGT and Serum Amyloid A (SAA; FDR < 0.05) as well as with corpulence and body composition variables (visceral fat mass, BMI, fat mass %; FDR < 0.05). The taxon was negatively associated with the circulating levels of Glucose-dependent insulinotropic polypeptide (GIP; FDR < 0.05; Figure R.II.4e).

Further association analysis revealed the abundance of *N. subflava* in USIM negatively correlated with various circulating markers, including triglycerides (FDR < 0.05), glucose control (fasting glucose and insulin, Hba1c, C-peptide and HOMA-IR; FDR < 0,05), inflammatory variables (low-grade inflammation Z scores, and components of this Z score: IL-6, ICAM 1; FDR < 0,05). In addition, a negative association was found between *N. subflava* and corpulence and body composition variables (visceral fat mass, BMI, fat mass; FDR < 0,05). On the other hand, this taxa showed a positive association with the circulating levels of ghrelin, HDL-cholesterol, adiponectin and the ASAT/ALAT ratio (FDR > 0,05). A positive association was also found between the taxon and wine consumption and the score from the AUDIT questionnaire (Figure R.II.4f; FDR > 0,05). No associations between *N. subflava* and clinical or environmental variables were found in OM and FM.

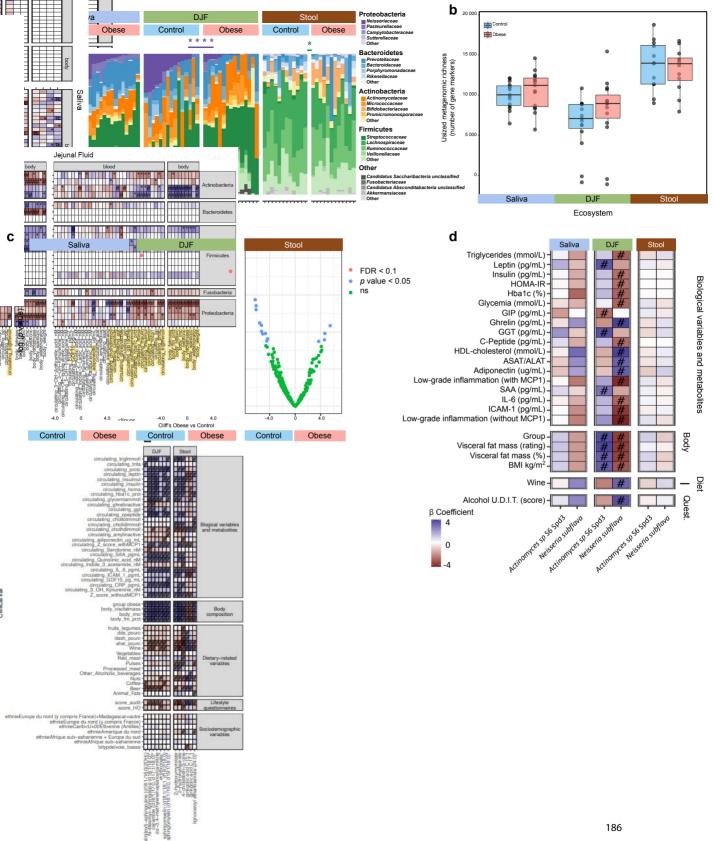


Figure R.II.4 - <u>Obesity-associated microbiome signature of the upper small intestinal</u> <u>microbiome obesity.</u>

a.) Relative abundance of bacterial Phyla and family in USIM and the two other ecosystems These barplots display the relative abundance per ecosystem (OM in blue, USIM in green, FM in brown), stratified per group (Ctrl group in blue; Ob group in red). The different Phvla are colour-coded (Purple: Proteobacteria, Blue: Bacteroidetes, Orange: Actinobacteria, Green: Firmicutes and Grey: Others), and within each Phyla, the bacterial families are coloured in different shades (From dark to light purple: Neisseriaceae, Pasteurellaceae, Campylobacteraceae, Sutterellaceae; from dark to light blue: Prevotellaceae, Bacteroidaceae, Porphyromonadaceae, Rikenellaceae, Other; From dark to *Micrococcaceae*, light orange: Actinomycetaceae, Bifidobacteriaceae, Promicromonosporaceae, other; From dark green to light green: Streptococcaceae, Lachnospiraceae, Ruminococcaceae, Veillonellaceae, other; From dark to light grey: Candidatus Saccharibacteria unclassified, Fusobacteriaceae, Candidatus Absconditabacteria unclassified, Akkermansiaceae, other). The relative abundance is expressed in percentage (y-axis). Each column represents a participant. P values result from between groups comparisons using W test. Legend: **** p < 0.0001; ** p < 0.01). b) Metagenomic richness between groups. This boxplot shows the marker gene count in each ecosystem: the USIM (in green), the OM (in blue) and the FM (in brown), stratified per group: the control group (in blue) and the Ob group (in red). c) Association between species and groups within each ecosystem. This Vulcano plot shows the Cliff's Delta effect size analysis between bacterial species and the participant's group within each ecosystem. P values are represented on the y-axis, and species' association to cohort group on the y-axis (ctrl in blue, Ob in red). Each dot represents a species and is colour-coded in regard to the level of significance of the association (green: not significant; blue: p < 0.05; red: FDR < 0.1). d) Association analysis between Actinomyces sp S6 Spd3 and Neisseria subflava and clinical and lifestyle variables. This heatmap reveals the associations between the two taxa and subsets of clinical and lifestyle variables in the whole cohort of 30 participants (red: negative association; blue: positive association). Legend: * p < 0.05, # FDR < 0.05).

Metabolome Signatures of the Upper Small Intestinal Microbiome in Obesity

We also investigated metabolomics differences between groups with an effect size analyses in Figure R.II.5. Several lipids were associated with the obese group in DJF (Figure R.II.5a). This was the case for various sphingolipids (N–stearoyl–sphingosine (d18:1/18:0), two ceramides (d18:1/17:0, d17:1/18:0), four sphingomyelins (d18:1/19:0, d19:1/18:0, d18:1/18:1, d18:2/18:0) and N –(2 –hydroxypalmitoyl) –sphingosine (d18:1/16:0(2OH)); FDR < 0.1) such as cis–3,4–methyleneheptanoylcarnitine. Erythronate, a metabolite from the carbohydrate pathway, was also higher in the obese group (p < 0.05; FDR < 0.1). No metabolites were associated with the control group after FDR corrections in DJF.

In stools (Figure R.II.5b), there was higher levels of four metabolites from the lipid pathway in the obese group after FDR correction (2-hydroxymyristate, 2-hydroxystearate,

lignoceroyl ethanolamide (24:0) and 4–cholesten–3–one) and two metabolites from the xenobiotic pathway were increased in the control group (ginkgolic acid C15:1, ginkgolic acid C17:1; p < 0.05; FDR < 0.1).

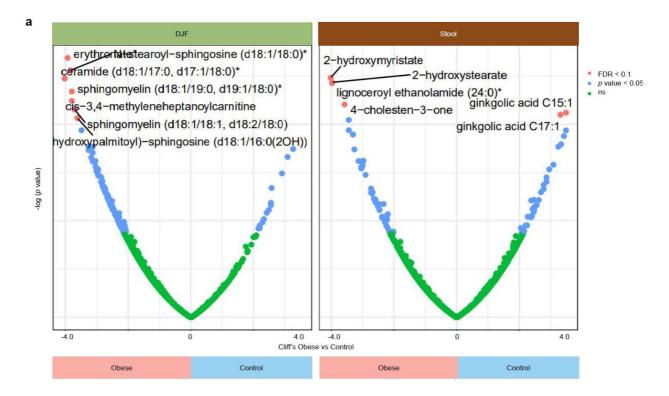


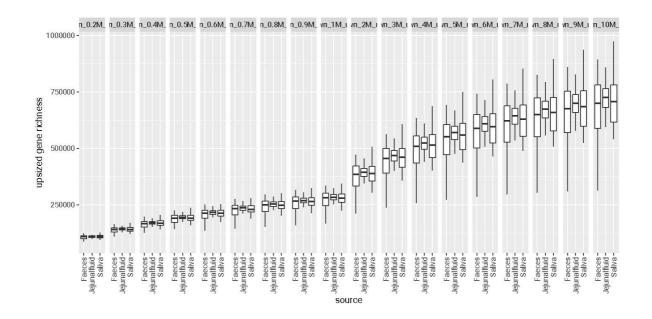
Figure R.II.5 - <u>Obesity-associated metabolome signature of the upper small intestinal</u> <u>microbiome obesity.</u>

a) Association between metabolites and groups within each ecosystem. These Vulcano plots show the Cliff's Delta effect size analysis between metabolites and the participant's group within each ecosystem: the DJF (in green) and stool (in brown). *P values* are represented on the y-axis, and associations with the cohort group on the y-axis (Ctrl in blue, Ob in red). Each dot represents a metabolite and is colour-coded in regard to the level of significance of the association (green: not significant - ns; blue: p < 0.05; red: FDR < 0.1).

In conclusion, obesity associates with a non-significant trend towards higher gene richness in the USIM and further analyses revealed that there is a higher abundance of bacterial species that associate with obesity in the USIM compared to the number of species augmented in the control group. This is in opposition to the results from FM in the current study. Metabolomics analyses also revealed that, although fewer metabolites were identified in the USIM compared to stools, there is a higher number of altered metabolites between the control group and the obese group in the USIM and that the great majority of altered metabolites in the USIM was increased in obesity. Finally, we showed that two bacterial species were altered between groups: *Actinomyces sp* S6 Spd3 and *Neisseria subflava*.

Additional results: *de novo* Assembly of a Metagenomic Catalogue Based on our Cohort

In our study, the duodenojejunal fluid microbiome had a lower metagenomic richness than the saliva and stool microbiomes with the MetaPhlan4 catalogue. However, preliminary results from *de novo* assembly of our catalogue suggest that metagenomic richness might be underestimated with the available catalogues. Indeed, we found that USIM gene richness was not different from the other ecosystem or even higher at higher sequencing depth (Figure R.II.5).



<u>Figure R.II.5 - *de novo* metagenome assembly of our own catalogue.</u> This figure shows the metagenomic richness for OM (saliva), USIM (jejunal fluid) and FM (faeces) at different upsized sequencing depths obtained from *de novo* assembly catalogue. This result is preliminary, and the assembly is still being developed by Dr. Eugeni Belda.

Gastroenterology

Comparative Analysis of the Duodenojejunal Microbiome with the oral and fecal microbiome unveils its role in Human Severe Obesity --Manuscript Draft--

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Dear Editors in chief, dear Dr Elena Verdu,

We are pleased to submit our letter entitled "*Comparative Analysis of the Duodenojejunal Microbiome with the oral and fecal microbiome unveils its role in Human Severe Obesity.*" for your consideration in Gastroenterology. The paper has not been previously published and is not under consideration for publication elsewhere.

The gut microbiota is recognized as a key player in the regulation of host metabolism, through direct effects on metabolism and through modulation of systemic and tissue inflammation, which in turn affects energy metabolism. However, our knowledge of the small intestinal microbiota is scarce compared to that of the faecal microbiota, despite the small intestine being the major site of nutrient absorption and enteroendocrine hormone secretion.

Therefore, we conducted a clinical investigation to explore this aspect and here we present an exploration of the intricacies of the gastrointestinal microbiome, using comprehensive whole metagenomic analyses across three distinct niches. We indeed recruited subjects with or without obesity, analyzed their oral, duodenojejunal and faecal microbiota and examined the duodenojejunal metabolome. Of note, we meticulously phenotyped these individuals with clinical and anthropometric variables, lifestyle questionnaires and dietary records. Our study reveals distinct compositional and functional aspects of the salivary, duodenojejunal and faecal ecosystems and we reveal that the microbiota of the small intestine is more closely associated with host clinical variables than the microbiota of distal segments of the digestive tract in the context of metabolic health. In addition, our research reveals the potential influence of two upper small intestinal taxa on metabolic health, with significant associations identified in glucose control, inflammation and body composition. We also show that the metabolome in the duodenojejunal lumen is altered in obesity, with an increase in sphingolipids.

Recent studies have documented the composition of the microbiota and the metabolome of the small intestine in a healthy human population. Herein, we document changes in the metagenome and metabolome associated with obesity phenotypes, that has never been done to our knowledge.

Thus, we believe that our findings, with data presented in a concise format, have significant implications for the field of gastroenterology and are likely to make a significant contribution to the scientific community. We anticipate that our letter will receive widespread attention and be highly cited due to its original and compelling results.







We sincerely appreciate your consideration of our submission and eagerly await your valuable feedback.

Thank you for your time and commitment to advancing scientific knowledge in the field.

Sincerely,

Prof. Karine Clément



Title page

Comparative Analysis of the Duodenojejunal Microbiome with the oral and fecal microbiome unveils its role in Human Severe Obesity.

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Conflict of interest statement

The authors declare that they have no competing interests that could appear to influence the content of this manuscript.

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Data Transparency Statement

Study data not presented within this article are available on request.

Abbreviations

ALAT, Alanine Transaminase
ASAT, Aspartate Transaminase
BMI, Body Mass Index
DJF, DuodenoJejunal Fluid
FM, Faecal Microbiota
GIT, GastroIntestinal Tract
HOMA-IR, Homeostatic Model Assessment for Insulin Resistance
NOB, Non-Obese Group
Ob, Obese group
OM, Oral Microbiota

USI, Upper Small Intestine

USIM, Upper Small Intestinal Microbiota

Main text

The gut microbiome (GM) plays a pivotal role in the development and maintenance of obesity, however previous research primarily focused on the fecal microbiome (FM). While these studies have provided valuable insights into the compositional and functional changes associated with obesity¹, the FM represents only a fraction of the gastrointestinal tract (GIT) microbiome. Particularly, the upper small intestine (USI) is of great interest due to its crucial functions in food digestion, nutrient sensing, absorption, enterohormone production, and metabolic homeostasis^{2,3}.

Distinct physicochemical conditions exist in each segment of the GIT, shaping microbial ecosystems⁴. Consequently, a comprehensive exploration of the GIT microbiome beyond the FM is warranted. While studies in rodents demonstrated the causal influence of the USI microbiome (USIM) on metabolic regulation, clinical investigations concerning the USIM in human obesity, remain limited and contradictory³. There is a pressing need for further research to elucidate the intricate interplay between lifestyle, the USIM, and metabolic health in humans^{2, 3}.

We investigated the USIM and associated metabolome in thoroughly characterized participants with (n=15, OB) or without (n=15, NOB) obesity matched for age and sex (Supplemental Table S1). We compared the duodenojejunal fluid (DJF) microbiome aspirated through gastroscopy at the Treitz Angle with the oral microbiome (OM) and FM. Our aim was to uncover the specificities between these microbiomes. Additionally, we conducted statistical analyses to explore potential associations between these microbiomes and participants' lifestyles and clinical phenotypes.

Metagenomic analyses showed that the USIM displayed a lower diversity than the OM and the FM (Supplemental Figure S1A) and was highly similar to the OM (Supplemental Figure S1B). This was confirmed by analyzing the distribution of dissimilarities between ecosystems, where dissimilarities between USIM and OM samples was significantly lower than the dissimilarities of both ecosystems vs. fecal samples (Supplemental Figure S1C). This was further confirmed with a Cliff's Delta analysis at the species level showing less species being significantly altered between the OM and USIM compared to the FM (Supplemental Figure S1D). Finally, the prevalence of species altered between the ecosystems showed the presence of

aerobic species belonging to the *Streptococcaceae*, *Veillonellaceae*, and *Prevotellaceae* families in the OM and USIM and the prevalence of strict anaerobes belonging to the *Lachnospiraceae* and *Ruminococcaceae* families in the FM (Supplementary Figure S1E).

Comparing within each ecosystem, the groups with or without obesity, we found that the relative abundance *Neisseriaceae* was lower in the USIM of the OB group (Figure 1A). In addition, the USIM in the OB group displayed higher metagenomic richness (Figure 1B) while the other ecosystems richness was similar in NOB and OB group. Coherently, we observed that body composition variables significantly explained the compositional variance of the USIM with a higher effect size than that observed in the OM and FM (Figure 1C) and correlated positively with metagenomic richness (Figure 1D).

At the species level, 18 species exhibited higher abundance in the OB group USIM, while only three species were more abundant in the NOB group (Figure 1E). Notably, across three ecosystems, 3 species significantly differed between groups after correction for multiple comparisons. In the USIM, *Actinomyces sp* S6-Spd3 was more abundant in participants with obesity, while *Neisseria subflava* was more abundant in the NOB group. In stools, *Ruminococcus lactaris* was more abundant in the NOB group. In stools, *Ruminococcus lactaris* was more abundant in the NOB group. To determine if USIM alterations in obesity are associated to metabolome changes, we performed a non-targeted metabolome analysis of the DJF and stools, revealing that several lipids and particularly sphingolipids, were increased in the USI of the OB group (Figure 1F). Coherently with the microbiome alterations with obesity within each ecosystem, the USI metabolome variance was more affected by body composition variables than the fecal metabolome (Figure 1C).

Association analyses showed that *Actinomyces sp* S6-Spd3 exhibited positive associations with BMI, fat percentage, visceral fat, along with higher circulating levels of Leptin, Gamma-glutamyl Transferase, and Serum-Amyloid A (Figure 1G). It also displayed a negative association with the circulating levels of gastric-inhibitory-polypeptide. Conversely, *N. subflava* showed negative correlations with corpulence and body composition variables and circulating markers such as glycemia, HbA1c, triglycerides, and inflammatory markers. Additionally, it exhibited positive associations

with circulating levels of ghrelin, HDL-cholesterol, adiponectin, and the ASAT/ALAT ratio, as well as lifestyle factors like wine consumption and the Alcohol-Use-Disorder Test score. Further adjusted association analysis highlighted *N. subflava*'s association with BMI even after controlling for alcohol consumption–(Figure 1D), suggesting a relationship between *N. subflava* and leanness while considering potential confounding factors.

Our findings support existing studies indicating the similarity between the oral microbiome and the upper small intestine microbiome^{5,6}. This contradicts a recent metagenomic analysis across the digestive tract⁴, which reported that the USIM, captured using ingestible capsules, is more akin to the fecal microbiome⁴. These discrepancies may be attributed to several factors, in particular differences in sampling location and variances in collection techniques between studies. Here, we precisely sampled DJF aspirate at the Treitz Angle, while Shalon and colleagues used capsules that may have collected luminal fluid at a less precise and presumably more distal location. In our study, DJF was immediately snap-frozen after sampling, whereas the luminal fluid in the capsules underwent an incubation period until defecation of the capsule. We cannot exclude that the enclosed USIM does not undergo compositional changes during this period-

Previous research has shown reduced diversity in the FM in obesity^{7,8}. However, our study indicates the opposite trend in the USIM, suggesting that increased richness is associated with obesity in the upper small intestine. Another report shows an elevated bacterial count in the duodenal mucosa-associated microbiome of hyperglycemic compared to normoglycemic individuals⁹. Further replications in larger cohorts are needed to establish associations between this newly explored microbiome and metabolic health.

Our study, first of its kind, employed whole metagenomic analyses across three digestive tract niches within a meticulously phenotyped cohort, including individuals with obesity and non-obese controls. This work opens avenues for investigating the potential causal impact of two upper small intestinal taxa on metabolic health, particularly in relation to glucose control, inflammation, and body composition.

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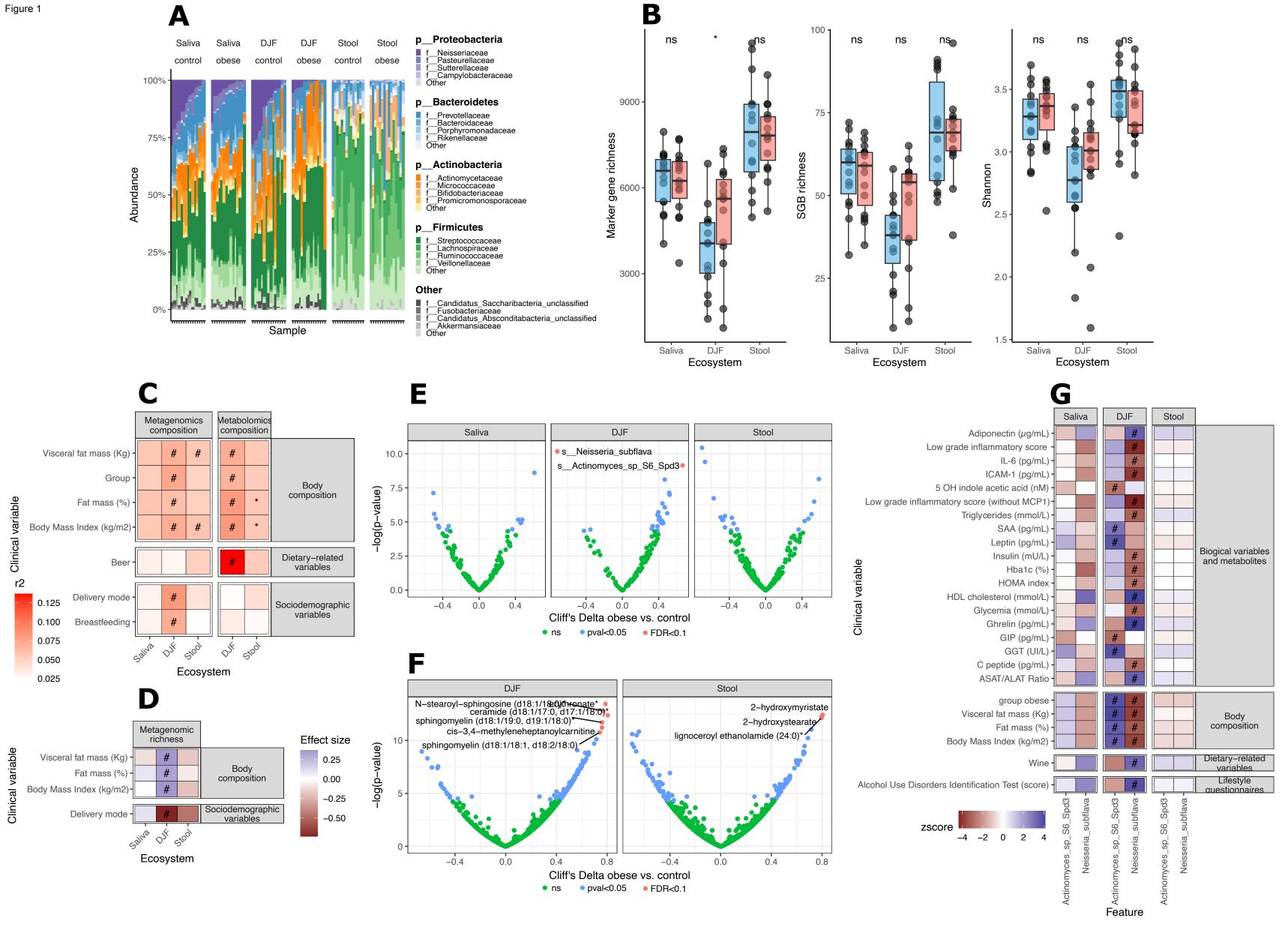
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Figure legend

Alterations of the oral, duodenojejunal microbiome and metabolome in obesity and their association with lifestyle and clinical phenotype.

(A) Relative abundance (%, y-axis) of bacterial Phyla and families per ecosystem (OM: blue, USIM: green, FM: brown), stratified per group (NOB: group: blue; OB: in red). The different Phyla are listed in bold and color-coded, and bacterial families are colored in different shades within each Phyla. Each column represents a participant. P-values result from between-group comparisons using the Wilcoxon test. (B) Metagenomic richness at the marker gene level (left panel), the species genome bin level (center) and the Shannon index (right panel; y-axis) in each ecosystem (x-axis), stratified per group (NOB group: blue; OB group: red). P-values result from betweengroup comparisons using the Wilcoxon test. Legend: *p < 0.05. (C) Proportion of compositional variance explained by different clinical or environmental variables. The Permutational Multivariate Analysis of Variance using the Adonis function was computed from the Bray-Curtis dissimilarity matrix for metagenomic data and the Euclidian distances for metabolomics data. The effect size of the determination is colour-coded from white (R2 = 0%) to red (R2 = 15%). Legend: # : p < 0.05 and FDR < 0.05. (D) Association analysis between metagenomic richness and lifestyle and clinical variables. This heatmap shows the association between metagenomic richness and lifestyle and clinical variables (listed on the left) in each ecosystem (listed at the bottom) using either Spearman's rank correlation coefficient, W test or Kruskal-

 Wallis test. Positive associations are coloured in blue, while negative values are coloured in red. Legend: Duodenojejunal fluid $(DJF)^* p < 0.05$; # FDR < 0.1. (E) Cliff's Delta effect-size analysis between bacterial species and participant's group. P-values are represented on the y-axis, and species' association to cohort group on the y-axis (NOB: blue; OB: red). Each dot represents a species and is color-coded in regard to the level of significance of the association (green: not significant; blue: p < 0.05; red: FDR < 0.1). (F) Association between metabolites and groups within each ecosystem. These Volcano plots show the Cliff's Delta effect size analysis between metabolites and the participant's group within each ecosystem: the DJF (in green) and stool (in brown). P values are represented on the y-axis, and associations with the cohort group on the y-axis (Ctrl in blue, Ob in red). Each dot represents a metabolite and is colourcoded in regard to the level of significance of the association (green: not significant ns; blue: p < 0.05; red: FDR < 0.1). (G) Association analysis between Actinomyces sp S6-Spd3 and Neisseria subflava and clinical and lifestyle variables in the whole cohort of 30 participants (red: negative association; blue: positive association). Legend: # p < 0.05 and FDR < 0.05.



1

- 2 Comparative Analysis of the Duodenojejunal Microbiome with the oral and fecal
- 3 microbiome unveils its role in Human Severe Obesity.
- 4 Emilie STEINBACH¹, Eugeni BELDA², Rohia ALILI¹, Jejunal Luminal and Colonic Mucosa-
- 5 Associated Microbiota in Metabolic Diseases (Je/Col-MiMe) Group, Benoit CHASSAING³,
- 6 Tiphaine LE ROY^{1*}, Karine CLEMENT^{1,4*}.

7 Abbreviations

- 8 ALAT, Alanine Transaminase
- 9 ASAT, Aspartate Transaminase
- 10 BMI, Body Mass Index
- 11 DJF, DuodenoJejunal Fluid
- 12 HDL, High Density Lipoprotein
- 13 IL-6/8, Interleukin 6/8
- 14 Je-MiMe, Jejunal Microbiota in Metabolic Diseases
- 15 LDL, Low Density Lipoprotein
- 16 MCP-1, Monocyte chemoattractant protein-1
- 17 TNFα, Tumor Necrosis Factor (alpha)
- 18 NOB, Non-Obese Group
- 19 OB, Obese group
- 20
- 21 Supplementary material and methods

22 **Population**

The Je-MiMe study, conducted at Hôpital-Privé des Peupliers, Ramsay-Santé, Paris, France, is an observational study. Prior to inclusion, informed written consent was obtained from all participants. The study adhered to the Helsinki Declaration and received approval from the local ethics committee (CPP IIe de France 8; approval number: 210648).

- 27 The study comprised 30 participants categorized into two groups: the **Non-Obese Group**
- (NOB): <u>composed</u> of individuals without obesity or known metabolic disorders for which
 gastroscopy was scheduled due to minor epigastralgia that did not necessitate medication.
- 30 ; **the Obesity Group (OB):** composed of candidates for bariatric surgery for which 31 gastroscopy was a prerequisite procedure.
- 32

The Je-MiMe study employed specific inclusion and exclusion criteria to select eligible participants and are listed on the Clinical Trial.gov website (NCT05186389).

35 Clinical and lifestyle data:

36 Clinical and lifestyle data were collected through online questionnaires. In addition to a 37 general medical questionnaire, various standardized questionnaires were used to evaluate 38 alcohol consumption (Alcohol Use Disorders Identification Test), nicotine dependence 39 (Fagerström Questionnaire), perceived stress (Perceived Stress Scale), anxiety (Hospital 40 Anxiety Depression Scale, only anxiety-items), depression (Beck Depression Inventory), 41 circadian rhythm (Horne and Ostberg Questionnaire), eating behavior (Dutch Eating 42 Behavior Questionnaire), and nutrition (Food Frequency Questionnaire).

Anthropometric measurements and body composition were measured (MC-780MA P,
Tanita, Amsterdam, The Netherlands). To avoid redundancy, the results only display a
subset of body composition and corpulence variables (fat mass %, visceral fat rating and %,
and BMI).

Except for stools, all samples were collected fasting before the gastroscopy, early in the morning. Participants had been fasting for at least 8 hours.

Girculating markers related to glucose metabolism (fasting glycemia, insulin, Hba1c), lipid profile (total cholesterol, LDL, HDL, triglycerides), liver function (aspartate transaminase -ASAT, alanine transaminase - ALAT, gamma-glutamyl transferase and alkaline phosphatase), thyroid function (ultra-sensitive measurement of thyroid-stimulating hormone), and inflammation (C-reactive protein) were measured (Alinity-Abbott; Cerballiance, Paris).

55 Quantification of circulating levels of Amylin, C-Peptide, Ghrelin, gastric-inhibitory-56 polypeptide, Glucagon-like Peptide-1, Glucagon, Interleukin-6 (IL-6), Insulin, Leptin, 57 Monocyte-chemoattractant protein-1 (MCP-1), Pancreatic Polypeptide, Peptide-YY, 58 Secretin, and tumor necrosis factor-alpha (TNFα) was performed on serum mixed with 59 dipeptidyl peptidase-4 inhibitors and protease inhibitors (MILLIPLEX® Human Metabolic 60 Hormone Panel V3, Millipore).

Quantification of inflammatory cytokines IL-6 and -8, C-reactive protein, serum-amyloid A,
 MCP-1, and TNFα were measured on serum (Meso-Scale Discovery's ultra-sensitive
 assay). Subsequently, a cumulative score of low-grade inflammation (Zscore) was
 calculated following the previously described methodology¹.

Guantitative determination of human High Molecular Weight Adiponectin(Human HMW Adiponectin/Acrp30 Immunoassay), human Growth Differentiation Factor-15, and human Fibroblast growth factor-21 were performed on serum (QuantikineTM, ELISA). Tryptophan metabolites were quantified through liquid chromatography coupled with high-resolution mass spectrometry from serum, as previously described².

70 Saliva sampling:

Participants were asked not to brush their teeth in the morning before saliva sampling, and they had been fasting for at least 8 hours. After collection, it was transported and aliquoted on ice and stored within two hours at -80°C.

74 **Duodenojejunal fluid sampling:**

After saliva sampling, participants thoroughly brushed their mouth and teeth to prevent (as much as possible) DJF contamination from oral cavities with a higher bacterial load³. Then, endoscopy was performed. The endoscope was washed in the stomach then DJF was aspirated between the second segment of the duodenum and 10 cm distal to the at the Treitz Angle and collected in a sterile tube. DJF was immediately aliquoted and placed within five minutes after sampling on dry ice, then stored at -80°C.

81 Stools sampling:

Total fresh stools were collected in a hermetic container at the patient's home. When the sample was collected, participants placed an anaerocult (bioMérieux, Paris, France) on the stools and hermetically closed the box. The sample was transported and aliquoted on ice in an anaerobic hood within two hours for different analyses, then stored at -80°C.

86 **Metabolomics**:

Untargeted metabolomics was performed using Ultrahigh Performance Liquid
Chromatography-Tandem Mass Spectroscopy on duodenojejunal fluid and stool
(Metabolon, Durham, North Carolina, United States).

90 Metagenomics:

Bacterial DNA extraction from saliva, DJF, and homogenized feces was performed using
NucleoMag DNA Microbiome kit (Macherey-Nagel, Vertrieb Gmbh & Co.Kg). Two cycles of
chemical- and mechanical-lysis were performed (Precellys[®], Bertin Technologies,
Montigny-le-Bretonneux, France). We also used an automated robot for DNA extraction and

purification using paramagnetic beads (Auto-Pure96, Nucleic Acid Purification System
 Hangzhou Allsheng Instruments CO., Ltd. Hangzhou, Zhejiang, China). Purity ratio and DNA
 quantity were controlled (NanoDrop and Qubit, ThermoFisher).

98 DNA was physically sheared to approximately 250-550 bp through then purified (QIAquick 99 Purification kit, Qiagen, Hilden, Germany). Library preparation for sequencing was 100 performed using the Invitrogen ColibriTM PS DNA Library Prep Kit for IlluminaTM 101 (ThermoFisher Scientific, Waltham, Massachusetts, United States). PCR amplification of the 102 purified adaptor-ligated DNA library was performed, followed by a third purification of the 103 amplified DNA library using reagents included in the Colibri kit. Sequencing was performed 104 with NextSeq 2000 (P2 300 cycles: 2x150 bp).

Metagenomic analyses were performed using The bioBakery tools⁴ for read-level quality control (KneadData, default settings), taxonomic profiling (MetaPhlAn4-catalog⁵ vs *mpa_vJan21_CHOCOPhlAnSGB_202103* reference database). To correct for variations in sequencing depth, Metaphlan4 normalized marker gene abundances (reads per kilobase, RPK) were divided by metagenome size (quality-filtered non-human read pairs) before robust average calculation of SGB abundances (0.2 default quantile value).

vegan v2.6.4 R package were used for ecological analyses of metagenomic profiles. Non-111 parametric statistics (Kruskal-Wallis tests followed by post-hoc pairwise Dunn tests for 112 113 ecosystem-comparisons; Wilcoxon rank-sum tests for group comparisons) were used to identify taxonomic and metabolomic features associated to different ecosystems and clinical 114 115 groups. Only features present in >20% of the samples were retained for analyses. P-values 116 derived from KW tests were corrected for multiple testing using the Benjamini-Hochberg method (Padi), only Padi<0.05 were reported as significant. Linear regression analyses 117 were used to evaluate the association of taxonomic and metabolomic markers with clinical 118 119 covariates unadjusted and adjusted by alcohol intake. All analyses were done on R v4.2.2.

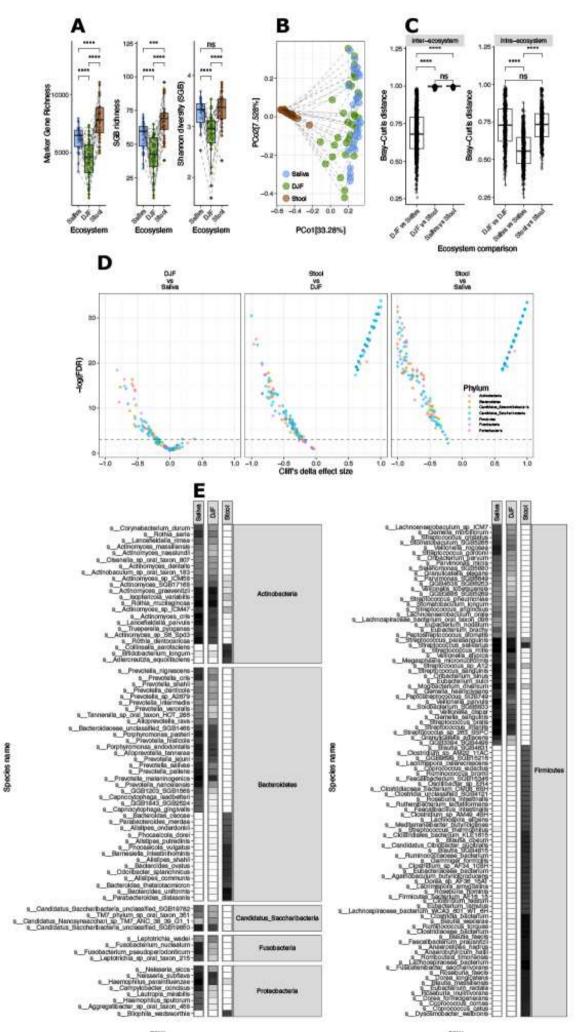
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LDL cholesterol (mmol/L) 2.5 (0.7) 3.1 (0.7) * Liver Enzymes * * * ASAT (U/L) 27.7 (6.9) 23.9 (5.1) ns ALAT (U/L) 28.3 (13.3) 34.3 (21.5) ns ASAL/ALAT 1.06 (0,22) 0.82 (0,26) ** GGT (U/L) 18.1 (7.7) 42.1 (23.3) *** Inflammation marker *** *** *** C-Reactive Protein (mg/L) 1.2 (0.4) 5.4 (4.2) *** Mutritional parameters *** *** *** Alcohol (%) 4.46 [2.53;5.38] 0.11 [0.01;0.98] **** Carbohydrates (%) 39.9 [36.1;43.0] 44.3 [41.6;46.8] * Proteins (%) 17.2 [16.5;19.3] 17.6 [15.0;19.8] ns Lipids (%) 37.2 [34.9;41.4] 38.1 [35.0;41.5] ns Diet quality scores *** *** aHE1% 52.4 [43.5;62.8] 31.0 [20.9;37.4] *** Food groups (Daily intake) 50.8 [51.0;64.8] 48.4 [41.8;56.3] *** <td>HDL cholesterol (mmol/L)</td> <td>1.6 (0.4)</td> <td>1.1 (0.2)</td> <td>***</td>	HDL cholesterol (mmol/L)	1.6 (0.4)	1.1 (0.2)	***	
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Nuts (g) 3.24 [0.81;9.94] 0.00 [0.00;1.62] ** Pulses (g) 21.4 [16.1;65.9] 10.7 [0.00;21.4] ** Sweetened beverages (mL) 0.00 [0.00;21.0] 71.0 [0.00;176] ** Processed meat (g) 3.89 [0.00;6.29] 19.1 [7.13;41.6] * Vegetables (g) 271 [243;424] 202 [135;276] *					
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	Processed meat (g)	3.89 [0.00;6.29]	19.1 [7.13;41.6]	*	
	Vegetables (g)	271 [243;424]	202 [135;276]	*	
	Oily fish (g)	8.83 [4.41;18.0]	4.41 [3.31;5.52]	ns	

127 Supplemental table 1. Characteristics of the Study Participants

128 This descriptive table summarizes the major clinical parameters; the scores obtained from various questionnaires; and some results from the Food Frequency Questionnaire of the Je-129 130 MiMe Cohort. Results are expressed as mean (SD) or median [min;max] for continuous data 131 and n (%) for categorical data. Alcohol consumption was evaluated by the Alcohol Use 132 Disorders Identification Test (AUDIT): Nicotine dependence by the Fagerström Test; Quality of Life and Depression by the Patient Health Questionnaire (PHQ-9): Perceived stress by 133 134 the Perceived Stress Scale (PSS-10): Anxiety by the Hospital Anxiety and Depression Scale (HAD, "A" items only); Depression with The Beck Depression Inventory (BDI); Chronotype 135 or 'circadian rhythm' by the Horne and Ostberg Questionnaire; Eating Behaviour was 136 137 assessed with the Dutch Eating Behaviour Questionnaire (DEBQ). When available, the corresponding score interpretation is mentioned below the scores. P values result from the 138 139 Mann-Whitney-Wilcoxon non-parametric test (W test). Legend: BMI: Body Mass Index; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance. ASAT: aspartate 140 transaminase; ALAT: alanine transaminase; GGT: gamma glutamyl-transpeptidase; aHEI 141 (Alternate Healthy Eating Index) and the DASH (Dietary Approaches to Stop Hypertension) 142 scores. ns P-value >0.05; * P-value ≤ 0.05; ** P-value ≤ 0.01; *** P-value ≤ 0.001; **** P-143 value ≤ 0.0001. 144



Supplemental Figure S1. <u>Microbiome patterns across three ecosystems of the digestive</u> <u>tract.</u>

(A) Metagenomic richness across the three ecosystems. This boxplot shows the metagenomic richness at the marker gene level (left panel), the species genome bin level (center panel) and Shannon diversity computed at the species genome bin level (right panel: y-axis) of each ecosystem (x-axis): saliva (OM, in blue), duodenojejunal fluid (USIM, in green) and stool (FM, in brown). P-values result from Wilcoxon tests. Legend: **** p<0.0001. (B) Dissimilarity matrix between the three ecosystems. This principal component analysis computed at the species genome bin level shows the distance between each sample (represented by a dot coloured in their corresponding ecosystem) in each ecosystem: saliva (in blue), duodenojejunal fluid (in green) and stool (in brown). Samples from the same patient are connected with a dotted line (PCo2). (C) Inter-ecosystem (left panel) and intra-ecosystem (right panel) bray-curtis dissimilarity index computed at the species genome bin level between pairs of ecosystems (left) and within each ecosystem (right). (D) Volcano plot of Cliff's delta association analysis between species and each ecosystem. Each dot represents a species, coloured in their corresponding Phylum. Statistical significance is displayed on the y-axis, and the horizontal dotted line indicates the p < 0.05 threshold. Cliff's delta is displayed on the x-axis. (E) Species prevalence across the three ecosystems. These heatmaps show the presence-absence (grey-shaded heatmap) of species across ecosystems. Phyla are colour-coded using the same legend as panel 1c of this figure. This graph only displays species significantly altered between ecosystems (Kruskall-Wallis: p<0.001). Abbreviations: duodenojejunal fluid (DJF) Fusobacteria (Fuso.) Candidatus Absconditabacteria (C.A.) Candidatus Saccharibacteria (Can. Sacc.).

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DISCUSSION

Throughout my doctoral thesis, I investigated the USIM in human obesity and its links with obesity-related metabolic and inflammatory traits. The hypothesis was that the USIM, similar to the FM, undergoes notable alterations in the setting of obesity and that, as it is the main site of food digestion, nutrient sensing and absorption, the USIM may eventually exhibit stronger associations with metabolic health compared to FM.

To assess our scientific goals, I conducted a clinical exploration in subjects with or without obesity from whom different microbial ecosystems were explored (duodenojejunal fluid, stool and saliva) to explore the association between metagenomic or metabolomic information and clinical variables.

The main results were the following: we found that the proximal small intestinal microbiome has a lower gene richness compared to the oral and faecal microbiomes. In terms of composition, the USIM was more similar to the OM than the FM, and it showed high compositional and metagenomic richness variability, which was explained by fat mass accumulation. We thus stratified the following analyses per group: the obese group versus the control group. These comparisons enabled the identification of two altered taxa between groups.

Firstly, we showed that the USIM is remarkably similar in composition to the OM compared to the FM, corroborating existing literature. Multiple studies have indicated that the first GIT segments are populated by bacteria initially identified from oral cavities (Chen et al., 2020b, 2020a; Villmones et al., 2022). Human adults swallow approximately 0.5 to 1.5 litres of saliva daily, containing around 10¹² bacteria (Humphrey and Williamson, 2001; Iorgulescu, 2009). This implies a significant influence of saliva on shaping the proximal USIM composition despite the numerous physical and chemical barriers these bacteria must navigate before colonising subsequent sections of the gastrointestinal tract, such as gastric and BAs (di Gregorio et al., 2021; Martinsen et al., 2005).

The stomach, until Robin Warren and Barry Marshall discovered *H. pylori* in 1982 (Ahmed, 2005; Dunn et al., 1997) was assumed to be 'sterile'. Subsequent research has indicated that, notwithstanding the deterrents of gastric pH and peristalsis, the stomach hosts genera found in saliva, such as *Prevotella, Streptococcus, Veillonella, Rothia*, and

Haemophilus (Nardone and Compare, 2015), with concentrations ranging from $10^3 - 10^4$ cfu/mL (Berg, 1996; Delbaere et al., 2023).

Additionally, bacteria known to inhabit oral cavities are also detected further in the duodenum. Sequencing analysis of 21 paired duodenum-saliva samples in healthy individuals showed that about 89% of bacterial taxa in the saliva samples were also found in corresponding duodenum samples (Barlow et al., 2021). Our study sampled the USIM at the Angle of Treitz, the anatomical divide between the duodenum and jejunum, and findings are in line with previous research indicating that duodenal and proximal jejunal microbiomes are primarily inhabited by the *Streptococcus* genus, followed by *Prevotella, Fusobacterium, Veillonella* and *Haemophilus* (Di Pilato et al., 2016; Nagasue et al., 2022; Sundin et al., 2017; Villmones et al., 2022).

Past research has demonstrated a likeness between the mouth and proximal SI microbiomes, with a composition differing from the FM. However, a recent study published in Nature (Shalon et al., 2023) contradicts these findings, including our still unpublished work. In our research, duodenojejunal fluid was aspirated during endoscopy and immediately snap-frozen in the surgery unit. In contradiction, Shalon and colleagues (2023) used small ingested capsules to capture the GM along the digestive tract and found all small intestinal samples more similar to stool than oral microbiome.

I question if using these capsules to collect the microbiome truly represents the sampled ecosystem. The authors claimed that there were no alterations to the captured microbiome. However, the results are not presented in their paper. These capsules must navigate through the GIT, be recovered in stools, and then transported, stored and frozen in the lab. It cannot be excluded that the enclosed microbiome and metabolome change during the 24 to 48 hours of incubation at approximately 37°C in the human GIT and that the ecosystem could be altered during this time. Bacterial action might slowly reduce oxygen and transform nutrients and other metabolites. These changing conditions might potentially promote the overgrowth of strict anaerobes, shifting the original duodenojejunal microbiome. These modifications could be particularly significant as the USIM, having a lower bacterial load compared to stool, might be more susceptible to alterations.

In our study, the duodenojejunal fluid microbiome had a lower metagenomic richness than the saliva and stool microbiomes, which aligns with previous results indicating the lowest alpha diversity in the USIM compared to other GIT microbiomes (Kashiwagi et al., 2020; Seekatz et al., 2019; Sundin et al., 2017; Vuik et al., 2019). However, we believe these findings could reflect this ecosystem's current lack of understanding and characterisation. These results may shift with the development of extended gene catalogues specific to the USIM. Eugeni Belda, a researcher in our lab, is currently working on the *de novo* assembly of the USIM, and our preliminary results showed that metagenomic richness might be underestimated with the available catalogues as unidentified taxa are removed from the analyses pipeline.

As reviewed elsewhere (Steinbach, Masi et al. submitted in *Metabolism*), USIM results vary between luminal and mucosa-associated microbiomes sampled in the same GIT segment. This variation is attributed to factors such as oxygen gradient changes: while the USI lumen is microaerophilic, the highly vascularised mucosa diffuses oxygen, thus altering the resident microbiome (Espey, 2013). Here, we report results from the luminal microbiome of the upper intestinal tract. It could be interesting in a following study to also investigate the mucosal-associated microbiome, which could, as it is in a closer relationship with both mucus and intestinal mucosa, and due to its potential to inhabit the mucus (Birchenough et al., 2023) with less fluctuations over time, be even more associated to metabolic health.

The initial findings revealed a greater inter-individual variability in the microbiome and metabolome across the digestive tract, specifically in the USIM, compared to the other two ecosystems. This variability encompassed both metagenomic gene richness and microbiome composition. Consequently, subsequent analyses aimed to identify the clinical or lifestyle factors that could explain the variance in gene richness and GM composition.

The variance analysis demonstrated that all variables related to body composition, such as the percentage of fat mass accumulation, significantly accounted for the compositional variability observed in the USIM. These variables were also positively associated with the metagenomic richness of the USIM, whereas no such relationship was observed in the OM and FM in our small group of subjects. As a result, we subsequently stratified the following analyses by groups and focused on uncovering the distinctions between the obese and the control groups.

A non significant trend towards a higher metagenomic richness in the USIM was observed in the group with obesity when contrasted with the control group, however this difference was not statistically significant. More interestingly, we showed an augmentation of the number of species found to be associated with obesity in the proximal intestine compared to those associated with the control group. This is the opposite in stool, where obesity associates with lower metagenomic richness (Cotillard et al., 2013; Le Chatelier et al., 2013). In prior studies examining USIM metagenomic gene richness in cohorts comparing a metabolically healthy group with an obese group, results were contradictory and lacked statistical significance. These results were discussed in the literature review inserted further in the introduction (Steinbach, Masi et al., submitted to *Metabolism*).

It is possible that in obesity, an interplay of various factors, such as lifestyle and particularly dietary changes, local inflammation and altered gut permeability, low-grade inflammation or other factors such as the alterations of USIM could alter the host's normal physiologic response including the functions of the mucus, the mucosal immune system, bile acids or antimicrobial peptides (AMPs) production. A 'lack of control' from the host on the USIM could account for the trend towards higher metagenomic richness we observed in the proximal intestine in obesity.

For example, a study found that a diet rich in fermentable fibres, increased the expression of Reg3 γ , an antimicrobial peptide and suggested the contribution of this peptide in the regulation of mucus, the microbiome and gut permeability in the proximal intestine (Shin et al., 2022). Another study found, in contradiction, that a Western-style diet could alter USI mucus integrity and disrupt its associated microbiome and colonisation resistance. The authors speculated that such alterations could promote small intestinal bacterial overgrowth (Birchenough et al., 2023).

Subsequent studies should examine if there is an alteration of metagenomic richness in obesity in the USIM. We should not draw conclusions and extrapolations yet as this result is not significant..

Subsequent analyses revealed two altered taxa between groups and only in the USIM: *N. subflava* and *Actinomyces sp* S6 Spd3.

Inter-group comparisons also revealed a heightened relative abundance of *Actinomyces sp* S6 Spd3, an Actinomycetota (previously Actinobacteria; <u>Oren and Garrity</u>, 2021) in the group with obesity compared to the control group. This taxon was negatively associated with the enterohormone GIP and positively associated with circulating GGT, Leptin, SAA (a marker of acute inflammation) and all body composition variables.

To our knowledge, *Actinomyces sp* S6 Spd3 has not been previously associated with obesity in the scientific literature. One investigation noted that the oropharyngeal abundance of *Actinomyces sp* S6 Spd3 correlated with COVID-19 patients developing the requirement for respiratory assistance, indicating COVID-19 severity (Bradley et al., 2022). Additionally, another study discerned that the faecal abundance of *Actinomyces sp* S6 Spd3, upon admission, was positively correlated with extended clinical manifestations of COVID-19 at six months post-recovery (Liu et al., 2022; Zhou et al., 2023). These studies did not report adjusting the association on participants' weight, although obesity has also been associated with COVID-19 severity. It is thus possible that this taxon associates with adiposity in this research. This gram-negative taxon could, for example, play a role in low-grade inflammation and aggravate health issues. However, there might be a confounding effect of the participant's AT accumulation might have a confounding effect on the need for respiratory support.

The second taxa, *N. subflava*, a species within the Proteobacteria phylum, was markedly higher in the control group, thus accounting for a portion of the Proteobacteria augmentation observed in this group. This species is a gram-negative known to colonise the human body, predominantly the oral cavity, upper gastrointestinal tract, and respiratory system (Hadfield and David, 2016). It was negatively associated with various circulating markers from the lipid profile, glucose control or inflammatory markers and all body composition and corpulence variables. On the other hand, this taxa showed positive association with the circulating levels of ghrelin, HDL-cholesterol, adiponectin, the ASAT / ALAT ratio, wine consumption and the score to the alcohol use disorder test.

Contrary to *Neisseria meningitis* and *Neisseria gonorrhoeae* (Corless et al., 2001; Unemo et al., 2014), *N. subflava* has rarely been discovered to be pathogenic or virulent when translocated to extraintestinal sites (Amsel and Moulijn, 1996; Baraldès et al., 2000; Bauer et al., 2004; Roberts et al., 2003). To our knowledge, no studies have associated *N. subflava* with obesity. However, other investigations have discovered associations between the oral abundance of this taxon with diabetes, hypertension, hepatic steatosis, bucco-dental health, and dietary fibre intake.

In an exploratory study investigating the correlations between the abundance of nitrate-reducing bacteria in the oral cavity and diabetes, a higher relative abundance of nitrate-reducing bacteria, including *N. subflava*, was associated with lower blood glucose levels and reduced insulin resistance (Goh et al., 2019). Additionally, a case-control study comprising normotensive or hypertensive subjects aged between 50 and 70 years discovered the subgingival relative abundance of *N. subflava* was significantly elevated in normotensive subjects compared with hypertensive patients (Barbadoro et al., 2021).

In the OM of patients with hepatic steatosis, an association was discerned between the abundance of *N. subflava* and alterations in plasma metabolites, although these results were presented in supplementary materials of this paper and are somewhat challenging to interpret (Zeybel et al., 2022). Moreover, *N. subflava* was found to be over-represented in the dental plaque of a 'caries-active' group compared to the 'caries-free' group (Peterson et al., 2013).

Dietary factors were linked to the relative abundance of *N. subflava* in two distinct studies. An observational investigation discovered an elevated relative abundance of *N. subflava* in a 'vegan' group compared to an 'omnivorous' group, which was associated with dietary fibre intake (Hansen et al., 2018). *N. subflava* has been identified as a saccharolytic bacterium capable of metabolising mono- and disaccharides via oxidative processes (Knapp, 1988) and may therefore respond to dietary fibre intake. Finally, a randomised, double-blind dietary intervention found the relative abundance of *N. subflava* in saliva trended towards an increase after four weeks of red beetroot juice consumption compared to placebo (Litwin et al., 2020).

Our control group displayed a non-significant increase on the Fagerström Test for Nicotine Dependence. A poster by S. Chattopadhyay (PHRM 2022) revealed that cigarette users (n=42) showed a significantly higher relative abundance of *N. subflava* compared to a group of 43 non-users, although this work is not yet published.

Our control group comprised participants with minor epigastralgia who were prescribed gastroscopy but who were not needing and had not undergone treatment for these symptoms over the past three months (including PPI and H2RA). In another investigation on 64 patients undergoing a gastric biopsy, the authors reported a positive association between co-infection of *Helicobacter pylori* and *N. subflava* in the human stomach. Additionally, colonisation by *Neisseria species*, particularly *N. subflava* was found to be associated with lymph follicle formation in the stomach (Nakamura et al., 2006). Another study found a co-infection or association between *N. subflava* and *H. pylori*. The researchers discovered that the lipopolysaccharides from *N. subflava* stimulated IL-8 secretion in MKN45 cells via TLR4 activation, suggesting that this species might also contribute to gastric inflammation during chronic *H. pylori* gastritis (Miyata et al., 2019).

Lastly, *N. subflava* was detected in gastric mucosal samples from 288 patients following *H. pylori* eradication. The researchers showed that the bacterium could induce both pro-oncogenic and pro-inflammatory responses in gastric epithelial cells, largely mediated by TLR4 signalling in a manner distinct from that of *H. pylori* (Niikura et al., 2023).

However, we could not confirm the association between *H. Pylori* and *S. subflava*. Indeed, in the present study, all immunohistochemical analyses from duodenojejunal biopsies from the 30 participants were negative for *H. Pylori*.

Even though we identified two taxa associated with obesity, our observational study only reports correlations and does not imply causation. In addition, the small effect size and the homogeneity of the distinct groups make it difficult to adjust confounding factors, such as wine or overall alcohol consumption, for example, which showed a negative linear association with BMI in this work. These limitations will be further discussed in the following section, such as the methods to test for the causal implication of the candidates we identified in the current work.

In addition to metagenomics analyses across three ecosystems of the digestive tract, we performed metabolomic analyses from DJF and stool.

In the present investigation, one of the findings indicated an elevation in sphingolipids, such as diverse sphingomyelin, sphingosine, and ceramides in the group with

obesity, compared to the control group, evident in both stools and USI fluid. This corroborates previous results. A clinical study involving 399 newborns explored the cord blood metabolomic signatures linked to rapid growth during infancy and early childhood overweight. This research identified a correlation between sphingosine levels circulating in cord blood and rapid growth and early life overweight in children (Handakas et al., 2021). Concurrently, elevated levels of cholestenone, a cholesterol derivative (a steroidal lipid within the cholesterol class) resulting from microbial catabolism, and two acylcarnitines were identified as predictive markers of rapid growth. Our cohort evidenced a similar trend, with higher levels of cholestenone in faecal samples and one acylcarnitine in DJF in participants with obesity.

Dietary factors might contribute to the observed upregulation of sphingolipids. For instance, rhesus macaques fed on a high-fat and high-fructose diet exhibited increased body weight and fat mass together with decreased insulin sensitivity. This deterioration in insulin sensitivity was associated with elevated plasma sphingolipids, ceramides, and dihydroceramides (Brozinick et al., 2013). Moreover, another investigation revealed that a lifestyle intervention targeting prepubertal obese children decreased circulating levels of multiple sphingolipid metabolites, including sphingomyelin, ceramide, glycosylsphingosine, and sulfatide species. The decline in these metabolites is associated with improved HbA1c levels (Leal-Witt et al., 2018). Therefore, strategies to reduce sphingolipid levels in obese individuals may represent a promising approach to enhancing insulin sensitivity.

In this line, the GM could be an interesting player to target. Indeed, our laboratory showed serum ceramides associated with alterations in FM composition and impaired glucose metabolism in obesity (Kayser et al., 2019). In the study from Kayser and colleagues (2019), 31 FM species, including *Bifidobacterium* and *Methanobrevicater smithii*, inversely correlated with ceramides. Lipopolysaccharide and flagellan synthesis metagenomic modules associated with ceramides levels and this was the opposite for methanogenesis and BAs metabolism modules. In our work, as sphingolipids were higher in the USI of participants with obesity, it confirms, once more, the particular interest in the upper small intestine and its microbiome in obesity.

In addition to these results, other metabolites were altered between groups in the GIT. Erythronate was higher in the obese group in USIM. This metabolite can be metabolised from erythritol, which can be synthesised endogenously from glucose via the pentose-phosphate pathway (Hootman et al., 2017) or provided from food intake as erythritol, a natural sweetener or sugar substitute (Bordier et al., 2022) which circulating level was shown to associate with baseline Hba1c % and most notably, to an increased weight gain in a 9-month longitudinal study (Hootman et al., 2017). The implications of the metabolisation of erythritol into erythronate for human health remain to be determined.

In stools, together with various metabolites from the lipid pathway that were increased in the obese group, we observed a higher levels of two ginkgolic acids from the xenobiotic pathway in the control group. These metabolites could be provided through the consumption of ginkgolic acids isolated from Ginkgo biloba leaves. However, I contacted the participants having the highest levels of those metabolites in their stools, and no participant reported the consumption of such food supplements. Ginkgolic acids have been proposed as potential candidates for treating type II diabetes (Kim et al., 2022) and obesity (Hosoda et al., 2020).

Indeed, a study proposed that treatment with a ginkgolic acid activates AMPK independently of insulin in 3T3-L1 adipocytes, stimulates insulin sensitivity and the basal translocation of GLUT4, and attenuates palmitate-induced insulin resistance in C2C12 muscle cells via the insulin-dependent Akt pathway (Kim et al., 2022). Another study proposed that ginkgo vinegar reduced HFD-induced weight gain in mice and that this effect could be explained by reduced adipocyte differentiation (Hosoda et al., 2020). However, more research is required to confirm the potential therapeutic effect of ginkgolic acids, and our two specific candidates have yet to be tested in this matter.

Limitations

The primary limitations of the present study encompass: 1) a small participant sample size, 2) the specific characteristics of the control group, and 3) the potential for cross-contamination during the collection of the USIM.

During endoscopy, the introduction of the endoscope to the small intestine could engender cross-contamination during sampling (Sundin et al., 2017). However, as detailed in the <u>Materials and Methods</u> section, we implemented measures to prevent contamination from the oral cavity, which harbours a significantly higher bacterial load than the USIM. I postulate

that when one sample from such inaccessible anatomical localisation in healthy humans, we inevitably encounter hurdles and must find compromises. I have reservations about utilising ingested capsules to collect the USIM, given the incubation time within the gastrointestinal tract. As previously discussed, I maintain that the encapsulated microbiome could undergo alterations during this period, and these changes could be particularly significant in contexts with low bacterial loads.

In our study, we compared participants with severe obesity to a control group devoid of any known metabolic health disorders. However, these participants were scheduled for a gastroscopy due to epigastralgia. I deliberately selected participants with minor symptoms and without the necessity for medication for symptom management. Future research endeavours should secure approval for including a control group presenting no health complaints.

Finally and most importantly, a significant limitation is the small effect size in the current study, which presents certain risks and major limitations. It may imply that certain observed changes or outcomes were minor and potentially not clinically significant. The small effect size can also make it challenging to determine whether any observed changes are due to confounding factors (i.e., smoking or alcohol consumption in the control group). The statistical power of the study is limited, making it more difficult to confidently detect and particularly interpret the associations between USIM and patient's phenotype. Consequently, we should be cautious and avoid generalising the current findings.

However, it is important to note that this was a pilot study, primarily aimed at assessing the feasibility of the project and establishing the methods to be used, including DNA extraction and sequencing from USIM, or culturomics, carried by my supervisor and which is a tremendous load of work for only 30 patients. The primary goal was not necessarily to achieve large effect sizes but to validate the study design, assess the practicality of the protocols, and identify any potential issues that might arise in larger-scale research. As such, the study provides a solid foundation for future, more comprehensive clinical research.

This pilot study will undoubtedly pave the way for broader clinical studies and more mechanistic research using other models, such as murine models, the use of organoids or even 'gut-on-chip' models. Lessons learned from this initial project will inform the design and conduct of these future studies, enhancing their reliability and potential for meaningful, impactful findings. Thus, while the small effect size of the pilot study may seem limiting, it represents a crucial stepping stone in the pursuit of a greater understanding of USIM and its potential clinical implications. This research journey is just beginning, and the potential for significant discoveries and advancements remains high.

Perspectives

Further Exploring the Interplay Between Duodenojejunal Bacterial Species and Intestinal Functions

Our current research is a pilot clinical study to investigate the associations between USIM and human obesity. The study of this cohort led to the constitution of a biobank that can further be exploited and still includes, for each participant, saliva, stool, plasma, serum, urines and duodenojejunal biopsies. These samples will enable the laboratory to answer further scientific questions; some are proposed below.

In addition to the results presented in this manuscript, we wanted to evaluate the associations between the USIM and the functions and integrity of the gut barrier.

Two duodenojejunal biopsies were sampled from each participant at the angle of Treitz for (immuno)-histological and transcriptomic analysis. The objectives were multifold and aimed to assess the functions and integrity of the epithelium and its associations with the resident microbiome. For example, I initially aimed to quantify the expression levels of tight junction proteins (such as claudins, occludins and zonulin). In a clinical study published in 2018 from our laboratory, obesity was associated with a reduction of occludin and tricellulin, such as an altered intestinal permeability of the jejunal epithelium. This is associated with circulating levels of zonulin and LPS-binding proteins (Genser et al., 2018). I also aimed to replicate the results of another study from our laboratory which revealed an increased mucosa surface due to lower cell apoptosis and, particularly as well as an infiltration of adaptative immune cells (i.e., CD8 T Lymphocytes) into the jejunal mucosa of patients with obesity compared to controls. Our team also showed that cytokine secretion impaired insulin signalling in enterocytes (Monteiro-Sepulveda et al., 2015). Both of these studies did not study the interaction with the resident USIM. It would have been interesting to replicate some

of these analyses carried out and investigate these associations with the resident microbiome to identify potential taxa that associate with the mucosa integrity and architecture in obesity.

I also wanted to investigate the associations between the USIM and the transcriptional activity of the USI mucosa. It would have been interesting to look at the association between specific taxa and the expression levels of various glucose transporters or enzymes implicated in fatty acid digestion, for example. Two fundamental studies from Bauer lab showed the USIM and particularly *Lactobacillus* species to impact the expression of SGLT1 and ACSL3 and, in turn, to alter hepatic glucose production in rodents and thus whole glucose homeostasis (Bauer et al., 2018a, 2018b). Unfortunately, due to limited time, I was not able to exploit all biobank samples.

As mentioned, our team is currently working on culturomics from the Je-MiMe project. The aim is to isolate and identify bacterial strains from duodenojejunal fluid samples. Using metagenomic sequencing, we identified two taxa that associate with clinical metabolic traits in the USIM. The laboratory objectives are, among others, to isolate the specific stains and test their causal impact on metabolic mechanisms. I will discuss these perspectives and others in the following sections.

Studying the Upper Small Intestinal Microbiome ex-vivo

Beyond correlations, we should test for the causal impact of the USIM on various physiological functions. In the present work, we identified two taxa that were altered between groups in the proximal intestinal microbiome. To our knowledge, these taxa have not been tested in regards to metabolic functions. If specific strains corresponding to the identified species were isolated, their causal impact could be first thoroughly investigated through ex vivo studies. In addition, two ginkgolic acids were higher in the stools of the control group, and these compounds were proposed to have a potentially beneficial effect on glucose control and adiposity, as discussed above. These metabolites could also be tested through ex-vivo studies.

For example, ex-vivo studies utilising enteroids derived from human duodenojejunal biopsies from gastric bypass could be employed to unravel the mechanistic implications of isolated USIM bacterial strains discerned on various cellular outcomes. These enteroids represent an interesting model for studying mechanistic pathways since they demonstrate cell differentiation, self-organisation, and morphogenesis potential that are physiologically more pertinent than standard cell cultures (Osinski et al., 2022; Sato and Clevers, 2013).

In our laboratory, Dr Céline Osinski and colleagues have developed the application of enteroids. Employing human jejunal crypt samples containing stem cells, she successfully generated enteroids comprising enterocytes, Paneth cells, and enteroendocrine cells, inclusive of GLP-1+ cells, during her doctoral research. These enteroids demonstrated functionality, as shown by the elevation of GLP-1 secretion in response to glucose concentrations. Results also suggest a functional role of the glucose transporter, SGLT1. Moreover, enteroids from patients with obesity, or with obesity and insulin resistance or T2D present an altered function. These alterations include a reduced secretion of GIP/GLP1 (unpublished results from Dr C. Osinski) in response to glucose.

We could test the causal impact of the strains associated with obesity in our study on GLP-1 secretion potential or glucose transport by investigating the expression of glucose transporters (SGLT- or GLUT-transporters) in such models (Poole et al., 2018). This model can also evaluate the effect of administering specific metabolites (Petersen et al., 2014). In our study, we noted higher levels of two Ginkgolic acids in the stools of the control group and higher levels in various sphingolipids in the duodenojejunal fluid in the obese group. These metabolites are worthy of testing in such ex-vivo models.

Nonetheless, the study of microbe-host interactions is hindered in organoids, given that the lumen is enveloped within the organoid body (Bhatia and Ingber, 2014). As static models, enteroids or traditional cell cultures based on 2D monoculture plates preclude mechanical cues crucial for organ regeneration (Shyer et al., 2013) or epithelial cell differentiation (Basson, 2003). In this line, additional models, such as Human gut-on-a-chip technology, could be employed (Lee et al., 2016). However, their establishment in our laboratory needs to be thoroughly validated first.

Human gut-on-a-chip devices, underpinned by microfluidics and cell biology, are designed to mimic the structure, functionality, and microenvironment of the human gut (Xiang et al., 2020). When contrasted with enteroids, these microfluidic chips offer a more physiologically relevant platform to examine the interplay between microorganisms and host

tissue in the human gut as they can incorporate numerous complexities such as oxygen gradients, a diverse human microbiome, cyclical mechanical deformations (peristalsis), and various patient-derived cells (Ashammakhi et al., 2020; "Increasingly microphysiological models," 2019; Marrero et al., 2021). Such models are versatile, capable of monitoring physiological parameters like gut barrier permeability, dissolved oxygen concentration, cytokines profile, and the production of microbial short-chain fatty acid (Marrero et al., 2021).

From ex-vivo to in vivo: studying the Upper Small Intestinal Microbiome using Animal Models

We showed that *N. Subflava* was inversely associated with fasting glycemia, Hba1c, the circulating levels of GIP, C-peptide, insulin and HOMA-IR. This suggests that this specific taxon may influence glucose regulation. Following the causal demonstration of the relationship and the identification of mechanistic pathways by which this specific strain potentially impacts metabolic health via ex-vivo studies, we should extend this investigation to preclinical studies.

Murine models provide the capacity to examine the causal impact and interactions between a controlled environment and microbiome on physiological processes. Various models are now well-developed in this context (i.e., germ-free, mono-colonised gnotobiotic, humanised gnotobiotic), despite pros and cons regarding the relevance of each model.

Germ-free (GF) mice, or gnotobiotic mice, enable the causal study of GM and transplanted microbes can be controlled and can easily colonise their GIT. A study recently published by Meier and colleagues in Nature Metabolism (2023) compared the metabolome along the gastrointestinal tract of conventional mice to GF mice. Such study design enabled the identification of USI metabolites' origins (Meier et al., 2023). Another study showed that GF mice, compared to wild-type mice present an impaired lipid digestion and absorption. There was a reduced Cholecystokinin (CCK) response. These alterations, in part, protected them from HFD-induced obesity. GF mice were then colonised with the jejunal microbiome from HFD mice. After colonisation, the mice fed either a low-fat diet or an HFD showed increased lipid absorption, thus highlighting the role of the GM in metabolic mechanisms such as host and diet interactions (Martinez-Guryn et al., 2018).

Even though this model is great in studying the causal role of the GM, in our laboratory, we do not rely on germ-free mice for various reasons, such as the required complex setting and its related high cost and particularly the altered physiology of this animal model. Indeed, even though animals raised in sterile conditions enable the study of the causal impact of the GM, they exhibit altered physiology, such as deficits in mucosal immunity development (Round and Mazmanian, 2009), reduced Paneth cell numbers in the mucosa, decreased AMPs secretion (Schoenborn et al., 2019). Such physiological alteration may impede the study of the causal role of the USIM.

Other models with fewer metabolic alterations are thus preferable. In our laboratory, colleagues frequently rely on 'conventional' or 'wild type' (WT) mice models such as C57BL/6, also known as black 6 (B6) mice, together with ob/ob mice, also known as B6 ob mice, characterised by mutation of the leptin gene. These mice are commonly used in research to model obesity as they tend to gain weight and store excess fat compared to conventional B6. Ob/ob mice display hyperphagia, transient hyperglycemia, glucose intolerance, and increased plasma insulin levels (Ingalls et al., 1950; Charles River, n.d.).

To facilitate the colonisation by transferred microbiome or specific taxon administered through gavage, for example, using B6 (WT or ob/ob), Dr Tiphaine Le Roy has developed a mice model with a depleted microbiome using a mix of laxatives and antibiotics (Le Roy et al., 2018). Such a model could thus be used further to test the impact of the USIM on rodents. However, additional precautions should be taken into account when studying this microbiome.

In most GM transplant research, faecal transplants from humans were commonly used. This prevents the study of the USIM. Indeed FMT should be avoided, and whole intestinal microbial transplants (WIMT) should be considered instead to reconstruct the microbiome across the GIT. Such transplants encompass microbiome from various segments of the intestine, including the USI, and were found to increase colonisation by small intestinal-related bacteria in this region (Li et al., 2020).

We could also only investigate the impact of direct USIM transplant through gavage directly in the proximal intestine from different groups of humans to rodents (i.e., from an Ob, ObD and a control group). We could compare the role of this ecosystem transplanted from

groups of participants with different metabolic health statuses on weight and fat mass gain, glucose metabolism, inflammatory markers and other markers of metabolic health. Finally, we could, in particular, study the administration of our two taxa of interest in the models described above. We could compare the study of the direct administration of each taxon with, without or only it's supernatant to differentiate the direct role of the taxon or its produced metabolites on rodent metabolism.

In USIM research using rodents, coprophagy should be avoided as it alters the small intestinal microbiome and shifts its composition closer to that of the colon (i.e., mice wearing tail-cups), such as alteration of BAs profile in mice small intestines. Such precautions showed an increased relative abundance of genus such as *Lactobacilli* in the USIM (Bogatyrev et al., 2020; Delbaere et al., 2023).

Conclusion: Hope from Thorough and Translational Research on the Upper Small Intestinal Microbiome

Obesity is a worldwide health priority due to its ever-increasing prevalence. This disease increases the risk of developing various comorbidities, some of which are the leading causes of death worldwide. Obesity is a complex and multifactorial disease, and treatment should target as many mechanisms as possible to reach at least 5% weight loss. The current therapies for obesity are still in need of development. Some of these therapies, such as Semaglutide, enable approximately 12.5 % weight loss in 1.25 years of treatment, while the bariatric surgery, Roux-en-Y, enables a 27 % over 5 to 7 years. However, these interventions come with risks and adverse effects.

In this line, the GM appears as a promising actor. This field of research sheds light on our understanding of the physiopathology of obesity and is an *additional* promising actor in the development of innovative treatments.

Our study showed the USIM as a potentially stronger indicator, compared to FM, of obesity. Other studies showed its causal impact on metabolic perturbations in animal studies.

Translational studies on the USIM could eventually lead to innovative treatments, such as developing specific probiotics. Suppose the taxa identified in the current project, such as *N. subflava*, was found to have a causal impact on metabolic regulation, after extensive studies in ex-vivo models, followed by in-vivo research, it could be delivered in the duodenum of patients through these capsules discussed above. However, we are far from such developments as we require much more mechanistic studies to unveil the impact of USIM on human health.

A better knowledge of the interactions between the USIM and diet and host interaction could lead to personalised interventions specifically targeting the USIM in obesity.

These interventions could target, for example, strategies to target the proximal intestinal microbiome gene richness or load by increasing the host's defence mechanism against pathogens. It could also target specific candidates known to influence digestion an

nutrient absorption. These strategies could increase nutrient sensing to promote satiety and glucose metabolism or promote gut health.

Valorisation of my Work

Publications

Steinbach, Masi et al., (Submitted). Upper Small Intestine Microbiome in Metabolic Health: a new field of investigation. Submitted in *Metabolism*. (Inserted in the introduction section, subsection 4).

Attaye, Bel Lassen et al., 2023. Isocaloric dietary protein intervention had neutral effect on glycemic parameters but altered gut 2 microbial diversity and-derived metabolites: Results from a multi-center randomized controlled trial 3 in type 2 diabetes. *iScience*. doi: 10.1016/ j.isci.2023.107471. (**Third author**, performed during the first year of my PhD, Annex 2).

Bel Lassen et al., 2021. COVID-19 and its severity in bariatric surgery operated patients. *Obesity* (Silver Spring). doi: 10.1002/oby.23026. (Contributor, performed during the lockdown, Annex 3).

Training, Congresses and Communications

Date	Training, congresses and communications	Hours
10.2020	Animal experimentation and its regulatory environment, Charles River $(1/2 \text{ day})$	4 hours
01.2021	37è journées scientifiques de l'AFERO, Association Française d'étude et de recherche sur l'obésité (2 days)	20 hours
11.2021	'Bonnes Pratiques Cliniques' (BPC), Groupement Inter-régional de Recherche clinique et d'Innovation d'Ile-de-France (5 days)	50 hours
05.2021	21 st edition of the JED, <i>ED394</i> (2 days)	20 hours
01.2022	38è journées scientifiques de l'AFERO, Association Française d'étude et de recherche sur l'obésité (2 days)	20 hours
05.2022	22 nd edition of the JED, <i>ED394</i> , oral communication (2 days)	20 hours
06.2022	1 st edition of the Journée des doctorants de la faculté de médecine, Sorbonne Université, oral communication , (1 day)	6.5 hours
10.2022	Formation en communication et innovation en sciences, <i>Innoviris State</i> <i>Institution of Région de Bruxelles-Capitale</i> (>5 days); Laureate of the Women Award in Technology & Sciences (10 000 euros prize)	50 hours
11.2022	MSSG meeting, A joint meeting with <i>AFERO</i> and the <i>French Association for the study of Obesity</i> , oral communication (2 days) Laureate of the "Young Fellow Prize"	20 hours
12.2022	Séminaire des doctorants/post doctorants FHU PaCeMM, oral communication (½ day)	5 hours
01.2023	39è journées scientifiques de l'AFERO, Association Française d'étude et de recherche sur l'obésité, poster, (2 days)	20 hours
02.2023	2ème journée scientifique annuelle de la FHU PaCeMM (1day)	8 hours
06.2023	23 nd edition of the JED (ED394); poster (2 days)	20 hours
09.2023	45 th espen congress on clinical nutrition & metabolism. Lyon, France; poster (2 days)	20 hours
Total of training and congresses: 283,5 hours		

Fundings

I wrote a grant proposal to Fondation de l'Avenir with the supervision of Professor Karine Clement and we obtained funding that covered the majority of the costs related to the clinical study I ran for my Ph.D. project (34 450€).

Supervision

I supervised Reda Mihoub, a First-year master's student in medicine (3-months internship, Master 1, Sorbonne Université).

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ANNEXES

Annex I: Clinical Protocol of The Je-MiMe Study



La science pour la santé _____ From science to health

SYNOPSIS RECHERCHE IMPLIQUANT LA PERSONNE HUMAINE (RIPH) VERSION N°2.0 DU 02/07/2021

- /	N° Inserm	C20-86			
Réser		RIPH du 1° médicament	RIPH du 2° à risques et contraintes		
ver au	Qualification	RIPH du 1° médicament thérapie innovante	minimes		
prom oteur	réglementaire	RIPH du 1° dispositif médical	RIPH du 3° non interventionnelle		
oleui		RIPH du 1° hors produit de santé			

Intitulé de la recherche Titre abrégé et/ou acronyme	Jejunal and Mucosa-Associated Microbiota in Metabolic Diseases: Methods, Identification and Causalities Microbiote jéjunal et associé au mucus du colon dans les maladies métaboliques: méthodes, identification et causalités JE-MIME / COL-MIME (JEjunal MIcrobiota in MEtabolic diseases / COLonic Mucosal Microbiota in MEtabolic diseases)>		
Investigateur principal / coordonnateur	Dr. Gianfranco DONATELLI		
Responsables scientifiques	Prof. Karine CLEMENT Dr. Benoit CHASSAING		
Recherche	 ☑ Monocentrique □ Multicentrique Nombre de centres : 	⊠ Française □ Européenne /Internationale Nombre de pays :	

OBJECTIFS DE L'ESSAI

Objectif principal:

Caractériser le microbiote jéjunal (JE-MIME, partie I de l'étude) et le microbiote associé à la muqueuse colique (COL-MIME, partie II de l'étude) et les comparer au microbiote fécal (évaluer la différence et les similitudes entre le microbiote jéjunal ou le microbiote associé au mucus et microbiote fécal).

Objectifs secondaires :

-Corréler le microbiote avec la santé métabolique et les marqueurs inflammatoires -Corréler le microbiote avec le mode de vie et la santé neuropsychologique

RESUME (ne pas dépasser la page 2) :

Le microbiote intestinal (GM) est un domaine de recherche important pour la compréhension et les progrès thérapeutiques de l'obésité et des maladies métaboliques. Le GM est considéré comme l'intégrateur des causes environnementales et endogènes (ex: génétique) de ces maladies mortelles. Les recherches sur le microbiote intestinal utilisent principalement les matières fécales, en raison de leur accessibilité. Cependant, bien que le microbiote fécal ait conduit à d'importantes découvertes physiopathologiques, nous pensons que le microbiote jéjunal est un indicateur potentiel (et éventuellement plus pertinent dans l'étude des maladies métaboliques) de l'interaction entre l'environnement et la santé métabolique de l'hôte. En effet, le jéjunum est une zone critique de l'absorption des nutriments et de la régulation de l'appétit par signalisation neuroendocrinienne.

Cependant, l'échantillonnage du microbiote jéjunal est plus invasif et requiert des interventions cliniques telles que l'endoscopie. Nous émettons l'hypothèse que l'étude du microbiote jéjunal peut améliorer notre compréhension de la pathogenèse et de la chronicisation des maladies métaboliques. De plus, les données du co-PI Dr Chassaing et d'autres démontrent un rôle central joué par le microbiote associé au mucus dans l'établissement, la promotion et la chronicité



des maladies inflammatoires chroniques, y compris les dérégulations métaboliques. Bien que ces données aient été principalement générées dans des modèles animaux, le Dr Chassaing a précédemment démontré que l'empiètement du microbiote sur la couche de mucus interne normalement stérile est une caractéristique commune du diabète de type 2 chez l'homme, et il est maintenant urgent d'identifier ces membres du microbiote sélectionnés capables d'altérer la santé de leur hôte.

Le protocole est organisé en deux parties:

1. **Partie I** : une investigation clinique pour collecter, analyser et comparer le microbiote jéjunal humain au microbiote fécal (intra-patients). La cohorte sera composée de trois groupes afin d'effectuer une analyse de corrélation entre la composition du microbiote jéjunal et la santé métabolique. Cette recherche portera sur 45 participants. Quinze participants non obèses (7 ou 8 femmes et 8 ou 7 hommes) considérés comme « métaboliquement sains » (groupe témoin), quinze participants atteints d'obésité sans DT2, le « groupe obèse » (Ob) et 15 participants étant atteints d'obésité et diagnostiqués avec un DT2 composé du « groupe obèse et diabétique » (ObD).

2. Partie II: une recherche clinique visant à collecter des biopsies de la muqueuse du côlon afin d'analyser et de comparer le microbiote associé au mucus au microbiote fécal (intra-patients). La cohorte sera composée de trois groupes afin d'effectuer une analyse de corrélation entre la composition du microbiote inséré dans le mucus colique et la santé métabolique. Cette recherche portera sur 45 participants. Quinze participants non obèses (7 ou 8 femmes et 8 ou 7 hommes) considérés comme « métaboliquement sains » (groupe témoin), quinze participants atteints d'obésité sans DT2, le « groupe obèse » (Ob) et 15 participants étant atteints d'obésité et diagnostiqués avec un DT2 composé du « groupe obèse et diabétique » (ObD).

Objectifs principaux:

- Échantillonner, décrire et comparer la composition du microbiote jéjunal collecté par endoscopie, chez l'homme.
- Comparer le microbiote jéjunal et le microbiote fécal.

• Étudier les relations entre le microbiote jéjunal et le phénotypage bioclinique des patients présentant différents états métaboliques, allant de « métaboliquement sain » à l'obésité sévère avec et sans diabète de type 2.

• Échantillonner, décrire et comparer la composition du microbiote associé aux muqueuses collecté lors des coloscopies de routine dans le cadre du dépistage du cancer au microbiote fécal, chez l'homme.

• Comparer le microbiote associé au mucus colique et le microbiote fécal

• Étudier les relations entre la composition du microbiote associé aux muqueuses et le phénotypage bioclinique des patients présentant différents états métaboliques, allant de « métaboliquement sain » à l'obésité sévère avec et sans diabète de type 2.

Les résultats générés dans le cadre de cette recherche permettront d'améliorer notre compréhension actuelle des maladies métaboliques et pourront conduire au développement de traitements innovants, par exemple, basés sur la découverte de bactéries spécifiques ou de métabolites bactériens, provenant des compartiments jéjunaux ou muqueux, qui sont importants pour la santé métabolique. De plus, cette étude est une approche préliminaire qui permettra d'explorer des méthodes pour capturer et identifier les bactéries jéjunales chez l'humain.

MOTS CLÉS (5): < Microbiote, Jejunum, Mucus, Obésité, Diabète de type II>



DOMAINES DE RECHERCHE :

Endocrinologie Gastroenterologie, hépatologie Immunologies, allergies Cardiovasculaires–cardiologie–angiologie Métabolisme – maladies métaboliques Neurosciences, neurologie Nutrition, obésité Santé mentale, psychiatrie Santé publique Dépendances : alcool, tabac, drogues... Systèmes de soins, SHS

TYPOLOGIE				
Essai portant sur un produit de santé (PS) :				
☐ Médicament ☐ Médicament de Thérapie Innovante (MTI)	Phase : 🗆 I 🛛 🗆 II 🗖 IV			
□ Dispositif Médical	Classe : I Ila invasif à long terme IAutre IIa IIb III DMIA			
□ Autre. Préciser :				
🖾 Essai hors produit de santé (HPS) (physiologie, physiopathologie, …)				
 □ Etude épidémiologique □ Etude cas témoin □ Etude de cohorte (longitudinale) □ Etude transversale 				

METHODOLOGIE (si applicable)		
□ Contrôlé □ Randomisé	□ Groupes parallèles □ Croisé	□ Double insu □ Simple insu □ Ouvert

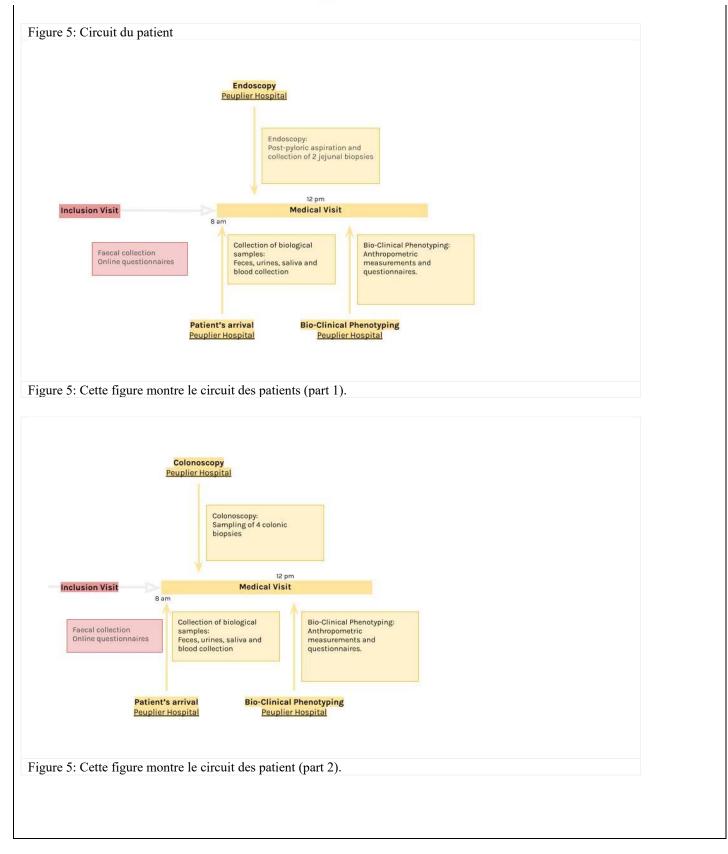
CALENDRIER PRÉVISIONNEL DE L'ESSAI

Durée d'inclusion : 24 mois

Durée de la participation du sujet : 1 mois (une semaine à un mois entre la visite d'inclusionV0 et la Visite V1, Durée totale de la recherche : 25 mois + 6 mois d'analyse

Pour chaque participant, l'étude est basée sur une journée et demie d'investigation clinique. Les visites de la recherche seront couplées aux visites de soins standard. Les patients doivent venir deux fois comme initialement prévu dans le cadre de leur prise en charge: pour leur inclusion dans le protocole (visite d'une heure) après la première consultation avec l'endoscopiste (c'est-à-dire avant l'endoscopie) et la visite d'investigation clinique (visite d'une journée, le jour de l'endoscopie / coloscopie, environ 8 heures).





POPULATION ÉTUDIÉE

Nombre et profil * de participants par groupe :

Les deux parties de cette étude sont composées de 3 groupes: 1) « Groupe contrôle, 2) Groupe obèse (Ob), 3) Groupe obèse avec diabète de type 2 (ObD). Chaque groupe est composé de 15 volontaires adultes humains pour l'étude JE-



MIME (partie 1) et de 15 participants pour l'étude COL-MIME (partie 2). Le nombre total de participants est de 45 par étude. Le nombre total de participants est de 90.

- Groupe témoin: sujets minces (JE-MIME: n = 15; COL-MIME: n = 15). L'endoscopie (JE-MIME) est prévue pour les soins de routine en raison d'épigastralgies mineures. La coloscopie (COL-MIME) est prévue dans le cadre du dépistage du cancer du côlon.

- Groupe Ob: sujets obèses, candidats à la chirurgie bariatrique (JE-MIME: n = 15; COL-MIME: n = 15). L'endoscopie (JE-MIME) est prévue comme soins de routine, avant la chirurgie. La coloscopie (COL-MIME) est prévue dans le cadre du dépistage du cancer du côlon.

- Groupe ObD: sujets obèses et diabétiques de type II candidats à la chirurgie bariatrique (JE-MIME: n = 15; COL-MIME: n = 15). L'endoscopie (JE-MIME) est prévue comme soins de routine, avant la chirurgie. La coloscopie (COL-MIME) est prévue dans le cadre du dépistage du cancer du côlon.

Modes de recrutement par profil :

Les participants seront identifiés par les cliniciens-investigateurs. Les participants de tous les groupes, et en particulier du groupe contrôle, seront recrutés dans le service d'endoscopie (Hôpital des Peupliers; Ramsay-GDS, Paris, France). Des brochures de recrutement seront également mises en ligne dans les centres de nos collaborateurs et sur leurs réseaux sociaux et les participants pourront postuler spontanément pour participer à l'étude en répondant à ces brochures de recrutement.

Les références seront transmises au coordinateur clinique avec la permission des patients. Les participants potentiels seront contactés par le coordinateur clinique et leur demanderont s'ils souhaitent participer à l'étude. Si le participant accepte de participer à l'étude, le processus d'inclusion sera tenu par le Dr Donatelli.

Justification du nombre de sujets :

La première partie est une étude pilote. Les calculs de puissance sont basés sur les résultats obtenus lors d'une précédente étude du laboratoire du Prof Karine Clément (UMRS 1269). Cette étude a comparé des sujets obèses à des sujets obèses atteints de diabète de type II (Vieira-Silva et al., 2020). Dans cette étude, les sujets obèses avec ou sans diabète de type II avaient une diversité du GM réduite de 30% par rapport aux témoins. Cette différence a été observée avec des échantillons de microbiote fécal. Notre hypothèse est que la perte de diversité GM sera également trouvée dans les échantillons jéjunaux dans la même mesure. Ainsi, pour obtenir une puissance de 90% avec un risque α de 0,05 (unilatéral) et un risque 1-1-ß de 0,9, nous avons besoin de 13 sujets par groupe (approximation Arcsin pour les proportions estimées). La perte de patients est faible puisque les patients sont en suivi de routine, nous compterons ainsi 15 sujets par groupe.

La deuxième partie est basée sur la littérature publiée par notre collaborateur concernant l'empiètement du microbiome dans la couche muqueuse chez les personnes atteintes d'obésité et de DT2. Ces études ont révélé une différence de 19 μ m (ET = 6,75 μ m) de distance entre les bactéries et les cellules épithéliales intestinales chez les personnes atteintes de DT2 par rapport aux personnes sans DT2. Sur la base des données de ce travail publié, une taille d'échantillon de 13 personnes par groupe (total = 52) sera suffisante pour détecter une différence moyenne de 10 μ m avec une puissance de 90% et le niveau de signification de 0,05 (en utilisant une ANOVA à un facteur modèle suivi de 3 comparaisons posthoc avec ajustement de Bonferroni). Nous conserverons une puissance de 80% pour détecter un changement d'empiétement de 10 μ m avec un taux d'abandon allant jusqu'à 20% (N = 10 par groupe). Compte tenu de la courte durée de l'étude, il est peu probable que nous ayons des abandons qui auront un impact sur le critère d'évaluation principal.



CRITERES D'ELIGIBILITE

Critères d'inclusion :

Table 1: Critères d'inclusion

Critères d'inclusions (tous groupes confondus)

- Agés entre 18 et 60 ans;
- Capacité de comprendre et recevoir le consentement éclairé;
- Capacité et motivation à remplir les exigences de l'étude.

Critères d'inclusion (par groupes):

Groupe contrôle

Les critères d'inclusion pour les participants du groupe témoin sont:

- Présence de symptômes de reflux gastro-œsophagien ou d'inconfort gastrique.
- IMC [19 kg / m² <IMC <25 kg / m²]
- Faire correspondre l'âge et le sexe des patients des groupes Ob et ObD.

• Seuls les patients ayant une tolérance au glucose normale (NGT), une tolérance au glucose normale (IGT)

et / ou une glycémie à jeun (IFG) normale et une HbA1c <6,5% seront inclus dans ce groupe.

Groupe Ob

Les critères d'inclusion pour les participants des groupes Ob et ObD sont:

- Candidat à la chirurgie bariatrique et répondant aux critères de la HAS (Haute Autorité de Santé, 2009):
 IMC ≥ 40 kg / m² sans comorbidité
- <u>**Ou**</u> IMC ≥ 35 kg / m^2 avec au moins une comorbidité liée à l'obésité (ex: hypertension, dyslipidémie, apnée obstructive du sommeil, maladie des articulations, stéatohépatite non alcoolique, à l'exclusion du T2D) • Poids stable pendant au moins 2 mois
- Pas de traitement des complications métaboliques de santé, pas de chirurgie de l'obésité antérieure, pas

de médicament pour traiter l'obésité.

• Pas de forme monogénique d'obésité (Hebebrand et al., 2017)

Groupe ObD

Les critères d'inclusion pour les participants des groupes ObD sont:

- Candidat à la chirurgie bariatrique avec DT2
- Plus de deux comorbidités liées à l'obésité, y compris le DT2
- Glycémie à jeun (FPG) ≥ 7 mM (= 1,26 g / l) ou
- Patients avec HbA1c ≥ 6,5% (48 mmol / mol)
- Tous les stades de l'albuminurie

Critères de non-inclusion :

Table 2: Critères de non-inclusion

Critères de non inclusion (tous groupes):

• Traitement durant les 12 semaines précédentes qui pourrait altérer

o la fonction motrice gastro-intestinale (p. ex. opioïdes, procinétiques, anticholinergiques, laxatifs),

- l'acidité (PPI, H2RA)
- population microbienne (ex.: antibiotiques, probiotiques)
- o immunosuppresseurs (ex: inhibiteurs de la calcineurine, corticostéroïdes, agents biologiques, etc.).
- utilisation d'un médicament amaigrissant ou d'une intervention diététique visant à perdre du poids;

• Anatomie altérée de l'œsophage, de l'estomac, de l'intestin grêle ou du gros intestin en raison d'une

chirurgie gastro-intestinale antérieure (les exceptions incluent l'appendicectomie ou la cholécystectomie plus de 3 mois avant l'inscription) ou d'autres raisons;

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- Toute chirurgie abdominale ou pelvienne au cours des 3 derniers mois;
- Diverticulite, sténose diverticulaire ou autres sténoses intestinales.
- Résection intestinale du tractus gastro-intestinal
- Antécédents de bézoard gastrique ou de gastroparésie
- Maladie inflammatoire intestinale aiguë ou chronique ou maladies infectieuses (ex: VHC, VHB, VIH, etc.)
- Radiothérapie abdominale ou pelvienne ou cancer abdominal
- Cancer colorectal connu ou non
- Dysphagie, œsophagite à éosinophiles, sténose œsophagienne ou autre trouble de la déglutition
- Transplantation d'organes et patients sous traitement immunosuppresseur
- Insuffisance rénale sévère et / ou patients sous dialyse (créatinine sérique> 150 µmol / I ou DFGe <60 ml / min pour 1,73 m2 de surface corporelle)
- MCV, maladie endocrinienne, rénale ou autre maladie chronique susceptible d'affecter la motilité.
- Préparation de nettoyage du côlon au cours du dernier mois
- Pas <3 selles par semaine
- Femmes en âge de procréer sans contraception et / ou sont enceintes ou allaitantes
- Participants non affiliés à la sécurité sociale
- Participants déjà inclus dans une étude clinique qui implique de tester n'importe quel médicament pharmaceutique.
- Participants qui ne sont pas en mesure de comprendre les procédures de recherche ou ceux qui sont institutionnalisés, ou incapables de donner leur consentement éclairé
- Patients sous mesure de protection jurididique
- Toxicomanies
- Antibiothérapie 3 mois avant l'endoscopie
- Variation de poids (diminution ou augmentation)> 5kg dans les 3 derniers mois

Critères d'exclusion spécifiques

Groupes Ob et ObT2D

• Contre-indications habituelles à la chirurgie bariatrique;

CRITERES DE JUGEMENT

Critère principal :

Les principaux objectifs sont de comparer le microbiote jéjunal au microbiote fécal, le microbiote associé au mucus au microbiote fécal,

• Pour la partie I (JE-MIME) - microbiote jéjunal

Le microbiote sera analysé par séquençage du gène de l'ARNr 16S ainsi que par séquençage métagénomique des différents échantillons obtenus à partir de 1) endoscopie (liquide jéjunal) et 2) échantillons fécaux.

• Pour la partie II (COL-MIME) – microbiote associé au mucus

Le microbiote sera analysé par séquençage du gène de l'ARNr 16S ainsi que par séquençage métagénomique des différents échantillons obtenus à partir 1) de la couche de mucus interne de la muqueuse du côlon, collectée par l'approche de microdissection par capture laser et 2) de l'échantillon fécal.



Rationnel du choix du critère principal : À ce jour, la plupart des recherches utilisent le 16S et le séquençage métagénomique pour l'analyse microbienne non ciblée (vs: DGGE, FISH). Alors que l'ARNr 16S est toujours utilisé, le génome complet également appelé analyse « shotgun » ou « métagénomique » fournit plus d'informations sur les fonctions bactériennes. MinION est une technique bien développée et maîtrisée dans les deux laboratoires (Laboratoires Chassaing et Clément).

Critères secondaires :

Les objectifs secondaires sont d'analyser la relation entre le microbiote jéjunal (partie 1) et le microbiote associé au mucus (partie 2) et la santé métabolique, l'inflammation, le mode de vie et la santé mentale qui seront évalués par phénotypage bio-clinique approfondi comme décrit précédemment (Alligier et al., 2020; Genser et al., 2018) et comprendra:

La santé métabolique et l'inflammation seront évaluées pour permettre une meilleure caractérisation de nos sousgroupes (patients maigres vs obèses vs patients obèses atteints de diabète de type II). Cela permettra d'explorer la corrélation entre la composition du microbiote et la santé. Les antécédents personnels et familiaux du mode de vie et la santé psycho-émotionnelle, comme discuté dans l'introduction, sont en corrélation avec la santé métabolique et la composition des GM et seront donc également évalués pour explorer la corrélation.

• **Phénotypage métabolique** (sérum): glycémie à jeun, insuline, Hba1c, phénotype lipidique (cholestérol total, LDL, HDL, triglycérides), substitut de la résistance à l'insuline (indice HOMA), évaluation cardiovasculaire

• Inflammation systémique et marqueurs entero-endocrins (sérum et biopsies): (multiplex humain: amyline, C-peptide, ghréline, GIP, GLP-1, glucagon, IL-6, insuline, leptine, MCP-1 / CCL2, PP, PYY, TNFa), taux de LPS circulants.

• **Mesures anthropométriques et composition corporelle** (c.-à-d. poids, taille, indice de masse corporelle) évaluation de la composition corporelle (impédancemétrie et DXA).

• **Analyses histologiques** de biopsies jéjunales obtenue lors de l'endoscopie et de la coloscopie: analyse immunohistochimique de l'expression des protéines à jonctions serrées dont l'expression est corrélée à une inflammation de bas grade et des taux de LPS (ZO-1, Occludin, Claudins) (Genser et al., 2018; Everard et Cani, 2014; Larraufie et al., 2018).

• Questionnaires du mode de vie collectés par des questionnaires through lifestyle questionnaires:

• Questionnaire d'historique médical: Historique pondéral, histoire familiale, obésité et diabète

• **Alimentation:** La nutrition et l'apport alimentaire seront évalués semi-quantitativement au moyen de questionnaires de fréquence alimentaire (FFQ) en ligne et de rappels alimentaires consécutifs auto-administrés en ligne de 24 heures.

• **Activité physique:** Activité physique grâce au questionnaire modifié sur l'activité physique récente (RPAQ).

• **Santé neuropsychologique**: Les paramètres neuropsychologiques seront évalués au moyen d'un questionnaire de santé court (SF-36), d'un questionnaire sur la santé du patient (PHQ-9), d'un questionnaire d'inventaire de la dépression de Beck (BDI-II), d'une échelle de stress perçu, d'une échelle d'efficacité générale (GSES), d'un questionnaire Stop-Bang, L'indice de qualité du sommeil de Pittsburgh (PSQI) et l'échelle de somnolence d'Epworth (ESS).



La science pour la santé _____ From science to health

RESEARCH PROTOCOL INVOLVING HUMAN PERSON

the	Inserm NO.	EudraCT NO. or RCB ID ¹	CPP or other Ethical Committee NO.	CNIL ou MR NO.	Clinical Trial NO	
for	C20-86	2021-A01074-37	21 06 48	MR001	<>	
Reserved sponsor	Regulatory Qualification	 RIHP 1° medical product RIHP 1° advanced therapy product RIHP 1° medical device RIHP 1° excluding health product 		 ☑ RIHP 2° minor risks and constraints □ RIHP 3° non-interventional 		

Jejunal luminal and Colonic Mucosa-Associated Microbiota in Metabolic Diseases: Methods, Identification and Causalities

JE/COL-MIME

VERSION NO.: 2.0 - DATE: 02/07/2021

CONFIDENTIAL

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Single-site trial				
Multi-centre trial	European/International study			
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¹In addition to registration with the E.M.A., this study may also be recorded in other international registries, like the ClinicalTrials.gov, and the International Clinical Trials Registry Platform (ICTRP) of the World Health Organisation. ² If different from the Principal Investigator



SIGNATURE PAGE

N° Inserm : C20-86

Version and date : V2.0_02/07/2021

The undersigned have read the following protocol and its documents.

They agree to conduct the research in compliance with the approved protocol and its appendices and will adhere to the principles outlined in the Declaration of Helsinki, the sponsor's procedure, and other regulatory requirement.

For and on behalf of the Study Sponsor:	
Sandrine COUFFIN-CADIERGUES Head of sponsor Pôle de Recherche Clinique Inserm, Institut Santé Publique Biopark, bâtiment, 8 rue de la Croix Jarry 75013 Paris	Date : Signature :
	By delegation CEO of Inserm.

Principal Investigator	
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HISTORY OF PROTOCOL VERSIONS

Section reserved to the sponsor

Version NO.	Version Date	Amendment Summary
1.0	09/04/2021	Initial version submitted to the EC
2.0	02/07/2021	Authorized Version



LIST OF ABBREVIATIONS

ANSM	Agence nation	onale de sécurité du me	édicament et de	es proc	duits de santé	
ARC	Attaché de r	echerche clinique				
BI	Brochure inv	/estigateur				
CIC	Centre d'inv	estigation clinique				
CNIL	Commission	Nationale de l'Informa	tique et des Lib	oertés		
CPP	Comité de P	Protection des Personne	s			
CRF	Case report	form/ Cahier observation	on			
CV	Curriculum v	vitae				
DM	Dispositif me	édical				
DTA/MTA	Data Transfe	ert Agreement /Materia	Transfert Agre	ement	1	
EI/EIG	Événement	ou effet indésirable / Év	vénement indés	sirable	grave	
ITMO		atique multi-organisme	S			
MR	Méthodologi	e de référence (CNIL)				
МТІ	Médicament	de thérapie innovante				
PRC	Pôle Recher	che Clinique de l'Institu	it de Santé Pul	olique o	de l'Inserm	
RCP	Résumé des	s caractéristiques du pr	oduit			
RIPH		mpliquant la personne				
RQRC	Cellule « Ré	glementation et qualité	en recherche o	clinique	e » du PRC	
VRB	Fichier natio	nal des volontaires (vo	ontaires reche	rche na	ationale)	
CVD	Cardio-vasc	ular disease				
LPS	Lipo-Polysa	ccharide				
Ob	Obese partic	cipants				
ObD	Obese partic	cipants with associated	type 2 diabetes	s		
T2D	Type 2 diabe	etes				
GM	Gut Microbio	ota				
JE-MIME	Jejunal Micro	obiota in Metabolic Dis	eases			
COL-MIME	Colonic	mucosal-associated	Microbiota	in	Metabolic	Diseases



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1. BACKGROUND, RATIONALE AND HYPOTHESIS

1.1. Context and rationale: Microbiota and Metabolic Diseases

• Epidemiology

Humanity is facing a constantly rising epidemic of obesity and associated chronic, morbid and interrelated metabolic disorders, such as Type II Diabetes Mellitus (T2D) or CVD Diseases (CVD) the main cause of mortality (Flegal et al., 2013; Nathan, 2008; Sturm and Hattori, 2013; WHO, n.d.). There is not only an increase in the number of obese individuals, with a prevalence as high as 30% in adults over age 50 in Europe, but also an increase in the average weight towards the upper extreme. This epidemic is a major public health threat overwhelming health care systems and economies worldwide (World Health Organisation, n.d.). Moreover, these metabolic diseases also present a personal threat with heavy economic, physical and mental health costs. However, to date, there is no solution for effective prevention and treatment of these 'nutrition-related' diseases. It is thus a top public health priority and a great challenge in dire need of investigation.

Causes: environmental triggers

Both environmental, and (epi)genetic factors are causal determinants in the development of metabolic diseases. The most frequent form of genetic obesity is explained by single or multiple alterations in the leptin/melanocortin pathway, which regulates food intake and energy expenditure (Clément et al., 1998), but it concerns a very small percentage of obesity representing only 2 to 3% of childhood and adult obesity (Bell et al., 2005; Hinney et al., 2006; Lubrano-Berthelier et al., 2003). As such, the increase in prevalence over the last decades is mainly explained by environmental factors. Indeed, industrialisation and economic growth of our "Western Civilizations transformed human's lifestyle and favoured a positive energy balance and the prevalence of metabolic diseases" (Heymsfield and Wadden, 2017; McAllister et al., 2009). The two most obvious causes are **decreased energy expenditure** and **increase energy intake**: our modern lifestyle is sedentary (i.e.: seated work conditions) with decreased leisure physical activities (i.e.: watching television) and there is an increase in the consumption of high-calorie food with a rise in availability, marketing and perceived convenience. However, it would be reductive to think obesity is only due to a dysregulation of the energy balance. Several other pathophysiological elements have been shown to be associated with the development and the maintenance of obesity over time.

For instance, some important psycho-emotional factors, deemed as hallmarks of our modern societies such as increased **stress** and **depression** but also **inappropriate sleep**, resulting in neuroendocrine perturbations that impact energy homeostasis, appetite control glucose tolerance and energy expenditure (Knutson et al., 2007; Mullington et al., 2003; Spiegel et al., 2004). It is important to note that these psycho-emotional factors can be causes but also consequences of metabolic diseases, thus, positive feedback loop mechanisms, further aggravating both mental and physical conditions (Faith et al., 2011; Heymsfield and Wadden, 2017).

Along with these myriad environmental triggers perturbing energy balance (Heymsfield and Wadden, 2017), biological maladaptation of organs and their altered communication contribute to the chronicisation, maintenance of obesity and its resistance to treatment. Many studies depict an altered molecular and cellular cross-talk among the immune system, adipose tissue depots and organs, such as the liver, muscle, intestine and the brain (Touch et al., 2017). Interestingly, some studies show some of these alterations to precede obesity (Chassaing et al., 2016, 2017b; Viennois et al., 2017). Recently the gut microbiota (GM) is increasingly being discussed and recognized as an important factor in the development of obesity.

Pathophysiology: The GM, integrator of environmental triggers

This complex microbial ecosystem that resides in human's gut plays important beneficial roles in the host's physiology, especially in digestion and immune system maturation. However, in metabolic diseases, the composition, richness and function of GM are altered (Cotillard et al., 2013; Le Chatelier et al., 2013; Yatsunenko et al., 2012). These alterations -which are encompassed under the term of "dysbiosis"- are associated with low-grade inflammation which is systematically observed in these diseases (Hotamisligil, 2006; Nathan, 2008; Tilg and Moschen, 2006).

Dysbiosis is thought to promote obesity and inflammation (Dao et al., 2016; Everard et al., 2013; Schneeberger et al., 2015) and can result in excessive activation of innate immune pro-inflammatory signaling that can damage the host insulin-sensitivity and intestinal homeostasis (Cani et al., 2008; Chassaing et al., 2017a, 2017b; Dao et al., 2019).

Dysbiosis and inflammation are tightly linked. Many mechanisms can account for the deleterious effect of the microbiota - under certain circumstances - on the immune system. For example, microbial encroachment in the intestinal mucus (Chassaing et al., 2017a; Viennois et al., 2017) or lowered abundance of specific bacteria (Everard et al., 2013; Schneeberger et al., 2015) are shown to alter the



intestinal barrier by disrupting both the protective mucus and tight-junction proteins. This can trigger an inflammatory cascade with the leakage of bacterial fragments, such as lipopolysaccharides (LPS) to the host system, driving low-grade systemic inflammation, through CD14/TLR4 activation. This inflammatory cascade further disrupts both the GM and barrier (Cani and Delzenne, 2009).

Importantly, low-grade inflammation leads to an elevation in numerous cytokines and other inflammatory markers, not associated with aggregates of immune cell infiltrates that classically define inflammation. Such inflammatory states are systematically found in obesity and other metabolic diseases. Inflammarion alters insulin signaling, and thus, stimulates excess caloric intake and further exacerbates preexisting glucose tolerance and insulin desensitization in metabolic diseases (Hotamisligil, 2006; Tilg and Moschen, 2006). Inflammation does not necessarily initiate after caloric excess and insulin resistance, showing that for instance, diet by itself could be an initial proinflammatory trigger (Chassaing et al., 2017b, 2016; Viennois et al., 2017).

The effect of the GM on metabolic health and inflammation has been shown. Dietary intervention, bariatric surgery, treatment with specific bacteria or treatment to prevent bacterial encroachment increased microbial richness and composition (Aron-Wisnewsky et al., 2012, 2019b; Cotillard et al., 2013; Zhang et al., 2015) or prevented encroachment in the mucus (Tran et al., 2019) and ameliorated metabolic phenotype and/or inflammation. And, the causal effect of the gut microbiota has been shown with faecal transplants in metabolic diseases (Kootte et al., 2017; Vrieze et al., 2012).

To conclude, **the importance of the GM in metabolic diseases has been repeatedly demonstrated** (Aron-Wisnewsky et al., 2019b; Cani, 2018; Cotillard et al., 2013; Dao et al., 2016; Wu et al., 2011). The microbiome can be seen as an integrator of environmental triggers, further altering the host immune response and metabolic state. Its exploration can provide not only cues for patient stratification but also the understanding of metabolic health. And it could ultimately lead to the development of effective treatments (Aron-Wisnewsky et al., 2019a; Dao and Clement, 2018; Plovier et al., 2017).

1.2. Microbial differences along the gastrointestinal tract

The distribution of microorganisms varies greatly along the length of the gastrointestinal tract, mainly due to different environmental conditions: pH, oxygenation, bile acid, antimicrobial peptides (Ahmed et al., 2007; Aron-Wisnewsky et al., 2012; Crespo-Piazuelo et al., 2018; Hayashi, 2005; He et al., 2018; Sundin et al., 2017; Wilkinson et al., 2017). Bacterial density increases from $10^1 - 10^3$ in the stomach and duodenum, to $10^4 - 10^6$ in the jejunum and ileum, and finally $10^{11} - 10^{12}$ in the colon. Furthermore, the acidic pH and oxygen-rich environment of the proximal small intestine favours microbial colonization with acid- and oxygen-tolerant bacteria (e.g. Lactobacillus, Streptococcus, Veillonella), whereas in the colon, oxygen-poor conditions and slower transit results in fermentation of complex polysaccharides, resulting in greater taxonomic diversity and dominance of saccharolytic anaerobic Bacteroidales and Clostridiales (Scheithauer et al., 2016; Sommer and Bäckhed, 2016).

As previously introduced, a shift from the normal GM to dysbiosis state with perturbation of microbial composition is thought to contribute to a variety of disease states, including obesity and T2D (Debedat et al., 2019b, 2019a). But no study has properly investigated jejunal dysbiosis in the context of metabolic diseases in humans.

1.3. Limitations pertaining to the microbiota field of research

Gut microbiology is a promising field of research for our understanding and treatment of metabolic diseases. In metabolic diseases research, it is, however, questionable whether the extrapolated conclusions of studies exclusively on feces are relevant. Indeed, the main site of digestion and nutrient absorption - particularly carbohydrates – occur in the proximal part of the small intestine, mainly the jejunum. Moreover, data from co-PI Dr. Chassaing and others demonstrate a central role played by the mucus-associated microbiota in the establishment, promotion and chronicity of chronic inflammatory diseases, including metabolic deregulations. While these data were mostly generated in animal models, Dr. Chassaing previously demonstrated that microbiota encroachment of the normally sterile inner mucus layer is a common feature of type 2 diabetes in human, and there is now an acute need to identify these select microbiota members that are able to encroach upon their host. We thus submit that both the jejunal and mucosa-associated microbiota are strong integrators of nutritional and intestinal health in dire need of further investigations

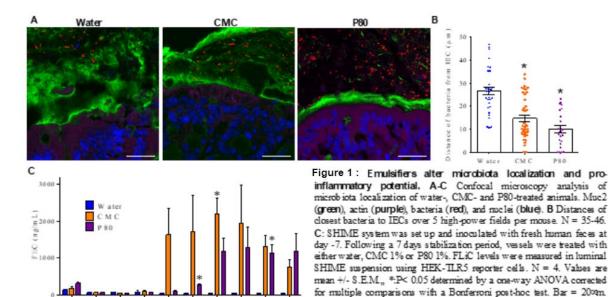


1.4. Challenge 1 to address: accessibility of the jejunal microbiota

The location and length of the jejunum has meant that direct microbial sampling is of importance but challenging. Endoscopically attained small bowel aspirate with a bacterial count > 10⁵ CFU/ml has historically been considered the "gold standard" for diagnosing SIBO with the Breath-Test (Sabate et al., 2017). A prospective study of 15 symptomatic patients who underwent jejunal aspirate with subsequent 16s rRNA gene sequencing and metagenomic analysis, demonstrated differing bacterial compositions between the jejunum, colon and oropharynx, which confirmed the findings of previous autopsy studies (Hayashi, 2005; Sundin et al., 2017). In this project, jejunal microbiota will be sampled through endoscopy as part of the patient's required medical examinations (causes: minor epigastralgia for lean patients, prerequisite for bariatric surgery for patients with obesity and/or Diabetes).

1.5. Challenge 2 to address: accessibility of the mucus-associated microbiota

Data from co-PI Dr. Chassaing previously demonstrated a central role played by the mucus-associated microbiota in the establishment, promotion and chronicity of chronic inflammatory diseases, including metabolic deregulations. For example, his team previously reported that consumption of synthetic dietary emulsifiers (found in many processed food items), carboxymethylcellulose (CMC) and polysorbate 80 (P80), altered microbiota composition, gene expression, localization, and pro-inflammatory potential and led to metabolic disorders in mice (Figures 1 and 2).



Chassaing et al, 2017 Gut.

IEC = intestinal epithelial cells. From Chassaing et al, 2015 Nature and



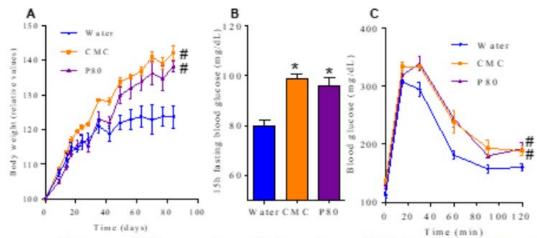


Figure 2: Dietary emulsifiers promote metabolic syndrome. WT Swiss Webster (A-B) and C57/B16 (C) mice were exposed to drinking water containing CMC or P80 (1.0%) for 12 weeks. A, Body weight over time; B, 15 hours fasting blood glucose levels; C, glucose tolerance test. Values are mean +/- S.E.M, N = 8. Significance was determined using one-way ANOVA corrected for multiple comparisons with a Sidak test (* P<0.05 compared to water-treated group) or two-way ANOVA corrected for multiple comparisons with a Bonferroni test (# P<0.05 compared to water-treated group). From Chassaing et al, 2015 Nature and unpublished observations.

While these data were generated in animal models, Dr. Chassaing also previously demonstrated that microbiota encroachment of the normally sterile inner mucus layer is a common feature of type 2 diabetes in humans, as presented in figure 3.

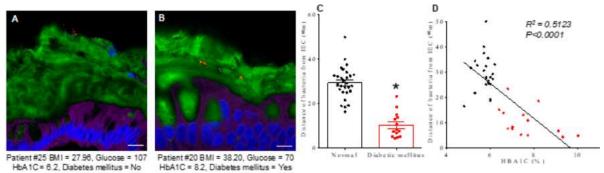
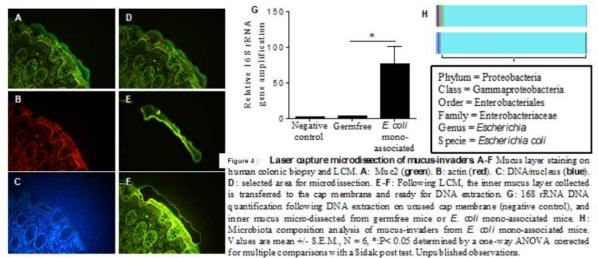


Figure 3: Metabolic syndrome correlates with microbiota encroachment in human. A-B. Colonic biopsies were collected during colonoscopy procedure and placed in methanol-Carnoy's fixative solution, followed by confocal microscopy analysis of microbiota localization. C. Distances of the closest bacteria to intestinal epithelial cells (IEC) per condition over 5 high-powered fields per subject were plotted vs. indicated metabolic parameter. Patients recognized as having type 2 diabetes are shown in red. Linear regression line was plotted and R² and P values were determined. N = 32. From Chassaing et al, 2017 Cellular and Molecular Gastroenterology and Hepatology.

However, this study presented a number of limitations, including that it only examined bacteria localization and did not assess bacterial instigators of disease. Hence, it remains crucial to identify microbiota members that are associated with these features. Here, we propose to use biopsies collected from healthy controls and patients with metabolic alterations in order to specifically identify select microbiota members able to encroach upon their host. Central to the research proposed here, is a new laser capture microdissection (LCM)-based approach that Dr. Chassaing recently developed. As outlined in Figure 4, this approach allows specific collection of inner mucus layer and discriminant isolation of mucus-infiltrating bacteria. It has been demonstrated that the amount of bacterial DNA obtained, although relatively low, is sufficient for sequence-based species identification. Specifically, application of this approach to mice mono-associated with an E. coli bacterium able to penetrate the mucus layer (Adherent-Invasive Escherichia coli, AIEC) revealed that more than 98% of the reads were assigned to E. coli (Figure 4), which was not detected in control mice, thus validating this new approach. Importantly, while the LCM technique was previously used for metagenomic analysis of mucosa-



associated microbial community and for analysis of crypt-associated bacteria, Chassaings' team is the first group to use this technique to specifically identify mucus-invaders. Hence, we are now poised to apply this technique here in order to identify which bacterial species encroach upon their host in humans with various metabolic health status.



1.6. Hypothesis

Both the jejunal microbiota and mucus-associated microbiota are strong integrators of nutritional environment and intestinal health status, respectively, compared to the fecal microbiota. Findings will help to better understand metabolic the physiopathology of diseases. This research could lead to finding specific microbiota members, either from the jejunal compartment or from the inner mucus layer, crucial for the promotion / protection of chronic intestinal inflammation and associated metabolic health.

1.7. Participants

This research protocol is organized in two parts. Each part of this study will recruit 45 participants that are only recruited in one of the parts of the protocol (JE-MIME or COL-MIME). Thus, in total, the study will include 90 patients. Each part of this study is composed of 3 groups: 1) "Control Group », 2) Obese group (Ob), 3) Obese and Type 2 Diabetes group (ObD). Control groups for part I (JE-MIME) and part 2 (COL-MIME) are composed of different participants. Each group is composed of 15 human adult volunteers for the JE-MIME study (part 1) and 15 participants for the COL-MIME study (part 2). Total number of participants is 45 for part 1, and 45 for part 2. Total number for this project is 90.

The first part (JE-MIME): consists of a clinical investigation to capture, analyse and compare human jejunal microbiota to faecal microbiota (within-patients). Importantly, patients with various metabolic status will be included in order to perform correlation analysis between jejunal microbiota composition and metabolic health. This research will be carried out on 45 participants. These 45 participants will not be included in the "second part" of the study. No participants, even from the control group will be enrolled in the second part of the protocol. 15 non-obese participants considered "metabolically-healthy" (control group), 15 severely obese participants but without T2D making up in the "obese group" (Ob) and 15 participants who are both severely obese and diagnosed with T2D making up the "obese and diabetic group" (ObD).

The first part is a pilot study. Power calculations are based on results obtained in a previous study from Karine Clément Laboratory. This study compared obese subjects to obese subjects with type II diabetes (Vieira-Silva et al., 2020). In this study, obese subjects with or without type II diabetes had a 25% decreased GM diversity compared to controls. This difference was observed with faecal microbiota samples. Our hypothesis is that GM diversity loss will also be found in jejunal samples. Thus, to obtain 90% power with an α risk of 0,05 (unilateral) and 1-1-ß risk of 0.9, we need 42 subjects (epiR test package 0.9-96). Assuming patient loss should be low, we will thus count 45 subjects.



The second part (COL-MIME): consists of clinical research to collect colonic mucosal biopsies in order to analyse and compare the mucus-associated microbiota to faecal microbiota (within-patients). Importantly, patients with various metabolic status will be included in order to perform correlation analysis between mucus-associated microbiota composition and metabolic health. This research will be carried out on 45 participants. These 45 participants will not be included in the "first part" of the study. No participants, even from the control group will be enrolled in the first part of the protocol. 15 non-obese participants considered "metabolically-healthy" (control group), 15 severely obese participants without T2D making up the "obese group" (Ob) and 15 participants who are both severely obese and diagnosed with T2D making up the "obese and diabetic group" (ObD).

The second part is based on published literature from our collaborator regarding microbiome encroachment into the mucosal layer in people with obesity and T2D. Those studies revealed a difference of 19 μ m (SD=6.75 μ m) of distance of bacteria from intestinal epithelial cells in people with T2D as compared to people without T2D. Based on the data from this published work, a sample size of 13 people per group will be sufficient to detect a mean difference of 10 μ m with 90% power and the significance level of 0.05 (using a one-way ANOVA model followed by 3 posthoc comparisons with Bonferroni adjustment). We selected a conservative estimate of the difference in encroachment as we expect smaller differences in people with obesity only versus type 2 diabetes. We will retain 80% power to detect a 10 μ m change in encroachment with up to a 20% dropout rate, hence justifying the need for 15 participants per group. Given the short duration of the study we are unlikely to have dropouts that will impact the primary endpoint.

Important note: no participants will be enrolled in both parts of the study, even for the control group. Thus our research will collect from samples either for an endoscopy or a coloscopy.

1.8. Microbiota collection:

Microbiota wil be collected during an endoscopy or coloscopy which is planned as routine care for the patients.

- Endoscopy: 1) Control Group: Endoscopy is planned for routine care due to minor epigastralgia.
 2) Ob Group and ObD group: adults with or without obesity, candidates for bariatric surgery (JE-MIME: n=20). Endoscopy is planned as routine care, before surgery.
- Coloscopy: All patients have a coloscopy as part of their cancer screening (routine care).



2. STUDY OBJECTIVES

2.1. Objectif principal

Characterize jejunal (JE-MIME, Part I of the study) and colonic mucosa-associated microbiota (COL-MIME, Part II of the study) and compare both microbiota to faecal microbiota (evaluate differences and similarities between jejunal microbiota or mucus-associated microbiota and faecal microbiota).

2.2. Secondary objectives

-Correlate microbiota with metabolic health and inflammatory markers -Correlate microbiota with lifestyle and neuropsychological health

3. STUDY ENDPOINTS

3.1. Primary study endpoint/outcome

The primary objectives are to compare jejunal and fecal microbiota, mucus-associated and fecalmicrobiota,

• For the 1st part of this project (JE-MIME) - jejunal microbiota

Microbiota will be analysed through 16S rRNA gene sequencing as well as shotgun metagenomic sequencing of the different samples obtained from 1) endoscopy (jejunal fluid) and 2) faecal samples.

• For the 2nd part of this project (COL-MIME) – mucus-associated microbiota

Microbiota will be analysed through 16S rRNA gene sequencing as well as shotgun metagenomic sequencing of the different samples obtained from 1) inner mucus layer from the colonic mucosa, collected through the laser capture microdissection approach and 2) the faecal sample.

3.2. Secondary study endpoints/outcomes

Secondary objectives are to analyse the relationship between jejunal microbiota (part 1) and mucusassociated microbiota (part 2) and metabolic health, inflammation, lifestyle and mental health that will be evaluated through extensive bio-clinical phenotyping as previously described (Alligier et al., 2020; Genser et al., 2018) and will include:

Metabolic health, and inflammation will be assessed to better characterise our subgroups (lean vs obese patients vs obese patients with type II diabetes). In this way correlation between microbiota composition and health can be explored. Lifestyle personal and familial history and psycho-emotional health, as discussed in the introduction, correlate to metabolic health and GM composition and will thus also be evaluated to explore correlation.

- **Metabolic phenotype** (serum): fasting glycemia, insulin, Hba1c, lipid phenotype (Total cholesterol, LDL, HDL, triglycerides), surrogate of insulin resistance (HOMA index), cardiovascular assessment
- Systemic inflammation status and Entero and Neuroendocrine markers (serum and biopsies): (human multiplex: amylin, C-peptide, Ghrelin, GIP, GLP-1, Glucagon, IL-6, Insulin, Leptin, MCP-1/CCL2, PP, PYY, TNFa), circulating LPS levels,
- Anthropometric measurements (i.e. weight, height, body mass index) and body composition, body composition assessed (impedancemetry and DXA).
- **Histological analysis** of the jejunal biopsy obtained during endoscopy and colonoscopy: immunohistochemical analysis of tight junction proteins expression which expression is



correlated to low-grade inflammation and LPS levels (ZO-1, Occludin, Claudins) (Genser et al., 2018; Everard and Cani, 2014; Larraufie et al., 2018).

- Lifestyle factors collected through lifestyle questionnaires:
 - o Medical history questionnaires: Weight history, family history of obesity and diabetes,
 - Dietary patterns: Nutrition and food intake will be assessed semi-quantitatively through online Food Frequency Questionnaires (FFQs) and consecutive self-administered webbased 24-hour dietary recalls. Emotional and binge eating behaviors will be assessed through Binge Eating Scale (BES), Dutch Eating Behaviour questionnaire (DEBQ). Alcohol use will be assessed with the Alcohol Use Disorders Identification Test (AUDIT)
 - **Physical activity:** Physical Activity through the modified Recent Physical Activity Questionnaire (RPAQ).
 - Smoking habits: Fagerstrom Questionnaire
 - Psycho-emotional health and Quality of Life: Neuropsychological Parameters will be assessed through the following standardized questionnaires: Quality of Life Questionnaire (QoL EQ-5D-5L), Beck Depression Inventory Questionnaire (BDI-II), Hospital Anxiety Depression (HAD) Perceived Stress Scale (PSS10), Stop-Bang questionnaire, Pittsburgh Sleep Quality Index (PSQI).

4. STUDY DESIGN

4.1. Study type

This research is a research involving humans with minor risks and constraints without medical product. The design is a non-randomized comparison among 3 groups: "Control Group », Obese group (Ob), Obese and Type 2 Diabetes group (ObD).

All participants are adult volunteers. The research is mono-centric and will be held at the Hôpital des Peupliers in France.

4.2. Experimental plan

This research is a non-randomized comparison between parallel groups followed in the context of their usual care.

4.3. Study duration (Flow Chart)

• For the 1st part of this project - jejunal microbiota

Figure 5: Study Timeline: Patient's Journey

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	Endoso Peuplier H			
		Endoscopy: Post-pyloric aspirati collection of 2 jejuna		
Inclusion Visit		12 pm Medical Visi	•	
inclusion visit	8 am	incurrent fior		
Faecal collection Online questionnaires	sample Feces, u	on of biological s: urines, saliva and ollection	Bio-Clinical Phenotyping: Anthropometric measurements and questionnaires.	
	Patient's arrival Peuplier Hospital		al Phenotyping ier Hospital	

Inclusion-period will last one year for the Ob and ObD group and two years for the control group. In total, the clinical research will last two years.

For each participant, the study is based on a day and a half of clinical investigation. Nevertheless, the patients are expected to come three times as expected by their routine care: for their inclusion in the protocol (V0; 1 hour-long visit) after the prior consultation with Dr Donatelli (i.e. before endoscopy/colonoscopy), the clinical-investigation visit (V1; one-day visit, approximately 8 hours), this V1 also corresponds to the end of the research for the participant. However, they will meet with Dr Donatelli in their care routine, two weeks after the procedure. During this follow-up visit the patient will receive results provided by the endoscopist. **Research investigation will be coupled to the standard care visits. No visits will be added for research purposes.**

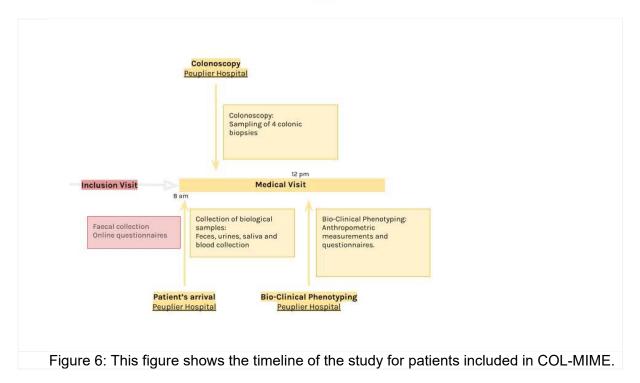
Groups of recruited patients will be matched as to age and sex.

In total, the clinical research will last 25 months.

Participants will be asked not to take part in the inclusion process of another study while participating in this study.

• For the 2nd part of this project – mucus-associated microbiota

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Inclusion-period will last one year for the Ob and ObD group and two years for the control group. In total, the clinical research will last two years.

For each participant, the study is based on a day and a half clinical investigation. The patients are expected to come twice: for their inclusion in the protocol (1 hour-long visit) and for the clinical-investigation visit (one-day visit, approximately 8 hours), depending on the patient's need). Research investigation will be coupled to the standard care visits.

Groups of recruited patients will be matched as to age and sex.

In total, the clinical research will last 25 months.

Participants will be asked not to take part in the inclusion process of another study while participating in this study.

Total duration of the study: 25 months and 6 months of analyses.

5. STUDY ENROLLMENT

5.1. Study population

Participants who will meet with Dr G.Donatelli for a clinical consultation either for an endoscopy (JE-MIME) or colonoscopy (COL-MIME). These patients can be referred by Prof. K. Clément* or Dr. L.Genser* for an endoscopy prior to bariatric surgery (Nutrition dept., Pitié-Salpêtrière Hospital, IE3M building). Dr G. Donatelli, ,however, will perform the inclusion.



JE-MIME (n=45)	COL-MIME (n=45)
Control group (n=15):	Control group (n=15):
Lean adults, care routine endoscopy	Lean adults, care routine
for minor epigastralgia.	colonoscopy for cancer screening.
Ob group (n=15):	Ob group (n=15):
Adults with obesity, care routine	Adults with obesity, care routine
endoscopy prior to bariatric surgery.	colonoscopy for cancer screening.
ObD group (n=15):	ObD group (n=15):
Adults with obesity and T2D, care	Adults with obesity and T2D,
routine endoscopy prior to bariatric	care routine colonoscopy for cancer
surgery.	screening.

Note: participants will only be recruited to one part or the protocol : JE-MIME *or* COL-MIME. This is also the case for participants from the control group.

5.2. Critères d'inclusion

- Affiliation with French national health scheme (securité sociale) or beneficiary of equivalent scheme. Additional Inclusion criteria are listed in table 1.

-

Table 1: Inclusion Criteria

General Inclusion Criteria:

- Aged between 18 and 60 years;
- Ability to understand and provide informed consent (in French);
- Ability and willingness to meet the required schedule and study procedures;

Group-Specific Inclusion Criteria:

Control Group

Inclusion criteria for the participants from the Control group are:

- Presence of Gastroesophageal Reflux Disease symptoms or gastric discomfort.
- BMI [19kg/m² < BMI <25 kg/m²]
- Match age and sex to the patients from the Ob and ObD groups.
- Only patients with normal glucose tolerance (NGT), glucose tolerance (IGT) and/or fasting glucose (IFG) and an HbA1c < 6.5 % will be included in this group.

Ob Group

Inclusion criteria for the participants from Ob and ObD groups are:

Candidate for bariatric surgery and meet the HAS criteria (Haute Autorité de Santé, 2009) : o IMC ≥ 40 kg/m² without comorbidities

<u>Or</u> IMC≥ 35 kg/m² with at least one obesity-related comorbidity (i.e.: Hypertension, Dyslipidemia, Obstructive Sleep Apnea, Joints Disease, non-alcoholic steatohepatitis, excluding T2D)

• Weight stable for at least 2 months



- No treatment for metabolic health complications, no previous obesity surgery, no obesity treatment drug.
- No monogenic form of obesity (Hebebrand et al., 2017)

ObD Group

Inclusion criteria for the participants from ObD groups are:

- Candidate for bariatric surgery with T2D
- At least one obesity-related comorbidity including T2D
- Fasting plasma glucose (FPG) ≥7 mM (=1.26g/l) or
- Participants with HbA1c \geq 6.5% (48 mmol/mol)
- All stages of albuminuria

5.3. Non-inclusion criteria

Exclusion criteria are listed in table 2.

Table 2: Non-Inclusion Criteria

General Non-inclusion Criteria:

- Treatment for the previous 12 week that could
 - o alter gastrointestinal motor function (e.g. opioids, prokinetics, anticholinergics, laxatives),
 - o acidity (PPI, H2RA)
 - microbial population (e.g.: antibiotics, probiotics)
 - o immunosuppressants (eg: calcineurin inhibitors, corticosteroids, biological agents, etc.).
 - o use of weight-loss drug or dietary intervention aiming to lose weight;
- Altered anatomy of the esophagus, stomach, small or large intestine due to prior gastrointestinal surgery (exceptions include appendectomy or cholecystectomy more than 3 months prior to enrollment) or other reasons;
 - Any abdominal or pelvic surgery within the past 3 months;
 - o Diverticulitis, diverticular stricture, or other intestinal strictures.
 - Intestinal resection of the gastrointestinal tract
- Previous history of gastric bezoar or gastroparesis
- Acute or chronic inflammatory bowel disease or infections diseases (i.e.: VHC, VHB, VIH, etc.)
- Abdominal or pelvic radiotherapy or abdominal cancer
- Colorectal cancer, either known or not
- Dysphagia, eosinophilic esophagitis, esophageal stricture, or other swallowing disorder
- Organ Transplantation and patients on Immunosuppressive Therapy
- Severe kidney failure and/or patients on dialysis therapy (serum creatinine > 150 µmol/l or eGFR < 60 ml/min per 1.73 m² body surface area)
- CVD, endocrine, renal or other chronic disease likely to affect motility.
- Colon cleansing preparation during the last 1 month
- No < 3 bowel movements per week
- Females of childbearing age who do not practice birth control and/or are pregnant or lactating
- Participants non-affiliated to the French national health scheme
- Participants who are already included in a clinical study which implies testing any pharmaceutical drug.
- Participants who do not understand the research procedures those who are institutionalized, or who unable to give informed consent
- Participants placed under legal protection
- Patients with drug addiction
- Antibiotherapy 3 months preceding the endoscopy
- Weight variation (diminution or increase) > 5kg in the last 3 months



Specific non-inclusion Criteria

Ob and ObT2D Group

• Usual contraindication for bariatric surgery;

5.4. Modalités de recrutement

- Usual consultation for endoscopy ; endoscopy and colonoscopy consultations with Dr Donatelli (Hôpital des Peupliers); Brochures in consultation rooms and social media
- Patients eventually referred by clinical collaborators and digestive surgeons: Dr Laurent Genser and Prof. Karine Clément (Pitié Salpêtrière hospital) with brochure given to the patient

6. STUDY PROCEDURES, SCHEDULE AND WITHDRAWAL

6.1. Selection

Participants from all groups who need an endoscopy (part 1) or a colonoscopy (part 2) for medical care will be recruited from the Endoscopy department (Hôpital des Peupliers; Ramsay-GDS, Paris, France). Recruitment brochures will also be posted in our collaborators' centres and on their social media and participants will be able to apply spontaneously to participate in the study by answering those recruitment brochures.

During the information consultation planned before the endoscopy or colonoscopy visit as planned for the patient care routine, Dr Donatelli will inform the participants of the protocol giving and referrals will be forwarded to the clinical coordinator with permission from the patients. Potential participants will be contacted by the clinical coordinator and asked if they would like to participate in the study. If the participant accepts to enrol in the study, the inclusion process will be held by Dr Donatelli.

6.2. Methods of information and participation agreement

All patients with obesity are candidate for bariatric surgery and their care includes endoscopy. These patients will be prospected and considered candidates as subjects in this study. The selection will be carried out by Pr. Karine Clément, Dr. Laurent Genser or Dr. Gianfranco Donatelli (i.e. generally seen for routine visit) or referred by other colleagues (PUPH). Spontaneous participants matching the profile will need to call Prof. Karine Clément or Dr Laurent Genser before the V0.

Together, the patient and the doctor will review the inclusion criteria, discuss the protocol and the participant will be able to ask questions. Afterwards, if the patient meets the criteria and is still interested to meet the doctor, they will schedule the V0 (i.e. Participant will then receive a copy of the information and consent form through email).

During the consultation (V0), which is also the consultation prior to their endoscopy / coloscopy, the participants will go through the inclusion process.

The inclusion visit will be held at Hopital Privé des Peupliers (Ramsay, Générale de Santé, 75013, Paris, France) by Dr Gianfranco Donatelli and Pr Karine Clément. The patient will be given final oral and written information, all potential risks will be discussed and any questions will be thoroughly answered by the doctor. Time will be given to the patient to make his/her decision.

If the patient agrees to participate in the study, he/she will sign the consent form and will be included in the clinical research.

Afterwards, the patients will be provided with all necessary information about the following topics:

- The protocol: reception and review of the information notices, planning the visits.
- **The different biological collections**: reception and reviewing of the written information for faeces collection. Reception of the appropriate kits to collect the samples at home.



- Fecal Sampling: The fecal sample kit will be given in advance as it is to be expected that these collections will be difficult to obtain on a unique day of investigation. Patients will be asked to sample one spoon of faeces in the two days preceding the medical visit and to store this faeces sample at home until their visit at the clinic. Moreover, the remaining of the stool sample produced by the participant will be collected and a courier will come to the participant's address and transport this sample to Dr. Benoit Chassaing's laboratory for appropriate aliquoting under anaerobic conditions and storage at -80C. On the day of the visit, we will collect total feces. All material needed for this faecal collection will be provided to the participants.
- Endoscopy procedure: Gastric/duodenal endoscopy will be performed as part of the patients' care examinations (epigastralgy or prerequisite for bariatric surgery) at the Hospital des Peupliers. Jejunal aspirate will collect all gastroduodenal fluid (2-3 mL). 2 mucosal biopsies will be collected and frozen at -80°C (1 biopsy) or placed in Carnoy fixative for histological examination (1 biopsy).
- **Bioclinical Phenotyping: He/she will receive** an anonymized unique link for connection on a dedicated patient-website where he/she will complete the questionnaires (survey). They will not have to create an account to keep their identity anonymous.
- A "**web food pattern diary**" (at home). Answers will be reviewed by the dietician with the patient during the medical visit.
- Online **environmental, psycho-social and physical activity questionnaires**, that will need to be completed from home

• For the 2nd part of this project – mucus-associated microbiota

The inclusion visit will be held at Hopital Privé des Peupliers (Ramsay, Générale de Santé, 75013, Paris, France) by Dr Gianfranco Donatelli and Pr Karine Clément, during a consultation day. The patient will be given final oral and written information, all potential risks will be discussed and any questions will be thoroughly answered by the doctor. If the patient agrees to participate in the study, he/she will sign the consent form and be included in the clinical research.

The "*dossier source*" will include the date at which the patient signed the agreement and the date of termination of his/her participation.

Afterwards, patients will be provided with all the necessary information about the following topics:

- The protocol: reception and review of the information notices, planning the visits.
- **Feces collections**: reception and reviewing of the written information for <u>faeces collection</u>. Reception of the appropriate kits to collect the samples at home.
- Fecal Sampling: The fecal sample kit will be given in advance as it is to be expected that these collections will be difficult to obtain on a unique day of investigation. Patients will be asked to sample one spoon of faeces in the two days preceding the medical visit and to store this faeces sample at home until their visit at the clinic. Moreover, the remaining stool sample produced by the participant will be collected and a courier will come to the participant's address and transport this sample to Dr. Benoit Chassaing's laboratory for appropriate aliquoting under anaerobic conditions and storage at -80C. On the day of the visit, <u>the fecal kit will be collected</u>. All material needed for this faecal collection will be provided to the participants at V0.Biological collection will be made during the colonoscopy procedure: reviewing of the written information for <u>the colonic mucosal biopsy collection</u>.
 - Colonoscopy procedure: Colonoscopy will be performed as part of the cancer screening protocol at the Hospital des Peupliers. During colonoscopy, 4 mucosal colonic biopsies will be collected and and placed in bacterial medium (1 biopsy), frozen at -80°C (1 biopsy), or placed in Carnoy fixative for histological examination (2 biopsies). All cancer positive patients will be excluded from the study.
- **Bioclinical Penotyping:** An anonymized unique link for connection on a dedicated patientwebsite to access the questionnaires:
 - A "**web food pattern diary**" (at home). Answers will be reviewed by the dietician with the patient during the medical visit.
 - Online environmental, psycho-social and physical activity questionnaires (cfr REDCAP mask), to be completed from home.



6.3. Inclusion and follow-up visits

6.3.1. Information consultation and Inclusion Visit (V0)

V0 corresponds to the **information consultation** before the endoscopy or colonoscopy visit as planned for the patient care routine.

Before this V0 visit Dr Donatelli will inform, the potential participants of the protocol details and referrals will be forwarded to the clinical coordinator with permission from the patients. Potential participants will be contacted by the clinical coordinator and asked if they would like to participate in the study. If the participant accepts to enrol in the study, the inclusion process will be held by Dr Donatelli during the information consultation (V0) prior to the endoscopy or colonoscopy.

Our PhD student will then meet the patient after she/he signs the informed consent form. She will share sampling materials and give them information for 1) faeces collection using the sampling kits provided and 2) transporting the fresh faeces through courier to our research units (the patient will have to inform the PhD student of the collection and she will organize the pick-up and transport of faeces to our unit). She will also give information regarding the questionnaires the patient will have to auto-complete between V0 and V1.

The V1 will be planned 1 week to 1 month after V0. During this time, the PhD student will follow up questionnaire completion and will be available through mail and phone calls to answer any questions the participants may have. She may spontaneously call a participant if he/she does not complete the questionnaires.

<u>6.3.2. V1: endoscopy or colonoscopy procedure and end of the research for the participant (V1)</u>

As planned for the patient care routine, the participants will arrive at the Hospital des Peupliers after a 12h fast. Patients will not brush their teeth on the morning of the procedure. Patients will handin the faeces sample in the kit with conservatives sampled 24h before the procedure. Blood sampling, urine collection body composition and anthropometric measurements will be made before the endoscopy or colonoscopy procedure. After the procedure, patients have to stay under medical surveillance for 4 hours. During this time, if the patient agrees, the PhD student will check the patient's questionnaires with them one last time will end the visit and hand in the information to receive her compensation.

	Visite 0	Visite 1
Place	Peupliers	Peupliers
Date	1 week to 1 month before V1	1 week to 1 month
		after V0
Consent	Yes	Given at V0
Biological samples	No	Yes
Questionnaires	No (between V0-V1)	No
Clinical examination	No	Yes



Visite 1: Clir	nical examination (post-endoscopy)
Care/Research	Medical Act: Type of clinical examination
Research	<u>Anthropometric measurements :</u> Neck circumference, Waist circumference, Hip circumference, sagittal diameter, weight, height, BMI
Research	Body compositione: Fat Mass / Fat Free Mass DER prediction and body composition with impedance
Research	<u>Cardiovascular evaluation :</u> - Blood pressure: average of 3 measurements on both right arm and left arm - Heart rate (beats / min) - hemostasis assessment (TP-TCA)

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Questionnaires auto-completed between V0 and V1 as part of the research
List of questionnaires (names of questionnaires , if standardized)
Environment and context
Personal and familial medical history of obesity and type II diabetes
General information that may affect the gut microbiota: mode of delivery, breastfeeding, ethnic origin (Reyman et al. 2019. Nature; Gaulke & Sharpton. 2018. Nature).
Drugs ((Bryrup et al. 2019))
Quality of Life and Handicap (Quality of Life, "QoL EQ-5D-5L")
Lifestyle and behavior
Nutrition:Food Frequency Questionnaire and Dietary habitsSelf-administered web-based 24h dietary recallsEmotional eating behaviour(Food Frequency Questionnaire, FFQ; Binge Eating Scale, BES; Dutch Eating BehaviourQuestionnaire, DEBQ)
Physical Activity (Paffenbarger Physical Activity Questionnaire, RPAQ)
Sleep Sleep apnea Sleep Quality Chronotype (STOP-BANG Questionnaire, Pittsburgh Sleep Quality Index, PSQI; Horne & Ostberg Questionnaire)
Smoking habits
Digestifs
Digestive symptoms Bristol Score
Stress and other psychological variables
Smoking habits Fagerstrom Questionnaire
Perceived stress (Perceived stress scale)
Anxiety and Depression (Hospital Anxiety and Depression, HAD; Beck Depression Inventory, BDI-II)

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			Type of analysis	CBC (NFS in french)	lonogram	Glycated Hemoglobin	Fasting glycemia	Lipid profile and abnormalities	Multiplex human metabolic hormone*	Leptin, Adiponectin		lonogram		metagenomics	Analysis on faecal water		metagenomics	Metabolic multiplex	Histology	ltérior). , TNFa)
		Blood test taken as part of the treatment, (before endoscopy, on an empty stomach)	Analysis (Immediate/Ulterior)	i (Peupliers)	i (Peupliers)	i (Peupliers)	i (Peupliers)	i (Peupliers)	u (UMRS1269)	u (UMRS1269)	λ	u (UMRS1269)		u (UMRS1269)	u (U1016)	ltine)	u (U1269)	u (U1269)/ (U1016)	u (U1269)/ (U1016)	: С (Care), R (Research), Y (Yes), N (No), I (Immediate), U (Ultérior). GLP-1, Glucagon, IL-6 Insulin, Leptin, MCP-1/CCL2, PP, РҮҮ, TNFa)
henotyping	Idoscopy	, (before endosco	Quantity	2mL	2mL	2mL	2mL	2mL	10mL	5mL	Urine sampling before endoscopy	4mL	Stool Sampling	1 spoon	Total	oscopy / Colonoscopy (Care routine)	3mL	1cm2	1cm2	Research), Y (Yes on, IL-6 Insulin, Le
V1 : Bio-Clinical Phenotyping	Before endoscopy	t of the treatment	tubes (n)	-	~	~	-	~	~	~	Urine sampling	-	Stool	~	-	Endoscopy / Colon	~	1/2	1/2	lu: C (Care), R (F , GLP-1, Glucago
		Blood test taken as part	on empty stomach	yes	yes	yes	yes	yes	yes	yes		oui		DU	no	ū	Yes	Yes	Yes	Abréviations spécifiques au tableau: * (amylin, C-peptide, Ghrelin, GIP, C
			Sample	blood	blood	Plasma	Serum	Serum	plasma	Serum		urine		stool	stool		Jejunal fluid (NA coloscopie)	Biopsies	Biopsies	Abrévia * (am)
			Care /Research	U	U	£	ĸ	£	к	£		ĸ		£	£		٣	ĸ	£	

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6.4. Discontinuation/Withdrawal from the research

Investigators will terminate the research if the endoscopy or colonoscopy is cancelled. The investigators might terminate the research if the patient is diagnosed with a disease or start a new treatment (cfr exclusion criteria) between V0 and V1. The participant will be excluded if a colorectal cancer is identified during the colonoscopy procedure planned at V1.

There is only one visit with biological sampling. Thus, after V1, patients can start new treatments. Participants can terminate the research without justification.

Following termination, investigators will include another patient. Two patients can be included to replace lost patients. Expectations for termination are low.

6.5. End of research

6.5.1. Definition of the end of the research

End of research corresponds to the last analysis: 6 months after the last visit of the last patient.

6.5.2. Description of the rules of definitive or temporary stop of a part or of all of the research

In accordance with article R1123-26 of the Public Health Code, if the research has not started (ie if inclusions have not started) within two years of obtaining the favorable opinion of the CPP and the authorization of the ANSM, the agreement of the committee / authorities will be considered null and void. However, on justification produced before the expiry of the said period, it may be extended by the committee concerned. This extension request, presented by the Promoter to the committee and to ANSM, must be accompanied by a letter justifying the delay in relation to the initial provisional schedule. In the absence of agreement on the extension request, the research must be resubmitted to the CPP and ANSM as part of an initial submission.

During the research, if the pace and number of inclusions appear insufficient, Inserm may decide to stop it if no other solution can be considered

The sponsor and the competent authorities may interrupt the research for any other justified reason (major deviations from the protocol that do not guarantee the safety of the participants, the quality of the data and the results of the research).

Bio-Clinical Phenotyping - Data collection Visit 1: Biological Samples (before endoscopy)) Blood Care/Resea Samples Biobank Storage Place of rch Storage С sampled for care routine 1 1 no С sampled for care routine 1 1 no R Plasma 1 1 no R Serum 1 1 no R Serum 1 no 1

7. COLLECTION OF HUMAN BIOLOGICAL SAMPLES



R	Plasma	yes	-80°C	UMRS 1269
R	Plasma	yes	-80°C	UMRS 1269
R	Serum	yes	-80°C	UMRS 1269
		Urines		
R	Urines	no	-80°C	UMRS 1269
		Microbiota		
R	Faeces (in conservative)	yes	-80°C	UMRS 1269
R	Faeces (fresh total)	yes	-80°C	UMRS 1016
V	isit 1: biological sampling duri	ng endoscopy, wh	ich is part of care r	outine)
		Microbiota		
R (waste)	Jéjunal fluid(waste)	yes	-80°C	UMRS 1269
	Bio	psies jéjunales		
R (waste)	Jéjunal/colic biopsies	oui	-80°C	u (U1269)/ (U1016)
R (waste)	Jéjunal/colic biopsies	oui	On paraffin - 20°C	u (U1269)/ (U1016)

7.1. Sampling conditions

A nurse will carry out the blood sampling. This act is part of the patient's routine care prior to endoscopy/coloscopy on V1. In addition to the tubes collected for routine care, we take 5 additional tubes.

The endoscopy/coloscopy is part of routine care. This act will be carried out by the endoscopist. We will collect waste (jejunal fluid). Two biopsies are already planned as part of the patient's routine care, we will collect two additional biopsies for the JE-MIME part of the research and four additional biopsies for the COL-MIME part.

Fecal and urinary samples are collected by the participant for research only.

All samples will be collected at the Hôpital Privé des Peupliers except for the faecal sample, which will be collected at the patients' home.

7.2. Sample coding and labelling procedures

Biological samples will be collected and labelled directly by our PhD student, Emilie Steinbach, following this pattern: the abbreviation JE (jejunum) or COL (Colon), the patient number (i.e.: 01 = First patient included), Name and Surname initials (i.e.: Steinbach Emilie= SE).

[JE/COL – patient number – first surname and main name initials]

Example: Emilie Steinbach, first patient of the cohort of the JE-MIME study is labelled: [JE-01-ES].

Etude JE/COL-MIME (C20-86)
Participant COL-[][]-[][]-V1
Date de prélèvement : L JL J/L JL J/L JL J
Promoteur: Inserm

,	Etude JE/COL-MIME (C20-86)
P	articipant JE-{]{]-[].]-V1
Date de	prélèvement : [] []/ [] []/ []/ []/ []/ []/
	Promoteur: Inserm

7.3. Sample processing procedure

Samples will be processed either immediately at the Hôpital des Peupliers or immediately transported, on ice, to the laboratories of Karine Clément and Benoit Chassaing for analysis.

7.4. Sample shipment procedure



Blood samples will be collected by a nurse at Peuplier hospital. Each biological sample collected for ulterior analysis will be transported directly by our PhD student to the UMRS 1269 in packed ice with a secured box.

Only the fresh fecal samples (to be transported from the participant's home by carrier) and colonic biopsies for the COL-MIME study will be transported to Chassaing Lab at Cochin (see table in the next section).

	Bio-Clinical Phe	enotyping - Data	a collection	
	Visit 1: Biological	samples (befo	re endoscopy)	
		Blood		
Care /Research	Type of sample	Biobank	Storage	Storage place
С	For care routine	no	/	/
С	For care routine	no	/	/
R	Plasma	no	/	/
R	Serum	no	/	/
R	Serum	no	/	/
R	Plasma (with inhibitor DPP4)	yes	-80°C	UMRS 1269
R	Plasma	yes	-80°C	UMRS 1269
R	Serum	yes	-80°C	UMRS 1269
		Urine		
R	Urines	yes	-80°C	UMRS 1269
		Microbiota		
R	Faeces (in conservative)	yes	-80°C	UMRS 1269
R	Faeces (fresh total)	yes	-80°C	UMRS 1016
Visit 1	: biological sampling durir	ng endoscopy, ^v	which is part of ca	re routine)
		Microbiota		
R (waste)	Jejunal Fluid	yes	-80°C	UMRS 1269
	Bioj	osies jéjunales		
R (waste)	Jéjunal/colic biopsies	yes	-80°C	U1269/1016
R (waste)	Jéjunal/colic biopsies	yes	On paraffin- 20C	U1269/1016



7.5. Sample storage procedure and/or destruction

Biobank condition and location are listed **above**. These samples are under the responsabilité of Prof. Karine Clément. These samples will be stored for 10 years and destroyed afterwards.

If at the end of the research, conservation of the remaining biological elements is planned, the CODECOH approach will be implemented in accordance with the provisions of Article L.1243-3 of the Public Health Code.

7.6. Storage safety procedure

- The preparation and packaging of samples is carried out in the U1260 UMRS room with a Microbiological Safety Station (PSM II). Each anonymized sample is packaged in cryotubes.

- The samples are prepared in such a way as to limit any risk of contamination and to meet the objectives of maintaining their quality in the long term.

- The samples are stored in a dedicated -80 ° C freezer located at 91 bd of the hospital (the cryotubes are stored in identified freezer boxes). An alarm system is installed and connected to a guard system. On-demand destocking is carried out under conditions allowing cryopreservation (use of dry ice) of the samples. Samples are always handled under PSM.

-The biological resources received, stored and made available are of human origin linked only to the cohort. The prepared collections are stored in the bank.

-The methods of preparation and storage of samples are validated and monitored.

-The quality management system is being considered. It is already periodically analyzed and updated in order to monitor the changing needs and expectations of potential users of biological resources.

-The staff working in the bank are competent and informed: the responsibilities of each are identified and known; activities are organized into monitored and constantly improving processes; the premises and equipment are adequate and supervised; the information system allowing the traceability of biological resources is secure; (Each staff member has his own username and password and can connect to the documentary database: anonymous Excel files);

regular and facilitated internal communication; the quarterly bioresource committee verifies these quality procedures.

-The -80 ° C storage room is air-conditioned (to avoid overheating which could lead to engine failure)

-An alarm system (ORTES company) equips each thermostatically controlled enclosure (-80 ° C and - 20 ° C freezer) of the unit. A temperature reading with calibrated probes is carried out live and alarm thresholds have been defined.

In the event of an alarm, the procedure triggers a message on 3 mobile phone numbers of identified personnel of the unit. These people can intervene on the spot as quickly as possible.

-Implementation of an empty emergency -80 ° C freezer on site (allows sample transfer in an optimized and satisfactory time, without affecting the quality of biological resources).

- The equipment is subject to regular maintenance thanks to a contract renewed each year.

-The contact details of the company responsible for the maintenance of -80 ° C are displayed in the storage room (Ortes SARL company).

All written data is locked in a locked shelf in Dr Donatelli office at the Hopital des Peupliers.

8. SAFETY ASSESSMENT

8.1. Definitions

A serious adverse event/reaction refers to any untoward medical occurrence or reaction that at any dose:

results in death;



• is life-threatening (means that the subject was at immediate risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe);

- requires hospitalization or prolongation of existing hospitalization;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect;
- is a grade 4 biological or clinical event;

• is an "important medical event" (medical events, based upon appropriate medical judgment, which may jeopardize the subject or may require medical or surgical intervention to prevent one of the above characteristics/consequences). Examples: allergic bronchospasm requiring intensive treatment at an emergency room or at home, blood dyscrasias, convulsions that do not result in inpatient hospitalization.

8.2. Responsabilities of the investigator

The investigator should report to the sponsor, without delay (pharmacovigilance.prc@inserm.fr) any serious adverse event possibly related to the trial procedures (i.e. blood, urine and feces samples) and any new fact, i.e. any safety data that could modify significantly the evaluation of the benefit/risk ratio of the clinical trial, likely to affect the safety of participant, the trial documentation or the conduct of the trial.

The investigator should notify adverse reactions related to any health product to the competent authoritie according to the applicable regulation (http://social-sante.gouv.fr/grands-dossiers/signalement-sante-gouv-fr).

8.3. Potential risks of the research and management guidelines in case of adverse event

This research does not include any risk already caused by the procedures planned as part of the patient care routine.

9. COLLECTION AND PROCESSING OF DATA

The sole purpose of processing the personal data of persons participating in this research is to carry out the research. This processing includes the management of data relating to persons suitable for research, with a view to enabling the collection, entry, checking of validity and consistency and statistical analysis of the data collected during the research.

Regarding the responsibilities related to the processing:

The processing of personal data necessary for the implementation of the study is placed under the responsibility of the National Institute of Health and Medical Research (Inserm). It responds to the performance of a public interest mission vested in Inserm (RGPD, art. 6.1.e) which justifies the processing of participants' personal health data for scientific research purposes (RGPD, art. . 9.2.j). This processing is carried out in accordance with the provisions of Law No. 78-17 on computers, files and freedoms and the General Data Protection Regulation (Regulation (EU) 2016/679).

Regarding the applicable standards, data processing is governed by:

- Regulation (EU) 2016/679 of April 27, 2016, known as the General Data Protection Regulation (Regulation (EU) 2016/679);
- Law n ° 78-17 of January 6, 1978 (78-17), relating to data processing, files and freedoms as well as by the law of June 7, 1951 on the obligation, coordination and secrecy in matters of statistics (n ° 51-711) which regulate the collection and use of personal data in the context of this study



- Decree No. 2019-536 of May 29, 2019, as amended, made for the application of the law of January 6, 1978 relating to computers, files and freedoms;
- the provisions of the public health code relating to research involving humans.

All the information required by the protocol will be recorded in an electronic logbook (e-CRF: redcap). Consistency tests are implemented according to a pre-established validation plan (i by the investigators at UMRS 1269. For any inconsistencies detected, requests for corrections are sent to the investigator. The corrections made by the investigator are verified by the clinical investigator and validated. Adverse events and medical history are coded using the MedDRA® dictionary and associated

treatments are coded using the WHO drug dictionary. Computer data files, as well as any changes made to them, are saved and retrievable on demand in a directly usable form. The "data management plan" is the reference document that describes all the procedures for data management.

The anonymised data are entered and stored in network information system of UMRS 1269 under the supervision of Mr Flavien Jacques (Bioinformaticien engineer).

All data recorded in the electronic logbook is reviewed by Florence Marchelli and Prof Karine Clément (nurse at the laboratory).

9.1. Description des données recueillies

No nominative data is collected as part of this research project.

Table 3: Core Phenotyping
Patient's clinical history (see Redcap mask)
1) Socio-demographic data
- Gender
- Year of birth
 Ethnicity, Country of parent's birth*
 Birth: type of delivery* (natural or C-section)
- Breast fed or not*
- Number of Children and Siblings
 Main professional occupation, retirement status
- Working physical activity
- Income /Year
- Years of education
*this can affect microbiota composition (Reyman et al. 2019. <i>Nature</i> ; Gaulke & Sharpton. 2018. <i>Nature</i>).
2) Weight History
- Obesity in childhood i.e. before 13 (Yes/No)
- Obesity in adolescence ie:13-18 (Yes/no)
- Weight at 20 years old
- Maximal weight reached during adulthood
- Number of diets (questionnaire)
3) Familial Medical History
- Focus on CVD risk factors
- Hypertension in the family
- Hyperlipidemia in the family
- Smoking in the family
- Diabetes in the family (type of diabetes, duration of diabetes, complications)
- Focus on family CVD events - Stroke
- Myocardial Infarction
4) CVD-risk Factors



Dishataa
- Diabetes
- Hypertension
- Smoking
- Dyslipidemia
 Early-onset CVD diseases in the family
5) Past Medical History
- Chronic diseases
- Past surgery, type of surgery
- Tumor of the Bowels
- Infectious diseases needing treatment with antibiotics
6) Current drug therapy (cf Redcao mask)
Lipid Lowering Agents:
Antidiabetics:
CVD acting Drugs:
Other drugs
7) Antibiotics
8) Stool Habits
- Stool Frequency
- Stool Regularity
- Bloating / Flatulence
- Bristol scale
9) Anthropometry and body composition
- Height (cm)
- Weight (Kg)
- BMI
- Waist circumference
- Hip circumference
- Recorded impedance (ohms)
- Fat mass (kg)
- Fat-free mass (kg)
- Fat-free mass (kg)
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm / mmhg Left arm: / mmhg
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn)
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest)
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires See REDCAP mask
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires See REDCAP mask
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 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires See REDCAP mask 12) Fasting Biological Blood Samples Usual Care General Samples: White Blood Count +Hb + platelets +TCA Liver function (ALT, ASAT, GGT)
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 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires See REDCAP mask 12) Fasting Biological Blood Samples Usual Care General Samples: White Blood Count +Hb + platelets +TCA Liver function (ALT, ASAT, GGT) Creatinin, urea TSH
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires See REDCAP mask 12) Fasting Biological Blood Samples Usual Care General Samples: White Blood Count +Hb + platelets +TCA Liver function (ALT, ASAT, GGT) Creatinin, urea TSH Serum albumin
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 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg Left arm:/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires See REDCAP mask 12) Fasting Biological Blood Samples Usual Care General Samples: White Blood Count +Hb + platelets +TCA Liver function (ALT, ASAT, GGT) Creatinin, urea TSH Serum albumin Basal Metabolic Profile Fasting lipids (CT, HDL-c, TG, calculated LDL), Uricemia
 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg Left arm:/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires See REDCAP mask 12) Fasting Biological Blood Samples Usual Care General Samples: White Blood Count +Hb + platelets +TCA Liver function (ALT, ASAT, GGT) Creatinin, urea TSH Serum albumin Basal Metabolic Profile Fasting lipids (CT, HDL-c, TG, calculated LDL), Uricemia Fasting glycemia, Glycated Hb
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 Fat-free mass (kg) 10) Clinical Evaluation Blood pressure: right arm/ mmhg Left arm:/ mmhg As defined by SOP that is going to be made Ankle Brachial blood pressure ratio Heart rate: (beat/mn) Oxygen Saturation in lying and seated position Symptoms of NYHA Oedema (legs, chest) 11) Questionnaires See REDCAP mask 12) Fasting Biological Blood Samples Usual Care General Samples: White Blood Count +Hb + platelets +TCA Liver function (ALT, ASAT, GGT) Creatinin, urea TSH Serum albumin Basal Metabolic Profile Fasting lipids (CT, HDL-c, TG, calculated LDL), Uricemia Fasting glycemia, Glycated Hb



Research
Adipokines
- Leptin,
- Adiponectin,
Inflammatory Markers
 Multiplex human metabolic hormone
(amylin, C-peptide, Ghrelin, GIP, GLP-1, Glucagon,
IL-6 Insulin, Leptin, MCP-1/CCL2, PP, PYY, TNFa)
Fasting Samples
- 1 tube EDTA (plasma) 7 ml
- 1 tube (serum) 7 ml
 1 tube with DPP4 inhibitor 3 ml (for the multiplex)
Samples done whatever the time of day
 Urine 5ml 2 tubes - Immediately stored at -80°c
Stool Sample: 6g
- 6 spoons of 1g – intermediately stored at -20°C and then at -80°C.

9.2. Data source definition

Source data is all the information appearing in original documents, or in authenticated copies of these documents, which relate to clinical examinations, observations or other activities carried out in the context of research involving the human person and which are necessary for the reconstruction and evaluation of the research. The documents in which the source data are recorded are called the source documents, whatever the medium used (paper, electronic, etc.).

The data generated as part of the treatment as well as the blood and urine results are in paper version in the source file, under the protection of Dr Donatelli. The metagenomic data generated as part of the research is entered in the patient's e-CRF file because there is no paper source possible (big data).

9.3. Data workflow

No identifying data is entered or stored.

At the V0 (inclusion visit), participants will follow a demonstration of the platform to answer the questionnaires. Participants will receive, through email, a personal link, protected with a password to access online questionnaires on their personal computer. The platform is easy to use. Participants will be able to complete the questionnaires between V0 and V1. At V1, after the endoscopy, the PhD student will go through the questionnaires with the participants to review any mistake or missing data.

Blood and urine test data have a hard copy in the source file. The analyzes are carried out at the Poplar Hospital using the analysis and treatment circuit. The results are then sent to Dr Gianfranco Donatelli who will encode the results in eCRF. The data related to the histological analysis of the tissues as well as to the metagenomic analysis are carried out on the NutriOmic servers and the results are entered by the doctoral student Emilie Steinbach in an eCRF file because there are no paper results.

Data protection has been detailed in the previous sections. The data is checked and there is a double entry made by Prof Clément and Dr Marchelli. All electronic data is protected and under the protection of Flavien Jacques (see above). Access to the eCRF is only possible for Dr Donatelli, Dr Clément, Dr Chassaing and Emilie Steinbach through an account created by Flavien Jacques. Access to eCRF is via a password.

The data collected from the questionnaires are collected directly via the eCRF. The participant completes them online.



Data collection and circuit

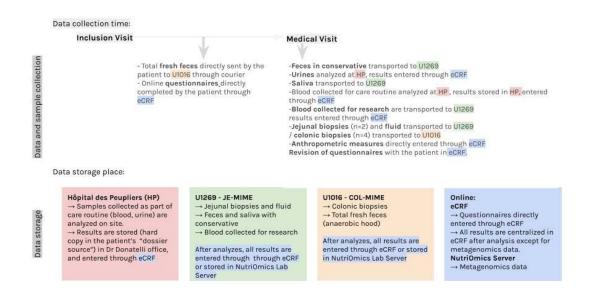


Table 6.3. : Data collection and circuit.

At the end of the search, the database is frozen. The automatic data backup takes place every month and is supervised by Flavien Jacques.

9.4. Conservation and archiving of documents and research data at the end of the research

The documents relating to the research are archived in accordance with the regulations in force. The sponsor and the investigators keep the documents relating to the research, which are specific to them for a period of 15 years.

The data are kept for 5 years in the active database and 15 years in archiving.

No removal or destruction may take place without sponsor agreement. At the end of the required period of retention, the sponsor will be consulted for destruction.

All data, documents and reports may be subject to audit or inspection.

9.5. Data transfer outside the European Union

No data transfer outside the European Union is planned as part of this research project. However, if such a transfer has to be done:

- It would be framed by appropriate and adapted guarantees in a contract / sharing agreement between Inserm and the recipient(s) of the data under conditions ensuring the confidentiality of the data concerned.
- The Ethics committee would be informed in the context of a substantial modification.



9.6. Summary of the categories of data collected, their sources, their recipients, their purpose, their retention periods

Data type	Sources	Recipient	Goal	Time of storage
Clinical date	Directly	K Clément	Statistical	15 years
and	entered through	B Chassaing	analysis	
questionnaires	I'ECRF during			
	consultation			
bio (blood,	Paper,	K Clément	Statistical	15 years
urine).	Peuplier center,	B Chassaing	analysis	
	of analysis, then			
	entered on eCRF.			
metagenomic	NutriOmique	K Clément	Statistical	15 years
s, histology	server, then	B Chassaing	analysis	
	eCRF			

10. STATISTICAL ANALYSES

10.1. Statistical analysis manager

The statistical analysis plan will be designed with Prof. Hedi Soula, professor of systems biology at Sorbonne University and member of UMRS 1269.

10.2. Study populations

The first part is a pilot study. Power calculations are based on results obtained in a previous study from Karine Clément Laboratory (UMRS 1269). This study compared obese subjects vs obese subjects with type II diabetes (Vieira-Silva et al., 2020). In this study, obese subjects with or without type II diabetes had a 30% decreased GM diversity compared to controls. This difference was observed with faecal microbiota samples. Our hypothesis is that GM diversity loss will also be found in jejunal samples to the same extent. Thus, to obtain 90% power with an α risk of 0,05 (unilateral) and 1-1-ß risk of 0.9, we need 13 subjects per group (Arcsin approximation for estimated proportions). Patient loss is low since patients are in routine follow-up, we will thus count 15 subjects per group.

The second part is powered based on published literature from our collaborator regarding microbiome encroachment into the mucosal layer in people with obesity and T2D. Those studies revealed a difference of 19 μ m (SD=6.75 μ m) of distance of bacteria from intestinal epithelial cells in people with T2D as compared to people without T2D. Based on the data from this published work, a sample size of 13 people per group (total=52) will be sufficient to detect a mean difference of 10 μ m with 90% power and the significance level of 0.05 (using a one-way ANOVA model followed by 3 posthoc comparisons with Bonferroni adjustment: healthy liver vs. fatty liver, mild fibrosis, and severe fibrosis). We selected a conservative estimate of the difference in encroachment as we expect smaller differences in people with steatosis only versus advanced fibrosis and to account for the expected increased encroachment in people with T2D. We will retain 80% power to detect a 10 μ m change in encroachment with up to a 20% dropout rate (N = 10 per group). Given the short duration of the study- we are unlikely to have dropouts that will impact the primary endpoint.

10.3. Statistical analysis plan

10.3.1. Analysis of the primary endpoint/outcome

For study 1. we will examine if jejunal microbiota abundance is decreased in subjects with obesity and obesity + type 2 diabetics compared to lean subjects. Based on metagenomic sequencing data, the



number of bacterial genes will be estimated and compared between groups by usual Anova test or non parametric test (Kruskall Wallis) in case of non parametric distribution of the variables

For study 2, as primary objective, we will examine the distance of bacterial layer in the mucus from intestinal epithelial cells in people with T2D as compared to people without T2D and lean subjects. A count of mucus-associated microbiota will also be performed. Anova test or non parametric test (Kruskall Wallis) in case of non parametric distribution the variables.

10.3.2. Analysis of the secondary endpoints/outcomes

- Quantitative metagenomics:

From study 1 and 2 generated data, we will i) compare fecal and jejunal microbiome within and between patient groups (PCA analysis) and, ii) examine links between these profiles and **information derived from Food questionnaire scoring (FFQ, 24h recall), lifestyle, biological, neuro-psychological scores**. This will be performed by association studies using pearson or spearman correlation depending of the distribution of variables. Multivariate models (Anova) will also be examined taking into account potential confounders. Obese patients with decreased microbial diversity and dysbiosis generally have a more severe metabolic phenotype. We will verify if we retrieve this phenotype in patient groups from study 2 by comparing median of richness (Anova or Kruskall wallis). We will examine if this holds true in the jejunum and which integrated bacterial signature (i.e. loss or enrichment of bacterial groups or metagenomics species) characterizes this feature in the jejunal area. Moreover, quantitative relationships will be examined between microbiome encroachment and bioclinical variables measured in the subjects as well as data from questionnaires, using pearson or spearman correlation depending of the distribution of variables. Multivariate models (Anova) will also be examined taking into account potential confounders. Based on these correlations, heat maps will be generated to visualize the data.

10.3.3. Exploratory analysis

- **Predicting functions in jejunal and fecal metagenome** Fine-tuned functional characterization of the microbiome is crucial for understanding the functionality of a given bacterial ecosystem. With the machine learning (ML) group in UMRS 1269, we will apply methods to improve the quantification of functions and relate it with environmental/lifestyle data. Our challenge will be to find subsets of features that will reveal functional modules or pathways that are associated with environmental health/nutrition information and reveal potential mechanistic hypothesis (different ML approach used as neuron networks for example).

11. CONFIDENTIALITY

11.1. Terms of participants' confidentiality

The confidential patient code will include: the abbreviation JE (jejunum) or COL (Colon) depending on their inclusion through endoscopy or coloscopy, the patient number (i.e.: 01 = First patient included), First Name and Surname initials (i.e.: Emilie Steinbach = ES).

[JE/COL – patient number – first name and surname initials]

Example : Emilie Steinbach, first patient of the cohort of the JE-MIME study is labelled: [JE-01-ES].

Only Dr Gianfranco Donatelli will keep printed results with the patient's name. He will keep the "dossier source" in a lock space in his office and under his supervision.

11.2. Terms of confidentiality

Direct access to clinical data and source documents will only be given to persons in charge of monitoring, sponsor funded audits and inspections performed by the relevant regulatory authority.



12. PUBLICATION AND VALORISATION

In compliance with the commitments made by Inserm and regulatory obligations, all research sponsored by Inserm is registered in a public site : EUDRACT for drug research, and in <u>www.clinicaltrials.gov</u> as a general rule.

12.1. Publication of results

All data collected during the present research are the property of the research sponsor and may not be communicated, without exception, to a third party without the written permission of the Sponsor.

The results will be published after the final analysis in the form of scientific articles in peer-reviewed journals, or as presentations at national and international conferences. All publications or communications (oral or written) shall be decided upon by mutual agreement between the coordinating and principal investigators, as well as the scientific officers and the sponsor, and will respect the international guidelines: **"Recommendations** for the Conduct, Reporting, Editing, and Publication of Scholarly work in Medical Journals" (<u>http://www.icmje.org/recommendations</u>).

All publications must respect the rules presented in the publications charter as defined by AVIESAN. The acknowledgments section must make mention of the source of financing, authorizations from competent authorities, and participant consent according the following model:

/Ethics statement //This research is part of clinical trial ****CXX-XX** sponsored by Inserm. It was granted approval by local Ethics Committee or "Comité de Protection des Personnes" on ---****DATE**---, authorized by the French authorities (****ANSM** ****NB**), and registered in a public trials registry (****CT XXXX**). All study participants gave their informed, consent (written or not) or non opposition to participation, in line with French legal guidelines.

In compliance with the commitments took by Inserm and the regulatory obligations, the results will be published on the public site on which the research had been recorded.

In order to respect the open science principle and to reinforce open access to Inserm publications, the authors are encouraged to deposit the published articles presenting the results of the research in HAL (national open archive intended for the collection, the provision and preservation of French scientific output). (<u>https://hal.archives-ouvertes.fr/</u>).

12.2. Final report

When the research is finished, a final study report is written within one year after the end of the research in all the countries where it was conducted.

The final study report is a written document, sufficiently detailed to understand the conduct of the research and to assess the quality of the research data. It is drafted, in collaboration, by the coordinator and biostatistician and it is submitted all investigators for opinion. When everyone agrees, the final version is signed by each investigator and made available to Inserm. It will only be sent to ANSM at their request.

This final study report includes a summary of the results written according to the reference plan of the competent authority. The summary is validated and sent by Inserm to the Competent Authority and the Ethics Committee according to the procedures established for each type of research. The summary is transmitted within one year following the end of the research in all the countries where it was performed.

12.3. Procedure for informing the study participants of the overall research findings

At the end of the research, participants have the right to be informed about the overall results of research, according to the modalities that will be communicated by the coordinating investigator.



12.4. Procedure for informing the study participants about their health data during and after the research

Participants may at any time be informed of their health data, upon a simple request from them to the coordinating investigator or, when appropriate, the physician or the qualified person who represent it. Any clinically significant abnormality detected in the assessments or analyses will be communicated to the participant and to the physician previously selected by the participant unless the participant has objected.

No medical information will be revealed to a participant by non-medical research staff.

12.5. Press communication procedure

The articles and abstracts, as well as oral presentations, from this research, will be sent before publication to the "Pôle Recherche Clinique" and the "Département de l'information scientifique et de la communication (DISC)" of INSERM.

13. PROTECTING RESEARCH PARTICIPANTS

13.1. Ethical justification

The research will be carried-out with respect to the French legislation in effect, in particular the sections regarding research involving human person as described in the French public health code, article L1121-2.

13.2. Adéquation du lieu à la recherche

According to Article L.1121-13 of the French Code of Public Health, the research will be carried out in a place equipped with the human, material and technical means adapted to the research and compatible with the security requirements of the people who lend themselves to it.

13.3. Ethical and regulatory considerations

The research will be carried-out with respect to the French legislation in effect, in particular the sections regarding research involving human person as described in the French public health code, article L1121-1 and those that follow, the laws of bioethics, laws regarding freedom of information, the Helsinki declaration and the present protocol.

The investigator agrees to conduct research in accordance with the ethical and legal guidelines. He is aware that all study documents and data related to the research could be subject to audit and inspection carried out with respect for professional secrecy, medical confidentiality may not be invoked to prohibit access to these documents. The investigator is aware that study results are the property of INSERM, the study sponsor.

13.4. Ethics Committee (EC)

Prior to start the research, the sponsor must submit the project to an ethics committee (EC) randomly selected as indicated in Article L. 1123-14 of the French Public Health Code. The sponsor will provide all necessary information (research protocol, documents used for data collection, case report forms, questionnaires, informed consent form) and all pertinent documents to the committee.

The research can only start when Inserm has been informed of the approval of the EC concerning the submitted protocol. This approval will include the title and protocol number assigned by the sponsor, the documents reviewed, as well as the date of review and the list of ERC participating members.



The sponsor will inform the EC of any further amendments to the protocol.

13.5. Competent Authority (CA)

Prior to the start of the research, the approval of the Ethics committee as well as the research synopsis will be relayed to the competent authority for information.

13.6. Data protection

This research is conducted in accordance with the reference methodology MR 001 approved by the Commission Nationale de l'Informatique et des Libertés (Cnil) on May 3, 2018 and to which Inserm is committed to comply (receipt n° 2211062 v 0 of January 15, 2019).

13.7. Insurance and Funding

INSERM, as the sponsor, has taken out an insurance contract of civil responsibility under the number SYB16899689A4, in accordance with the French legal and regulatory guidelines regarding category 1° and 2° of research involving the human person.

The insurance certificate relating to the present protocol can be found in Appendix.

13.8. Fichier VRB

The individuals participating in this research will receive a compensation for constraints incurred for 50 euros / and are not allowed to simultaneously participate in another research. Their participation in this protocol is recorded on the national file of persons suitable for biomedical research, managed by the Ministry of Health.

14. GOVERNANCE AND COMMITTEE

A steering committee will meet every 3 months to monitor the progress of the study. This committee is composed of investigators and co-investigators: Dr Gianfranco Donatelli, Dr Benoit Chassaing, Pr Karine Clément and Dr Laurent Genser.

15. QUALITY ASSURANCE

15.1. Description

Quality assurance contributes to the safety of research participants and the credibility of research data. The investigator is responsible for the quality of the research process. The research is conducted in accordance Inserm's standard procedures and specific study procedures as appropriate. All specific procedures must be validated by the sponsor.

15.2. Monitoring (research quality control)

The study monitor, sponsor's representatives, will visit the investigating center based on the rate of inclusions and the level of risk that has been attributed to this protocol. An initiation visits with protocol presentation will be carried out by representatives of the sponsor. Monitoring visits will be carried out by the study monitor according to the SOP. At the end of the research, a close out visit will be carried out. At the end of each visit a report will be written by the study monitor.

A monitoring plan is drawn up by the sponsor, approved by the investigator and, where appropriate, by the data manager. It specifies how to open, visit and close the investigative centers, and the rules to monitor the research. It takes into account the risk assessment of the research by the sponsor.



The Sponsor's representatives will visit the center according to the frequency of the inclusions and the monitoring plan.

16. SUBSTANTIAL AMENDMENTS TO THE PROTOCOL

Any request for substantial modification of the protocol should be submitted by the coordinating investigator to the sponsor. The submission guidelines to Inserm are available on the Inserm.fr website:

If approved, the sponsor (Inserm) will submit the amended protocol to the EC and/or competent authority.

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18. ANNEXES



La science pour la santé _____ From science to health

DOCUMENT D'INFORMATION ET FORMULAIRE DE CONSENTEMENT RECHERCHE IMPLIQUANT LA PERSONNE HUMAINE Version N°2.0 du 02/07/2021

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1. INFORMATION A L'ATTENTION DU PARTICIPANT

Madame, Monsieur,

Vous êtes invité(e) à participer à une recherche impliquant la personne humaine intitulée « **Le microbiote jéjunal luminal dans les maladies métaboliques : méthodes, identification et causalités**" (JE-MIME), dont l'Institut National de la Santé et de la Recherche Médicale (*Inserm - Pôle Recherche Clinique – Biopark, Bâtiment A 8 rue de la Croix Jarry 75013 Paris*), est le promoteur¹.

Ce document a pour but de vous fournir les informations écrites nécessaires à votre décision. Nous vous remercions de le lire attentivement.

L'investigateur² dont les coordonnées sont **Dr Gianfranco Donatelli** (Service d'Endoscopie, 8 Place de l'Abbé Georges Hénocque, 75013 Paris, +33 (0)6 64950070, donatelligianfranco@gmail.com), ou son représentant désigné vous a présenté l'objectif de la recherche et la façon dont vous pouvez y participer. Il est à votre disposition pour répondre à toutes vos questions et pour vous expliquer ce que vous ne comprenez pas. Vous pouvez prendre le temps pour réfléchir à votre participation à cette recherche, et en discuter avec votre médecin traitant et vos proches.

Votre participation est libre et volontaire : vous êtes libre d'accepter ou de refuser de participer à cette recherche impliquant la personne humaine.

Si vous acceptez de participer, vous serez invité(e) en fin de document à cocher tout ou partie des cases selon votre souhait, à indiquer vos nom et prénom(s) et à apposer votre signature et la date à laquelle vous signez dans l'emplacement qui vous est réservé. Sachez que vous pourrez retirer à tout moment votre consentement à la recherche, sans encourir aucune responsabilité ni aucun préjudice de ce fait. Cela ne changera en rien les rapports que vous avez avec l'investigateur qui vous propose directement ou via une personne le représentant de participer à la recherche. Nous vous demanderons simplement de l'en informer. Vous n'aurez pas à justifier votre décision et la qualité des soins dont vous devez/pourriez devoir bénéficier n'en sera pas modifiée.

Si vous décidez de ne pas participer à la recherche, vous n'aurez pas à vous justifier et la qualité des soins dont vous devez/pourriez devoir bénéficier n'en sera pas modifiée.

1.1. CADRE GENERAL ET OBJECTIFS

Il est actuellement fortement suspecté qu'il existe un lien entre l'environnement (alimentation, origine géographique...), le microbiote (l'ensemble des micro-organismes qui peuplent naturellement votre intestin) et les maladies dites « métaboliques » comme l'obésité et le diabète de type 2.

¹ Le Promoteur est une personne physique ou morale qui prend l'initiative d'une recherche portant sur la personne humaine.

² L'investigateur désigne la personne qui dirige et surveille la réalisation de la recherche sur un lieu donné.



Les bactéries du tube digestif produisent des molécules particulières (appelées métabolites) à partir des aliments que nous mangeons. Ces métabolites passent dans le sang à travers la barrière intestinale. Une fois absorbés, ils ont un impact sur l'ensemble de l'organisme et donc un impact sur notre santé.

Les études cliniques précédentes sur les liens entre microbiote et maladies biologiques ont été focalisées sur l'étude des bactéries du tube digestif à partir d'échantillons de selles. Mais la partie haute de notre intestin a été négligée (zone duodéno-jéjunal).

L'objectif global de cette recherche est d'étudier le microbiote duodéno-jéjunal qui est particulièrement intéressant car c'est le site d'absorption et de digestion des nutriments, notamment des lipides et glucides dont le métabolisme est perturbé dans les maladies métaboliques. Cette première étude devrait permettre de faire des liens entre le microbiote jéjunal des patients et leur santé métabolique.

Si vous acceptez de participer à cette recherche, quatre biopsies de la paroi intestinale, supplémentaires à celles prévues dans le cadre du soin habituel, seront effectuées pour pouvoir étudier les bactéries de votre paroi intestinale. Ces biopsies seront effectuées lors de votre endoscopie (examen servant à visualiser l'intérieur de la partie haute de votre intestin) effectuée dans le cadre du soin. Entre les 2 visites vous aurez à remplir un journal de votre alimentation trois fois par semaine.

Ce type de recherche permettra d'améliorer notre compréhension du développement des maladies métaboliques et pourra, à plus long terme, déboucher vers des traitements innovants et des recommandations nutritionnelles agissant sur le microbiote jéjunal.

Il est prévu d'inclure 45 adultes dans cette recherche, réparties dans 3 groupes :

- Groupe contrôle : 15 personnes ne souffrant ni d'obésité, ni de diabète,
- Groupe obésité : 15 personnes atteintes d'obésité,
- Groupe obésité DT2 : 15 personnes atteintes à la fois d'obésité et de diabète de type 2.

1.2. CADRE REGLEMENTAIRE

Cette recherche est réalisée conformément aux articles L.1121-1 et suivants du Code de la Santé Publique, relatifs aux recherches impliquant la personne humaine. Elle a reçu l'avis favorable du Comité de Protection des Personnes IIe de France 8 le 27/07/2021.

Cette recherche est menée conformément à la méthodologie de référence MR-001 homologuée par la Commission Nationale de l'Informatique et des Libertés (CNIL) le 3 mai 2018 et à laquelle l'Inserm s'est engagé à se conformer (récépissé n° 2211062 v 0 du 15 janvier 2019).

Conformément à la loi, l'Inserm promoteur de cette recherche, a contracté une police d'assurance garantissant sa responsabilité civile et celle de tout intervenant auprès de la compagnie NEWLINE INSURANCE COMPANY Ltd, dont l'adresse est Corn Exchange – 55 Mark Lane – London EC3R7NE – Royaume-Uni, sous le numéro SYB16899689A4

Pour pouvoir participer à cette recherche impliquant la personne humaine, vous devez être affilié(e) à un régime de sécurité sociale ou bénéficiaire d'un tel régime.

Au cours ou en fin de recherche, des assistants de recherche clinique et des auditeurs mandatés par le promoteur, ainsi que des inspecteurs des autorités de santé peuvent accéder aux données cliniques de votre dossier médical aux seules fins de vérification des données recueillies par l'investigateur ou son équipe. Ils sont soumis au secret professionnel, c'est-à-dire au respect de la confidentialité de vos données personnelles.

1.3. DEROULEMENT

Les deux visites du protocole, se feront à l'Hôpital Privé des Peupliers (8 Place de l'Abbé Georges Hénocque, à Paris) au moment de vos visites effectuées dans le cadre du soin. Il n'y aura donc pas de visite spécifique pour la recherche.

Première visite : Inclusion et consentement



Cette visite d'une durée d'environ une heure se déroulera lors de la consultation pour l'endoscopie dans le cadre du soin. Avant de vous questionner sur votre désir de participer à la recherche, l'investigateur principal ou son représentant désigné vous expliquera la recherche et répondra à toutes les questions que vous souhaitez : sur votre implication dans cette recherche et la recherche proprement dite. Après un délai de réflexion d'au moins une heure, et si vous acceptez d'y participer, votre consentement écrit sera recueilli par l'investigateur.

Toute prise de traitement antibiotique ou protecteur gastrique devra impérativement être signalée au médecin qui vous suit dans le cadre de la recherche car ils peuvent impacter les résultats des examens. En fonction de la durée de votre traitement, le médecin décidera de votre participation à l'étude. Toute prise de traitement antibiotique ou traitement par inhibiteurs de la pompe à protons (ou anti-acides) devra impérativement être signalée au médecin qui vous suit dans le cadre de la recherche car ils peuvent impacter les résultats des examens. En fonction de la durée de votre traitement par inhibiteurs de la recherche car ils peuvent impacter les résultats des examens. En fonction de la durée de votre traitement, le médecin décidera de votre participation à l'étude.

Afin que nous puissions comparer les bactéries de la paroi de votre intestin et celles situées dans vos selles, deux kits pour le prélèvement de selles vous seront remis.

<u>Recueil de selles</u>: Nous vous demanderons de réaliser deux prélèvements de selles à votre domicile 48h avant la visite pour l'endoscopie. Le premier prélèvement sera envoyé à notre laboratoire directement de votre domicile. Nous prendrons en charge l'organisation de cet envoi via un coursier.

Recueil de salive : Nous vous demanderons de réaliser un frotti buccal au réveil, le jour de votre visite pour l'endoscopie, afin de collecter votre salive. Vous pourrez nous remettre le kit lors de la visite. Ce jour là il ne faudra pas se brosser les dents.

Questionnaires :

Dans le cadre de cette étude nous vous demanderons de répondre à des questionnaires sur votre mode de vie et votre santé. Des identifiants personnels et un lien d'accès vous seront envoyés sur votre adresse e-mail pour vous donner accès aux différents questionnaires auxquels vous pourrez répondre en ligne de manière sécurisée. Ces informations vous seront envoyées au plus tard une semaine avant la visite pour la coloscopie. Ces questionnaires porteront sur votre activité physique, votre alimentation, votre humeur et votre qualité de vie, votre sommeil et sur vos symptômes digestifs. Nous vous demanderons aussi de faire un recueil de données relatives à votre alimentation 3 fois par semaine. L'ensemble de ces questionnaires prendront deux heures à compléter seul.

A la suite de la visite d'inclusion et jusqu'à l'endoscopie, vous recevrez également trois appels téléphoniques hebdomadaires de notre doctorante pour répondre à vos éventuelles questions et pour recueillir vos données sur l'alimentation. Ces entretiens téléphoniques dureront environ 15 minutes donc au total une heure et trente minutes d'appel. Vous pourrez aussi la contacter directement si vous avez des questions. Dans le cadre de cette recherche, les données concernant votre origine ethnique et géographique seront collectées. En effet, les habitudes alimentaires et la composition du microbiote intestinal peuvent varier en fonction de vos origines.

Première visite : Consultation médicale et Endoscopie : Cette visite se déroulera entre 1 semaine et 1 mois après la première visite et durera 4 heures, soit une demi-journée.

Pour la recherche médicale, vous devrez rapporter votre deuxième prélèvement de selles lors de cette visite. Vous serez reçu(e) par l'investigateur qui réalisera les examens suivants :

- Mesure tour de taille/hanche,
- Pression artérielle,
- Mesure de la composition corporelle par une balance à impédance (permettant de connaître le pourcentage de graisse dans votre corps).

Lors de l'endoscopie, nous allons récupérer :

- Le reste de votre liquide jéjunal qui n'est pas utilisé dans le cadre du soin (il est collecté afin de mieux observer votre tube digestif par la caméra et est considéré comme un déchet et jeté à la poubelle des déchets biologiques). Si vous êtes d'accord, nous pourrons analyser ce liquide et réaliser les analyses du génome des bactéries de votre tube digestif (analyses métagénomiques) et étudier la fabrication par celles-ci de métabolites.

- Deux biopsies duodéno-jéjunales (1 millimètre carré de surface) qui s'ajouteront à celles prévues dans le cadre du soin. Ces biopsies n'ajoutent pas de risques supplémentaires à l'examen initialement prévu. Ces biopsies permettront d'analyser les caractéristiques de votre tube digestif.



Après votre endoscopie, vous devrez rester sous surveillance à l'hôpital des peupliers pendant 4 heures. Lors de ces 4 heures la doctorante s'entretiendra avec vous pour vérifier la bonne complétion des questionnaires. Si vous acceptez, la doctorante s'entretiendra avec vous à ce moment pour vérifier la bonne complétion des questionnaires.

La durée prévisionnelle de la recherche est de 25 mois et votre participation sera de 1 mois maximum.

1.4. BENEFICES ATTENDUS

En participant à cette recherche, vous bénéficierez d'une indemnité de 50 euros. Les médecins vous donneront également les résultats de l'impédance et des analyses sanguines réalisées dans le cadre de la recherche.

Cette recherche a pour objectif d'améliorer les connaissances des mécanismes qui causent l'obésité et le diabète de type 2, de l'implication du microbiote intestinal dans ces maladies. Il n'y aura donc aucun bénéfice direct pour vous.

1.5. CONTRAINTES

Les prélèvements sanguins et les biopsies seront réalisés à la fois dans le cadre du soin habituel et pour la recherche. Il n'y aura aucune contrainte supplémentaire liée à la recherche en dehors du recueil de selles et de salive et de la complétion de différents questionnaires qui n'entraîneront aucun risque. La salive sera recueillie le matin avant l'endoscopie afin de mesurer votre cortisol salivaire qui est un indicateur du stress.

Si vous acceptez de participer, vous devrez respecter les points suivants :

- Etre disponible durant les appels téléphoniques avec le médecin de l'hôpital des Peupliers et les rendez-vous en consultation, examens, et post -examens (surveillance d'au moins 4H après l'endoscopie)
- Suivre les recommandations de votre médecin relatif à votre participation à l'étude
- Informer le médecin de la recherche, de l'utilisation de tout traitement ainsi que de tout événement survenant pendant la recherche (hospitalisation, grossesse, prélèvement sanguin réalisé au cours du dernier mois, ...)
- Ne pas consommer plus que 10g d'alcool (un verre de vin) trois jours qui précèderont l'endoscopie. Vous devrez être à jeun au moins 12 heures après le dernier repas avant l'endoscopie II vous sera également demandé de ne pas vous être brossé les dents 12 heures avant, comme c'est habituellement le cas lors des recommandations pour les endoscopies.
- Avoir à proximité un PC et un lien internet pour compléter les questionnaires en ligne, et les recueils alimentaires
- Compléter les différents questionnaires en ligne et le questionnaire sur ce que vous avez mangé pendant la journée, 3 fois par semaine.
- Effectuer le recueil de selles, prendre contact avec la doctorante pour l'envoi du premier recueil par coursier et rapporter le kit de selles lors de la visite pour l'endoscopie.
- Ne pas prendre part à un autre projet de recherche sans l'accord de votre médecin pendant toute la durée de participation à la recherche

Être affilié(e) à un régime de sécurité sociale ou être bénéficiaire d'un tel régime.

1.6. RISQUES PREVISIBLES

Aucun risque, supplémentaire à la procédure prévue dans le cadre de votre soin, n'est attendu dans le cadre de cette recherche.

1.7. INFORMATION CONCERNANT VOTRE SANTE

Vous avez le droit d'avoir communication, au cours ou à l'issue de la recherche, des informations concernant votre santé, détenues par l'investigateur ou son représentant, conformément à l'article L.1122-1 du Code de la Santé Publique.

Si une ou plusieurs anomalies sont identifiées dans vos résultats d'examen ou d'analyse, l'équipe investigatrice vous en informera et vous conseillera si tel est votre souhait. Ces résultats seront transmis au médecin de votre choix. Il est



possible que ce dernier décide d'interrompre votre participation à la recherche. L'investigateur ou son représentant peut également prendre cette décision.

1.8. CONSERVATION DE VOS ELEMENTS BIOLOGIQUES AU COURS DE LA RECHERCHE

Pendant toute la durée de la recherche, vos éléments biologiques seront conservés à l'UMRS1269 (91, Boulevard de l'Hôpital 75013, Paris) et l'UMRS1016 (24 rue du faubourg Saint-Jacques, 75014)

Si au cours de la recherche vous souhaitez ne plus y participer, vos éléments biologiques recueillis avant le retrait de votre consentement pourront être conservés et utilisés dans le cadre de la recherche, sauf si vous vous y opposez. Dans ce cas, ces derniers seront détruits.

1.9. CONFIDENTIALITE ET TRAITEMENT DE VOS DONNEES A CARACTERE PERSONNEL

Dans le cadre de la recherche impliquant la personne humaine dont l'Inserm est responsable et à laquelle il vous est proposé de participer, un traitement de vos données personnelles va être mis en œuvre pour permettre d'analyser les résultats de la recherche au regard de l'objectif de cette dernière. Ce traitement de vos données est placé sous la responsabilité de l'Institut National de la Santé et de la Recherche Médicale (Inserm) (situé au 101 rue de Tolbiac, 75 013 Paris - https://www.inserm.fr/) et répond à l'exécution d'une mission d'intérêt public qui justifie le traitement de vos données personnelles à des fins de recherche scientifique.

Votre participation à la recherche implique de collecter des données à caractère personnel vous concernant. A cette fin, vos données médicales y compris, les données relatives à vos habitudes de vie, ainsi que, dans la mesure où elles sont nécessaires à la recherche, les données relatives à vos origines ethniques seront transmises à l'investigateur et/ou à l'équipe coordinatrice de la recherche ou aux personnes agissant pour le compte de l'Inserm, en France ou à l'étranger. Ces données seront identifiées de façon confidentielle par un code confidentiel non directement identifiant. Ces données pourront également, dans des conditions assurant leur confidentialité, être transmises à des tiers français ou étrangers publics ou privés (autorités de santé, autres organismes de recherche...) à moins que vous ne vous y opposiez.

La durée de conservation et d'archivage de vos données issues de cette recherche

Vos données seront conservées dans les systèmes d'information sécurisé du responsable de traitement et du centre dans lequel vous avez été inclus pendant une durée de 5 années à compter de la date de fin de cette recherche. Ensuite, vos données seront archivées pendant une durée de 15 années à partir de la fin de la période de conservation.

Vos droits

Conformément aux dispositions de la loi n°78-17 relative à l'informatique, aux fichiers et aux libertés et au Règlement Général sur la Protection des Données (Règlement (UE) 2016/679), vous disposez des droits suivants :

- droit d'accès aux informations vous concernant, afin d'en vérifier l'exactitude et, le cas échéant, afin de les rectifier, de les compléter, de les mettre à jour.
- droit d'opposition : droit de vous opposer à tout moment, à la transmission de vos données et d'obtenir que vos données ne soient plus collectées pour l'avenir. L'exercice de ce droit entraîne l'arrêt de votre participation à la recherche.
- droit à la limitation du traitement des données : droit de bloquer temporairement l'utilisation de vos données : aucune opération ne peut être réalisée sur celles-ci.
- droit de retirer votre consentement à votre participation à la recherche à tout moment sans avoir à vous justifier. Sachez toutefois que les données utiles recueillies préalablement à l'exercice de votre droit d'opposition ou au retrait de votre consentement pourront continuer à être traitées de façon confidentielle pour ne pas compromettre la réalisation des objectifs de la recherche.

Si vous souhaitez exercer ces droits et obtenir communication des informations vous concernant, vous pouvez vous adresser à l'investigateur ou son représentant désigné qui vous suit dans le cadre de la recherche et qui connaît votre identité (**Dr Gianfranco Donatelli**, , par téléphone: +33 (0)6 64950070 ; par email: donatelligianfranco@gmail.com).



En cas de difficulté pour exercer vos droits, vous pouvez également contacter notre Délégué à la Protection des Données par mail (dpo@inserm.fr) ou par voie postale (Délégué à la Protection des Données de l'Inserm, 101 rue de Tolbiac, 75 013 Paris).

Dans l'hypothèse où vous vous ne parviendriez pas à exercer vos droits "Informatique et Libertés" tels que cités cidessus ou si vous estimez subir une atteinte aux règles de protection de vos données personnelles, nous vous informons que vous disposez également du droit d'introduire une réclamation auprès de la Commission Nationale de l'Informatique et des Libertés - CNIL- l'autorité française de protection des données personnelles, 3 Place de Fontenoy - TSA 80715, 75334 PARIS CEDEX 07 ou en ligne sur <u>https://www.cnil.fr</u>

Vous trouverez ci-dessous un tableau récapitulatif des personnes auprès desquelles vous pourrez exercer vos droits

Responsable de traitement	Responsable de la mise en œuvre	Délégué à la protection des données	Autorité de contrôle	
Qui assume la responsabilité de la recherche ?	Auprès de qui exercer vos droits	En cas de difficultés pour exercer vos droits	Pour déposer une réclamation	
Institut national de la santé et de la recherche médicale (Inserm)	Dr Gianfranco Donatelli	DPO Inserm	CNIL	
101 rue de Tolbiac, 75 013 Paris	Service d'Endoscopie, 8 Place de l'Abbé Georges Hénocque, 75013 Paris, +33 (0)6 64950070, donatelligianfranco@gmail .com	101 rue de Tolbiac, 75 013 Paris dpo@inserm.fr	3 Place de Fontenoy, TSA 80715, 75334 PARIS CEDEX 07 <u>https://www.cnil.fr</u>	

1.10. PERIODE D'EXCLUSION

Vous ne pourrez pas participer une autre recherche pendant toute la durée de votre participation à la présente recherche.

1.11. INDEMNITES

Une indemnité de 50 euros vous sera versée en compensation des contraintes subies dans le cadre de cette recherche. Cependant, aucun éventuel avantage financier ne peut être tiré de la participation à la recherche, même dans l'éventualité où celle-ci mène à la réalisation de produits commercialisés.

Cette indemnité vous sera intégralement versée uniquement si vous participez à l'ensemble des actes et examens prévus par la recherche.

En cas d'interruption de votre participation à la recherche par décision du médecin investigateur, cette indemnité vous sera intégralement versée.

1.12. INSCRIPTION AU FICHIER NATIONAL DES PERSONNES QUI SE PRETENT A DES RECHERCHES

Nous vous informons que vous serez inscrit(e) dans le fichier national des personnes qui se prêtent à des recherches prévues à l'article L. 1121-16 du Code de la Santé Publique. Ce registre confidentiel est administré par la Direction



Générale de la Santé et pour objectif de garantir votre sécurité : il mentionne la durée pendant laquelle vous ne pouvez pas participer à une autre recherche ainsi que le montant des indemnités que vous percevez.

1.13. INFORMATION DES RESULTATS GLOBAUX

Vous avez le droit d'être informé(e) des résultats globaux de la recherche à l'issue de celle-ci, conformément au dernier alinéa de l'article L.1122-1 du Code de la Santé Publique auprès de l'investigateur ou de son représentant qui aura recueilli votre consentement. Deux semaines après l'endoscopie, vous aurez rendez-vous avec le Dr Gianfranco Donatelli, comme cela est initialement prévu dans le cadre du soin. Les résultats d'examen ou d'analyse obtenus dans le cadre de la recherche vous seront communiqués en addition des examens réalisés dans le cadre du soin.

Les résultats de cette recherche peuvent être présentés à des congrès ou dans des publications scientifiques. Les données recueillies vous concernant seront codées de sorte qu'il sera impossible de vous identifier. Aucune information permettant de vous identifier ne sera diffusée.

1.14. DEVENIR DE VOS ELEMENTS BIOLOGIQUES A L'ISSUE DE LA RECHERCHE

Si vos éléments biologiques ne sont pas utilisés en totalité à la fin de cette recherche, ils seront conservés 10 ans à l'UMRS1269 (91, Boulevard de l'Hôpital 75013, Paris) et l'UMRS1016 (24 rue du faubourg Saint-Jacques, 75014) afin d'être utilisés ultérieurement pour d'autres recherches portant sur le microbiote et les maladies métaboliques, dans le respect de la confidentialité de votre identité. Vous pouvez vous y opposer et dans ce cas vos éléments biologiques seront détruits à la fin de la présente recherche.

Vos éléments biologiques pourront également être cédés à titre gratuit à d'autres équipes de recherche privées ou publiques, nationales ou internationales, sauf si vous vous y opposez.

Vous pouvez ainsi librement, et à tout moment, sans conséquence pour votre participation à la présente recherche ou à votre prise en charge médicale, vous opposer à cette utilisation ultérieure à but de recherche, en vous adressant à l'investigateur ou à son représentant qui a recueilli votre consentement.

1.15. RE-UTILISATION DE VOS DONNÉES DANS LE CADRE DE NOUVELLES RECHERCHES

Afin de permettre la réalisation de nouvelles recherches portant sur le microbiote et les maladies métaboliques, une partie des données qui vous concernent pourra être transférée à d'autres équipes de recherches, organismes ou institutions, publics ou privés, en France ou à l'étranger (appelés tiers destinataires).

Si vous acceptez le partage de vos données dans les conditions ci-dessus mentionnées, soyez assuré(e) que ces éventuels transferts à des tiers destinataires seront réalisés dans le plus strict respect de la réglementation en vigueur et des bonnes pratiques applicables. Ainsi, nous vous précisons que :

- toutes les mesures seront prises pour assurer un transfert de vos données de façon sécurisée
- vos données ne seront envoyées au(x) tiers destinataire(s) que sous forme codée et ne permettront pas de vous identifier directement dans la mesure où ni votre nom, ni votre prénom ne lui/leur seront transmis
- préalablement à tout transfert de vos données au(x) tiers destinataire(s), nous nous assurerons que ce(s) dernier(s) a/ont obtenu les avis et autorisations réglementairement requis pour mener sa/leurs recherche(s)

Vous disposez du droit d'obtenir des informations complémentaires, telles que la finalité de ces nouvelles recherches, auprès du médecin investigateur.

Vous disposez du droit de vous opposer à toute ré-utilisation de vos données dans le cadre de recherches futures.

🌐 Inserm

2. RECUEIL DU CONSENTEMENT DU PARTICIPANT

J'atteste avoir bien lu et pris connaissance des informations relatives à ma participation à la recherche intitulée **C20-86** « Le microbiote jéjunal luminal dans les maladies métaboliques : méthodes, identification et causalités » « JE-MIME » exposées par écrit sur les pages précédentes et avoir été informé(e) de l'objectif de cette recherche par l'investigateur ou son représentant, de la façon dont elle va être réalisée et de ce que ma participation va impliquer pour moi.

J'ai obtenu toutes les réponses aux questions que je lui ai posé.

J'ai bien compris les contraintes qui seront les miennes au cours de ma participation à cette recherche qui durera 1 mois.

J'ai eu suffisamment de temps pour réfléchir à ma participation à cette recherche impliquant la personne humaine.

J'ai bien pris note que je ne pourrai pas participer simultanément à une autre recherche pendant la durée de ma participation à l'étude.

J'ai pris connaissance des risques prévisibles et je suis conscient(e) que ma participation pourra être interrompue par l'investigateur en cas de nécessité.

J'ai été informé(e) que pour toute anomalie détectée au cours de la recherche et concernant ma santé, j'en serai averti(e) via le médecin de mon choix sauf si je m'y oppose.

J'ai été avisé(e) qu'une indemnité de 50 euros est prévue pour ma participation si je remplis l'ensemble des actes et examens prévus par la recherche.

J'ai compris que je peux retirer à tout moment mon consentement de participation à cette recherche quelles que soient mes raisons et sans avoir à m'en justifier, sans supporter aucune responsabilité et sans encourir aucun préjudice. J'en informerai simplement l'investigateur ou son représentant qui a recueilli mon consentement

J'ai bien noté que mes droits d'accès de rectification et d'opposition à mes données, prévus par la loi du 6 janvier 1978 relative à l'informatique aux fichiers et aux libertés modifiée et au Règlement Général sur la Protection des Données (Règlement (UE) 2016/679), s'exercent à tout moment auprès de l'investigateur ou de son représentant qui a recueilli mon consentement et qui connaît mon identité.

J'ai été informé(e) que mes données recueillies au cours de cette recherche seront conservées à l'issue de celle-ci afin de pouvoir les réutiliser dans le cadre de recherches ultérieures portant sur le microbiote et les maladies métaboliques dans le respect de la confidentialité de mon identité. Ces recherches pourront être menées par d'autres équipes de recherche, privées ou publiques, nationales ou internationales.

J'ai été informé(e) que mes éléments biologiques recueillis au cours de cette recherche seront conservés à l'issue de celle-ci, afin de pouvoir les réutiliser dans le cadre de recherches ultérieures portant sur le microbiote et les maladies métaboliques. Ces recherches pourront être menées par d'autres équipes de recherche, privées ou publiques, nationales ou internationales.



CONSENTEMENT POUR LA RECHERCHE

TRAITEMENT DE MES DONNEES PERSONNELLES

J'accepte que mes données personnelles relatives à mes données de santé y compris, mes habitudes de vie, mes origines ethniques, soient collectées et traitées par le Promoteur ou pour son compte afin de répondre aux objectifs de la recherche.

J'accepte que l'ensemble de mon dossier médical puisse être consulté par les personnes habilitées dans le cadre de cette recherche, dans le respect de la confidentialité de mes données et de mon identité.

CONSERVATION ET UTILISATION DE MES ELEMENTS BIOLOGIQUES

□ J'accepte □ Je refuse

le prélèvement, la conservation et l'utilisation de mes éléments biologiques tels que prévus dans le cadre de cette recherche.

CONSENTEMENT DE PRINCIPE POUR LES RECHERCHES ULTERIEURES

CONSERVATION ET UTILISATION ULTERIEURE DE MES DONNEES PERSONNELLES

□ J'accepte □ Je refuse

la conservation de mes données afin qu'elles soient utilisées dans le cadre de recherches ultérieures.

CONSERVATION ET UTILISATION ULTERIEURE DE MES ELEMENTS BIOLOGIQUES

□ J'accepte □ Je refuse

la conservation et l'utilisation de mes éléments biologiques dans le cadre de recherches ultérieures.

A compléter de la main de la personne donnant son consentement :	Le / /
Je soussigné(e) (Prénom	— — Signature du participant
NOM) accepte librement et volontairement de participer à la recherche décrite.	
A compléter par l'investigateur ou son représentant désigné :	Le / /
Je soussigné (e), (Prénom NOM) confirme avoir expliqué le but et les modalités de cette recherche ainsi que ses	 Signature de
risques potentiels. Je m'engage à faire respecter les termes de ce formulaire de	l'investigateur ou de son représentant désigné
consentement, conciliant le respect des droits et des libertés individuelles et les exigences d'un travail scientifique.	
Nom du service :	
Téléphone :	
Courriel :	

Etablir le document en deux exemplaires originaux.

Exemplaire participant : à remettre à la personne se prêtant à la recherche

Exemplaire investigateur : à conserver par l'Investigateur pendant la durée légale de conservation des documents de la recherche.



Investigateur Principal

Docteur Gianfranco DONATELLI Hôpital Privé des Peupliers Service d'Endoscopie 8, place de l'Abbé Georges Hénocque 75013 – PARIS **Promoteur** Inserm *Madame Sandrine COUFFIN-CADIERGUES* Institut Santé Publique Pôle Recherche Clinique Biopark, Bâtiment A 8, rue de la Croix Jarry 75013 – PARIS

Réf CPP : 21 06 48 (*A rappeler dans toutes vos correspondances*) **Réf CNRIPH :** 21.04.29.67842 **Réf. Protocole :** C20-86 / Étude JE/COL-MIME **N° ID RCB :** 2021-A01074-37

Documents Transmis					
Dossier Administratif	Dossier de la Recherche (suite)				
Courrier de demande d'avis au CPP, accompagné de la liste des documents					
transmis, signé et daté du 29 avril 2021	Questionnaires :				
Courrier de réponse à la délibération incluant un document détaillant les	AUDIT (Alcohol Use Disorders Identification Test), version française				
réponses aux commentaires du Comité, signé et daté du 12 juillet 2021	BDI II, version française				
Formulaire de demande d'avis au CPP pour une recherche mentionnée au 2° de	Échelle d'autoévaluation « Binge eating scale » (BES), version française				
l'article L. 1121-1 du code de la santé publique ne portant pas sur un produit	DEBQ, version française				
mentionné à l'article L. 5311-1 du même code, signé et daté 29 avril 2021	Échelle Bristol, version française				
Document additionnel, signé et daté du 29 avril 2021	Test de Fagertröm, version française				
Bordereau d'enregistrement de l'étude à l'ANSM, daté du 9 avril 2021.	<i>Questionnaire alimentaire (FFQ), version française</i>				
	Échelle HAD: Hospital Anxiety and Depression scale, version française				
Dossier de la Recherche	d'octobre 2014				
Protocole de recherche, version anglaise 2.0 du 2 juillet 2021	Échelle de Stress Perçu (Perceived Stress Scale) de Cohen et al. (1983), version				
Page de signature du protocole (v. 1.0), par le promoteur, le 29 avril 2021, et	française				
par l'investigateur principal, le 28 avril 2021	Index de Qualité du Sommeil de Pittsburgh (PSQI), version française				
Résumé du protocole, version française 2.0 du 2 juillet 2021	Questionnaire Activité Physique (RPAQ), version française				
Justification de l'adéquation des moyens, signée et datée du 29 avril 2021	Stop Bang Questionnaire, version française				
CNIL : Récépissé de déclaration de conformité à la méthodologie de recherche	Questionnaire de typologie circadienne de Horne et Ostberg, version française.				
MR-001 n°2211062 v0, signé et daté du 15 janvier 2019					
Note d'information et formulaire de consentement – COL-MIME, version 2.0					
du 2 juillet 2021 Note d'information et formulaire de consentement – JE-MIME, version 2.0 du					
2 juillet 2021					
Affiche de recrutement, version non datée					
Liste des investigateurs, version 1.0 du 9 avril 2021					
CV des investigateurs, datés et signés					
Attestation d'assurance, signée et datée du 22 avril 2021.	Dossier téléversé sur la plateforme de la CNRIPH le 29 avril 2021.				

Microbiote jéjunal et associé au mucus du colon dans les maladies métaboliques : méthodes, identification et causalités.

Notre Comité s'est réuni en séance le 8 juin 2021 et a examiné votre projet de recherche mentionnée au 2° de l'article L. 1121-1 du code de la santé publique, ne portant pas sur un produit mentionné à l'article L. 5311-1 du même code, ci-dessus référencée.

Comité de Protection des Personnes Ile de France VIII Hôpital Ambroise Paré - 9, avenue Charles de Gaulle 92100 Boulogne-Billancourt

Président : Franck LE MERCIER Responsable administrative : Catherine Avanzini Tél. 01 49 09 58 14 -Fax 01 49 09 53 52 <u>cppidf8@orange.fr</u> Agrément Arrêté Ministériel du 16 mai 2018



Conclusion:

Compte tenu de l'apport des précisions et des modifications apportées dans le protocole, le résumé du protocole, les notes d'information et de la transmission du CV du Dr Steinbach, en réponse à la délibération du 8 juin 2021.

Notre Comité émet un avis favorable sur votre projet de recherche, conformément aux dispositions du Code de la santé publique.

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Franck LE MERCIER *Président du C.P.P. Ile de France VIII.*

Liste des personnes ayant participé à la délibération.

Comité de Protection des Personnes Ile de France VIII Hôpital Ambroise Paré - 9, avenue Charles de Gaulle 92100 Boulogne-Billancourt

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Séance du mardi 8 juin 2021

Comité restreint

Ont participé à la délibération *

Collège 1

Docteur AUBERT-FOURMY Madame BERDOUGO Monsieur LE MERCIER Madame LECOEUR Madame MARTINS Monsieur MUSSETTA Madame RAHO Médecin Généraliste (Titulaire) Qualifiée en matière de Recherche impliquant la personne humaine (Suppléante) Pharmacien (Titulaire) Pharmacien (Suppléante) Infirmière (Titulaire) Biostatisticien / épidémiologiste (Titulaire) Qualifiée en matière de Recherche impliquant la personne humaine (Suppléante)

Collège 2

Madame AGAR Madame BOISGONTIER Madame LECLERC Monsieur RADET Psychologue (Titulaire) Psychologue (Suppléante) Représentante des associations agréées d'usagers du système de santé (Titulaire) Représentant des associations agréées d'usagers du système de santé (Titulaire)

* La séance s'est tenue en visioconférence.

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Annex II: The Microdiet Clinical Trial

iScience

Article

Protein supplementation changes gut microbial diversity and derived metabolites in subjects with type 2 diabetes

Ilias Attaye,^{1,11,13,*} Pierre Bel Lassen,^{2,3,11} Solia Adriouch,² Emilie Steinbach,² Rafael Patiño-Navarrete,² Mark Davids,¹ Rohia Alili,² Flavien Jacques,² Sara Benzeguir,² Eugeni Belda,² Ina Nemet,^{4,5} James T. Anderson,^{4,5} Laure Alexandre-Heymann,⁶ Arno Greyling,⁷ Etienne Larger,³ Stanley L. Hazen,^{4,5,8} Sophie L. van Oppenraaij,¹ Valentina Tremaroli,⁹ Katharina Beck,⁹ Per-Olof Bergh,⁹ Fredrik Bäckhed,^{9,10} Suzan P.M. ten Brincke,¹ Hilde Herrema,¹ Albert K. Groen,¹ Sara-Joan Pinto-Sietsma,¹ Karine Clément,^{2,3,12,13,*}

SUMMARY

High-protein diets are promoted for individuals with type 2 diabetes (T2D). However, effects of dietary protein interventions on (gut-derived) metabolites in T2D remains understudied. We therefore performed a multi-center, randomizedcontrolled, isocaloric protein intervention with 151 participants following either 12-week high-protein (HP; 30Energy %, N = 78) vs. low-protein (LP; 10 Energy%, N = 73) diet. Primary objectives were dietary effects on glycemic control which were determined via glycemic excursions, continuous glucose monitors and HbA1c. Secondary objectives were impact of diet on gut microbiota composition and -derived metabolites which were determined by shotgun-metagenomics and mass spectrometry. Analyses were performed using delta changes adjusting for center, baseline, and kidney function when appropriate.

This study found that a short-term 12-week isocaloric protein modulation does not affect glycemic parameters or weight in metformin-treated T2D. However, the HP diet slightly worsened kidney function, increased alpha-diversity, and production of potentially harmful microbiota-dependent metabolites, which may affect host metabolism upon prolonged exposure.

INTRODUCTION

Cardio-metabolic diseases (CMD) represent an umbrella term encompassing, among other, type 2 diabetes (T2D) and cardiovascular diseases.¹ CMD are currently on the rise globally and are the leading causes of morbidity and mortality.^{2–4} The pathophysiology of CMD is complex, but one important factor recognized to be involved is insulin resistance, which is a hallmark trait in the onset of T2D and glycemic control in individuals with T2D.⁵ A key element in management of patients with T2D is to provide diet guidance as multiple dietary interventions have shown the potential of diet manipulation to positively affect metabolic health.^{6,7}

Recently, a large focus has been put on modulation of dietary protein levels,^{8–10} due to its associations with cardiometabolic risks.¹¹ Protein, in particular animal protein consumption, has been linked with increased risk of T2D development in observational prospective and meta-analysis studies.^{12–15} On the other hand, several studies have reported the potential benefit of high protein (HP) diets by inducing weight loss and associated improvement of metabolic parameters.^{16,17} However, in intervention studies with modulation of protein intake the effects on metabolic and glycemic control whereas other studies showed a detrimental effect.^{18,19} Other studies reported little to no effects of increased protein intake on metabolic parameters in individuals with T2D.²⁰ Importantly, most HP dietary interventions include a caloric restriction and, therefore, weight loss is an important confounder making it difficult to truly distinguish the effect of protein modulation from the metabolic effect of weight loss.^{6,21–23}

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Continued







The pathophysiology behind these conflicting results remains complex. However, in recent years, it has become clear that the gut microbiota, an endocrine organ that encompasses trillions of microbes, plays a key role in metabolic health.^{24–27} The gut microbiota itself is dynamic in its composition and function and can be modulated via several routes (e.g., medication use and host ethnicity lifestyle including diet).^{28–30} One of the main mechanisms by which the gut microbiota can influence host health is through the generation of microbial derived metabolites, which can be produced from the host diet.^{31–33}

As such rodent and clinical studies have shown the potential of fiber intake to promote the production of beneficial short-chain fatty acids (SCFA) that can improve insulin sensitivity and positively affect multiple metabolic pathways.³⁴⁻³⁶ Conversely, several detrimental microbial metabolites can be formed from dietary (animal) protein. These metabolites include, among others trimethylamine (TMA), which is generated by bacteria from dietary carnitine, choline, and betaine. In the liver, TMA is converted to trimethylamine-N-oxide (TMAO), which has been extensively linked to poor cardiovascular outcome.^{37,38} Other important metabolites are phenylacetylglutamine (PAG) and imidazole propionate (ImP). PAG is formed from the essential amino acid phenylalanine and has been linked to increased platelet reactivity and higher prevalence of CMD.³⁹ ImP is produced by bacteria using the essential amino acid histidine as a substrate and has been linked to increased insulin resistance, T2D status, and reduced effect of the anti-diabetic drug metformin.^{40–42}

Some studies have investigated the effects of an HP diet on the gut microbiota composition;^{43,44} however, most data describing the interaction between dietary protein intake and metabolite production come from animal or observational studies.⁴⁵ Thus, evaluating the effect of HP consumption on microbial metabolites and the interaction with host metabolism merits consideration.

We therefore performed a 12-week randomized, multi-center, isocaloric dietary intervention comparing high protein (HP; protein: 30% of energy intak[(En%]) vs. low protein (LP; protein: 10% En%) intake in a multi-ethnic population of individuals with T2D on stable metformin. Our primary objective was to investigate the effects of this isocaloric protein modulation on glycemic control and insulin resistance markers in individuals with T2D. Secondary objectives were to analyze the impact of an increase in protein consumption on gut microbiota composition and functional metabolic output as monitored by plasma levels of microbiota-derived metabolites.

RESULTS

Baseline characteristics

A total of 171 subjects were randomized to either the HP or LP group. The study design is displayed in Figure 1. Out of these subjects, 151 (78 HP and 73 LP diet) finalized the clinical trial and were included in the analyses. Most common reasons for dropout were antibiotic usage during the trial (N = 3), failure to adhere to the diet (N = 6), or personal problems (N = 6) (Figure 2 for CONSORT flow chart). Baseline characteristics are shown in Table 1. At baseline there were no statistically significant differences in biochemical or anthropometric parameters. Subjects had a mean age of 58.2 ± 7.7 years in the HP group and $59.1.1 \pm 7.0$ years in the LP group. The groups mainly consisted of women, with 56.4% in the HP group and 58.9% in the LP group. All subjects were on stable metformin therapy and a subset also used other diabetes lowering drugs, such as sulfonylureas. However, these subjects were equally distributed among the HP and LP groups. Of note, there were also no statistically significant differences at baseline when subjects were stratified according to center (Table S1).

Compliance to the diet

The self-reported compliance with dietary recalls was good in order to match the target diet (Figures 3A and 3B). Baseline protein intake did not differ between the two groups with 19.7 \pm 4.3 En% in the HP group and 19.4 \pm 4.5 En% in the LP group. At the end of the intervention, protein intake reached 27.2 \pm 5.0 En% in the HP vs. 13.8 \pm 4.3 En% in the LP group (p < 0.001). The main other excepted change in macronutrient composition of the diet was carbohydrate intake which decreased from 39.5 \pm 9.8 En% to 34.0 \pm 6.2 En% in the HP group and increased from 41.9 \pm 10.3 to 47.5 \pm 7.1 in the LP group (p < 0.01). Fat and fiber intake remained stable throughout the study (Figures 3B and 3C). Importantly, the increase in protein was mainly due to an increase in animal protein consumption in the HP group. Saturated fat intake was slightly higher in HP vs. LP group. Plant protein intake did not differ during the intervention between the two groups. The

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12 weeks isocaloric high protein (HP) vs. low protein (LP) dietary intervention in T2D subjects treated with metformin

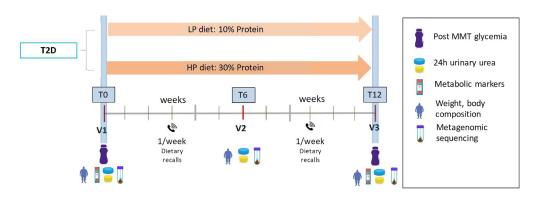


Figure 1. Study design MICRODIET trial

Subjects were randomized to follow either a high protein (HP) or low protein (LP) diet for 12 weeks. Study visits were performed at week 0 (baseline), week 6, and week 12 (end of intervention). A mixed-meal test (MMT) was performed at week 0 and week 12 and plasma for metabolomics was also obtained. Dietary adherence was observed through weekly contact with a dietician and the use of weekly food diaries. Before each study visit subjects collected 24-h urine and as well as 24-h fresh feces.

increase in carbohydrates in the LP group was mainly related with higher starch intake with no difference in sugar intake between the two groups (Table 2).

Urea/creatinine ratio was not different between the two groups at baseline, but increased significantly in the HP group from 31.8 \pm 7.4 at baseline to 36.6 \pm 8.0 in week 6 (p = 0.03) and 36.4 \pm 8.9 mmol/24-h at week 12 (p < 0.01) compared to baseline. In the LP group, urea/creatinine ratio decreased significantly from 32.9 \pm 7.8 at baseline to 26.7 \pm 7.3 in week 6 (p < 0.01) and 27.8 \pm 7.2 mmol/24 h at week 12 (p < 0.01). There were no differences in urea/creatinine ratios between week 6 and week 12 when comparing the LP and HP groups. (Figure 3D). Moreover, BMI remained stable throughout the intervention (Figure 3E).

An isocaloric high vs. low protein dietary intervention for 12 weeks does not affect glucose metabolism in metformin treated type 2 diabetes patients

We found that neither an HP or LP diet, when administered for 12 weeks, significantly affected glucose metabolism as area under the curve (AUC) after a mixed-meal test (MMT), HbA1c and HOMA-IR did not differ between baseline and end of the intervention (Figures 4A–4D). Moreover, post-prandial insulin response, c-peptide levels as well as results from the continuous glucose monitor performed throughout the study showed large interindividual differences and, therefore, no significant effect (p > 0.1 for all outcomes) of the dietary intervention, even when excluding extreme outliers (Figures S1 and S2).

Analyses were also performed after stratification to baseline gut microbiota diversity (high vs. low Shannon diversity), which did not affect the primary outcome (Figure S3).

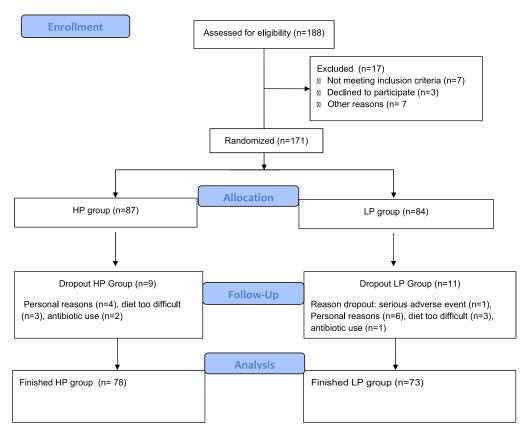
A 12-week isocaloric high vs. low protein dietary intervention does not affect BMI, body composition or biochemical parameters with the exception of renal function

As previous studies have reported both beneficial (increased satiety and weight loss), as well as detrimental (renal damage) effects of a HP diet, we next determined the effects of an HP vs. LP diet on body weight, body composition, and biochemical parameters (Figure 3E; Table 3). This study did not find significant weight changes or body composition changes following the isocaloric intervention. Moreover, lipid levels, inflammation markers, and energy expenditure were not affected. However, estimated renal function decreased in the HP group by 1.67 \pm 15.3 mL/min/1.73m2 and improved in the LP group 3.0 \pm 12.98 mL/min/1.73m2 (p = 0.03).





MICRODIET flow chart





Dietary protein intake affects gut microbiota composition and plasma metabolite profile

We next determined the effects of the dietary intervention on gut microbiota composition and diversity using metagenomic approaches (Figure 5A). This 12-week HP vs. LP intervention did elicit changes in the gut microbiota composition albeit with a modest effect; the explained variance of the beta-diversity was 0.146% (p < 0.001) (Figure S4). Moreover, alpha diversity, increased in the HP group by 2.6% and decreased in the LP group by 0.2% (HP vs. LP p = 0.01) (Figure 5A). Interestingly, the effects of the protein intervention on gut microbiota composition were not driven by large changes in individual taxa (Figure S5). Moreover, the respective diets did not induce significant functional changes in the gut microbiota (Figure S6).

As gut microbiota composition is related to microbiota-derived metabolite production, we next measured if the protein interventions elicited changes in the measured plasma metabolite profile (Figure 5B). We therefore determined fasting and postprandial (t = 240 min during MMT) plasma levels of several (proteinderived) metabolites. Interestingly, a majority of the observed metabolite changes were driven by increases of the metabolite levels in the HP group. The main metabolites that increased in the HP group were PAG (log fold change: 0.32 i.e., 38% increase); indoxyl sulfate (log fold change: 0.21 i.e., 23% increase), indole-3-acetic acid (log fold change: 0.15 i.e., 16% increase), homocitruline (log fold change: 0.15 i.e., 16% increase), and propionyl carnitine acid (log fold change: 0.13 i.e., 14% increase) in the fasting metabolites. In the postprandial samples phenylacetic acid, PAG, indoxyl sulfate, homocitruline, and propionyl carnitine were significantly affected by the dietary intervention. In the LP group, changes were more limited but we found an increase in indole 3 propionic acid (log fold change: 0.43 i.e., 44% increase). All previously described shifts in metabolites were statistically significant after correction for baseline value, center, and estimated glomerular filtration rate (eGFR). No changes on ImP levels were induced by the protein modulation.

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Table 1. Baseline characteristics			
Total	HP	LP	р
n	78	73	
Age (years)	58.22 (7.72)	59.06 (7.03)	0.488
Sex = M (%)	34 (43.6)	30 (41.1)	0.885
Ethnicity = Caucasian (%)	40 (51.3)	36 (49.3)	0.937
BMI (kg/m2) (mean (SD)	30.74 (4.17)	31.46 (4.60)	0.314
Fat (%)	31.73 (11.48)	34.01 (11.23)	0.228
Free Fat Mass (%)	60.88 (17.80)	59.57 (13.55)	0.622
Creatinine (mmol/L) (mean (SD))	71.00 (19.59)	67.97 (17.13)	0.319
eGFR_MDRD (mL/min) (mean (SD))	106.03 (30.61)	109.78 (33.16)	0.474
Fasting glucose (mmol/L) (mean (SD)	8.45 (2.33)	8.03 (1.74)	0.206
Fasting insulin (pmol/L) (mean (SD)	71.47 (47.68)	71.89 (36.62)	0.953
HbA1c (mmol/mol) (mean (SD)	57.28 (11.97)	54.35 (8.37)	0.088
LDL (mmol/L) (mean (SD)	2.49 (1.10)	2.44 (0.84)	0.726
HDL (mmol/L) (mean (SD)	1.30 (0.34)	1.36 (0.38)	0.371
Triglycerides (mmol/L) (mean (SD)	1.51 (1.19)	1.51 (0.85)	0.99
CRP (mg/L) (mean (SD)	2.61 (2.08)	4.30 (7.49)	0.106
REE (kcal) (mean (SD)	1566.50 (353.16)	1561.03 (283.03)	0.927
SU derivatives = y (%)	19 (24.4)	26 (35.6)	0.182
GLP1 agonists = y (%)	6 (7.7)	7 (9.6)	0.901
DPP4 inhibitors = y (%)	16 (20.5)	11 (15.1)	0.509
Statin = y (%)	47 (60.3)	39 (53.4)	0.495
Anti-hypertensive drug = y (%)	44 (56.4)	36 (49.3)	0.478

Data are expressed as mean \pm SD or %. Student t-test was used for comparison between the groups.

eGFR, estimated glomerular filtration rate; REE, Resting Energy Expenditure; SU derivatives, sulfonylurea derivatives; GLP-1 agonist, glucagon-like-peptide 1 agonist; DDP-4 inhibitors, Dipeptidyl peptidase-4 inhibitors.

To determine the relationship between taxa- and plasma metabolite levels, we tested associations with linear mixed effect models and found several relationships between individual taxa and an increase in the associated metabolite (Figure 6). The results show cross-sectional associations displaying the relationship between individual taxa and associated serum metabolites. However, when taking the dietary intervention into account, only four taxa-metabolite relationships remained significant, and most associations were therefore driven by the dietary intervention. The HP diet increased the Dorea sp.CAG:105, which was correlated with an increase in p-cresol sulfate; Firmicutes bacterium CAG:102 and Oscilibacter sp (both correlated with an increase in PAGIn) and Roseburia sp (which correlated with an increase in Indoxyl sulfate). The LP diet was associated with decreased levels of aforementioned species and metabolites, indicating again a diet-driven response (Figure S7).

DISCUSSION

In this 12-week randomized HP vs. LP dietary intervention without caloric restriction in individuals with T2D treated with metformin, we did not observe any significant effects of protein modulation on post prandial glycemic excursions, HbA1c, and other metabolic parameters. However, protein supplementation induced a small increase in microbial diversity and significant changes in gut microbial-derived metabolomic profiles.

We found no effect of a short-term HP vs. LP dietary intervention on glycemic markers despite detailed phenotyping using post-prandial glycemic excursion after a MMT, HbA1c, HOMA-IR, and real-world data with continuous glucose monitoring. Importantly, long-term observational studies have shown an association between increased animal protein intake and T2D incidence, whereas plant protein consumption had a neutral effect.^{12–15} These results are not contradicted by our study performed in individuals with installed T2D but should not be extrapolated to individuals without T2D for whom protein intake has been





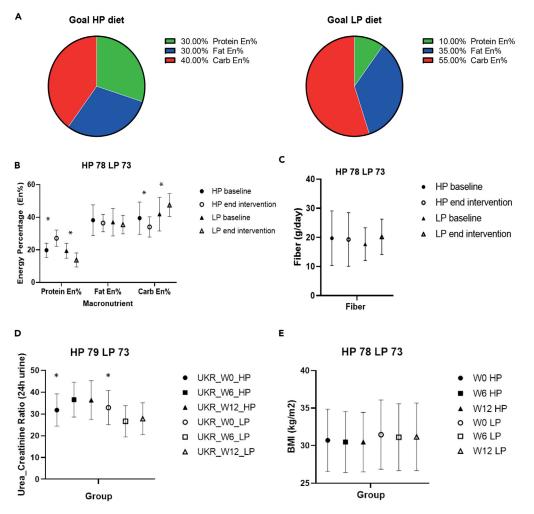


Figure 3. Overview dietary adherence and BMI throughout the study period

(A) Shows the target diet composition in the high protein (HP) and low protein (LP) group.

(B) Self-reported macronutrient consumption at baseline and end of the study period. Significant differences in protein energy percentage (En%) and carbohydrate intake between baseline and end of intervention period in HP and LP group. The effect of the intervention (HP vs. LP) on changes from baseline (delta between week 12 and week 0) was analyzed in a linear regression model adjusting for baseline values and center.

(C) Fiber intake between HP and LP group throughout the intervention (ns).

(D) 24-h urine urea/creatinine ratios. In the HP group statistical significant increase between week 0 and week 6 and week 0 and week 1, no statistical significance between week 6-week 12. In the LP group statistical significant decrease between week 0 and week 0 and week 0 and week 12, no statistical significance between week 6-week 12. Data were analyzed using a linear-mixed effects model with post-hoc Dunn's correction.

(E) BMI (body-mass index) between HP and LP group throughout the intervention (ns). All data are presented as mean \pm SD. A p < 0.05 was considered statistically significant (indicated with an *).

associated with increased insulin secretion and hepatic production of glucose, which ultimately may lead to increased insulin resistance and T2D development.⁴⁶ On the other hand, in individuals with T2D, low-calorie HP dietary interventions reported improved metabolic markers^{16,17} contrary to our results. However, in these studies, the effects of HP diet may have been confounded by the caloric-restriction induced weight loss, which was more important in HP diets and is a confounding factor when studying metabolic parameters. Here, we carefully controlled this potential confounder by providing isocaloric diet in both HP and LP groups. Consequently, the main change was the modulation of macronutrients as shown by the little observed weight loss (approximately 1 kg), which was not different between the two groups. Therefore, this study allows to interpret solely the effect of protein modulation without the bias of differences in weight loss between the groups.



	Total	HP	LP	р
n	150	78	72	
Kcal(mean(SD))	1456.5(417.0)	1551.4(432.4)	1395.3(386.4)	0.02
Total protein(gr) (mean(SD))	74.2(37.2)	63.9(28.0)	47.9(15.2)	<0.01
Animal protein(gr) (mean(SD))	45.7(30.0)	98.5(34.8)	26.2(17.2)	<0.01
Plant protein(gr) (mean(SD))	19.1(7.7)	19.0(9.4)	19.1(5.4)	0.93
Carbohydrates (gr) (mean(SD))	148.4(49.6)	135.8(41.1)	162.0(54.6)	<0.01
Fiber (gr) (mean(SD))	18.78(7.8)	19.29(9.2)	20.18(6.1)	0.25
Sugar(gr) (mean(SD))	33.4(28.4)	30.5(24.0)	36.4(32.3)	0.27
Fat (gr) (mean(SD))	60.8(21.2)	67.7(21.8)	56.6(19.9)	0.02
Saturated fat (gr) (mean(SD))	20.6(8.6)	22.9(8.4)	18.1(8.2)	< 0.01

The 24 h recall data were collected on 3 randomized days per week during the intervention. Data are expressed as mean \pm SD. Student t-test was used for comparison between the groups. A p < 0.05 was considered statistically significant.

Renal function was significantly changed by the protein intervention with a decrease in the HP group opposed to an improvement in the LP group. The effect was rather small with little clinical relevance, but this result questions what may be the outcomes of longer-term HP diets on renal function. Previous meta-analysis showed that indeed, an LP diet may improve renal function in T2D, without effecting glycemic parameters.⁴⁷ Whether an HP diet significantly and clinically worsens renal functions remains a topic of discussion⁴⁸

In this controlled setting, the HP vs. LP intervention induced an increase in gut microbiota diversity, due to the HP group. Previous studies in individuals without T2D have reported that a HP, caloric-restricted dietary intervention was associated with an increase in microbiota diversity sometimes reaching 30% increase.^{49,50} Our result suggests that HP diet per se (e.g., without calorie restriction) could be associated with increased alpha diversity albeit with a low magnitude. Indeed, the observed increase in diversity is moderate (2.4% increase in Shannon index) and probably not sufficient to restore microbiota richness in subjects with severe dysbiosis which has been linked with metabolic diseases.⁵¹ This suggests that to significantly and more dramatically increase microbial diversity, combining caloric restriction with macronutrient changes might be more efficient.

Furthermore, this study found that protein modulation did result in changes in plasma metabolite profile, mainly driven by the HP group. Of note, these changes were seen without large effects on gut microbiota composition or function as evaluated via metagenomics analysis. One possible explanation for this, is the potential of diet to induce post-translational changes,⁵² i.e., changes that can be more pronounced in the transcriptome which is undetectable via metagenomics.

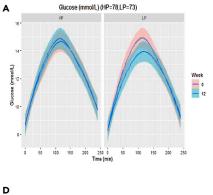
The main metabolites monitored that increased due to the HP diet were PAG and indoxyl sulfate, both of which have been clinically and mechanistically associated with cardiovascular risk.^{39,53,54} PAG is a gutderived metabolite produced from the essential amino acid phenylalanine. Increased PAG levels have been associated with cardiovascular disease and increased thrombosis potential.³⁹ Indoxyl sulfate is a gut-derived uremic toxin which has been linked to chronic kidney disease and cardiovascular disease, possibly via increased inflammation, endothelial dysfunction, and higher levels of cardiac fibrosis.⁵³

In the LP group the main metabolite change was a significant increase in indole-3-propionic acid. This gut microbiota generated metabolite of the essential amino acid tryptophan may be a protective factor against atherosclerosis by promoting reverse cholesterol transport and is down-regulated in patients with atherosclerotic cardiovascular disease.⁵⁵ Moreover, indole-3-propionic acids has been clinically and mechanistically been linked to diabetes and other metabolic disorders.⁵⁶ The protein content of the diet had a neutral impact on ImP, a metabolite of the essential amino acid histidine. Although this result may be surprising, it confirms the absence of link between histidine consumption and ImP levels previously reported in an observational study from the European MetaCardis population.57



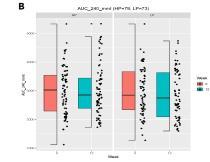
NVA_B





HOMA_IR (HP=78; LP=73

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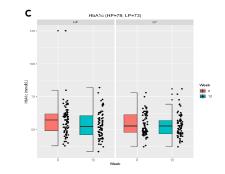


Figure 4. Effect of dietary intervention on glycemic parameters

(A) Glucose excursions following a mixed-meal test (MMT) at week 0 and week 12 for 240 min. Data are represented as mean ± SD, no significant changes between the HP or LP group.

(B) Area under the curve (AUC) of MMT test performed at baseline and week 12 (ns).

CON 10

(C) No significant effect of dietary intervention on HbA1c between week 0 and week 12.

Week

(D) No significant changes in Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) due to the dietary intervention at baseline and week 12. The effect of the intervention (HP vs. LP) on changes from baseline (delta between week 12 and week 0) was analyzed in a linear regression model adjusting for baseline values and center.

This further supports the hypothesis that dietary protein may be metabolized in the small intestine but that other macronutrients, e.g., dietary fiber, can modulate the gut microbiota structure and function thus affecting enzyme activities and metabolite production where substrates originate from host or microbial proteins.⁴⁰

On the other hand, we did not observe significant metagenomic functional changes with the intervention whereas metabolites change significantly with a relatively important effect size. This suggests that for these metabolites the most modification of the gut environment with the protein modulation was sufficient to change their production without changes in abundance of metagenomic functions. It is also possible that duration of the intervention was not sufficient to induce significance metagenomic functional shifts and that a longer intervention would have resulted in both metagenomic functional changes and metabolites changes.

Since diet induced small, but significant, changes in gut microbiota diversity and also led to a change⁵⁸ in plasma metabolite profile, we next determined associations between microbial taxa and plasma metabolites. This study identified several associations between taxa and metabolites, such as *Oscillibacter* and indoxyl sulfate levels, which has been previously reported.⁵⁸ However, when specifying to the intervention in this study, only four associations remained. The HP diet increased the *Dorea* sp.*CAG:105*, which was correlated with an increase in p. cresolsulfate; *Firmicutes bacterium CAG:102*, *Oscilibacter* sp (both correlated with an increase in PAGIn), and *Roseburia* sp (which correlated with an increase in Indoxyl sulfate). The LP diet decreased aforementioned species and metabolites. More research is warranted in these specific taxa-metabolite relation-ships, as these can serve as potential targets of specific dietary interventions.

In conclusion, this multi-center, 12-week, randomized-controlled isocaloric, dietary protein intervention in individuals with T2D subjects on metformin treatment showed that a short-term protein modulation does not

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Total	HP	LP	Delta HP	Delta LP	р
n	78	73	78	73	
BMI (kg/m2) (mean (SD)	30.55 (3.96)	31.17 (4.50)	-0.29 (0.84)	-0.38 (0.75)	0.63
Fat (%)	31.83 (10.55)	33.42 (10.82)	-0.15 (5.14)	-0.99 (3.67)	0.43
Free Fat Mass (%)	60.77 (16.38)	60.06 (14.08)	-0.66 (5.36)	0.58 (4.28)	0.24
Creatinine (mmol/L) (mean (SD))	73.15 (23.13)	66.73 (16.77)	1.82 (10.13)	-1.57 (7.09)	0.02
eGFR_MDRD (mL/min) (mean (SD))	104.08 (30.60)	110.90 (29.76)	-1.67 (15.32)	3.00 (12.98)	0.03
Fasting glucose (mmol/L) (mean (SD)	8.26 (2.27)	7.83 (1.74)	-0.23 (1.64)	-0.16 (1.33)	0.76
Fasting insulin (pmol/L) (mean (SD)	72.31 (47.19)	73.57 (38.16)	0.83 (38.63)	1.26 (24.97)	0.90
HbA1c (mmol/mol) (mean (SD)	53.96 (10.00)	53.16 (8.67)	-3.27 (9.15)	-1.09 (6.35)	0.42
LDL (mmol/L) (mean (SD)	2.36 (1.09)	2.35 (1.19)	-0.13 (0.66)	-0.09 (0.94)	0.76
HDL (mmol/L) (mean (SD)	1.33 (0.39)	1.32 (0.42)	0.02 (0.16)	-0.02 (0.15)	0.06
Triglycerides (mmol/L) (mean (SD)	1.35 (0.88)	1.51 (0.90)	-0.15 (0.71)	0.01 (0.55)	0.07
CRP (mg/L) (mean (SD)	3.79 (7.09)	3.27 (4.39)	1.18 (7.37)	-1.16 (7.81)	0.44

Data are expressed as mean \pm SD or %. The effect of the intervention (HP vs. LP) on changes from baseline (delta between Week 12 and Week 0) was analyzed in a linear regression model adjusting for baseline values and center. eGFR= estimated glomerular filtration rate.

affect glycemic parameters, but an HP diet results in a small increase in serum creatinine. Moreover, the HP diet vs. the LP diet leads to changes in gut microbiota composition and production of (gut-derived) metabolites which are themselves known to be associated with cardiovascular risk. Furthermore, this study identified several taxa-metabolite associations that were diet driven. More research is needed with longer duration and study population in order to investigate and validate potential causal effects of these findings in CMD.

Limitations of the study

This study has limitations and strengths. A first limitation is the relatively short duration of the study and exposition to the dietary modification. Indeed, it is possible that with a more prolonged exposure, the dietary intervention may have had a significant effect on metabolic outcomes. Another limitation is that our sample size may have not been large enough to detect smaller metabolic changes. However, our power analysis did show sufficient power for the primary outcome. Moreover, the population studied was heterogeneous and used several antidiabetic drugs, which could have influenced gut microbiota composition and function as shown in previous metagenomic analysis.⁵⁹ Although half of the diet was directly provided to the patients, we did not control all of the dietary and beverage intake of the participants during the study, as this was not feasible for such a relatively large group. Another limitation is the fact that, by design, protein was not the only macronutrient modified by the dietary intervention study, as carbohydrates were also significantly changed in order maintain an isocaloric diet. However, we can note that the main difference in terms of dietary intake between the 2 groups is the protein content which is double the amount in HP vs. LP when the difference in carbohydrates or fat intake between the two groups is much less pronounced. Moreover, fiber intake was kept constant throughout the study to avoid confounding effects. Moreover, this study only investigated post-prandial metabolite changes after 240 min at baseline and after a 12 weeks intervention. However, this study did identify several metabolites that had altered post-prandial levels after 12 weeks of protein intervention compared to baseline. However, studies are needed investigating longer post-prandial timepoints to ensure that these changes are not transient, but can, with prolonged duration of intervention, affect host metabolism.

Strengths of this study included the detailed phenotyping on both metabolic, as well as microbial level. Moreover, this study investigated a heterogeneous, real world, multi-ethnic population which increases the generalizability of the findings. The results of this study add to the ongoing debate whether animal protein intake can be associated with negative metabolic outcomes.¹¹ This is of importance as this is one of the first studies investigating the effect of protein modulation in an isocaloric fashion taking also the effect on gut microbiota composition and gut-derived metabolites into account. Lastly, this study was not confounded by weight changes, as the majority of dietary intervention studies are which allows a proper interpretation of macronutrient modulation.



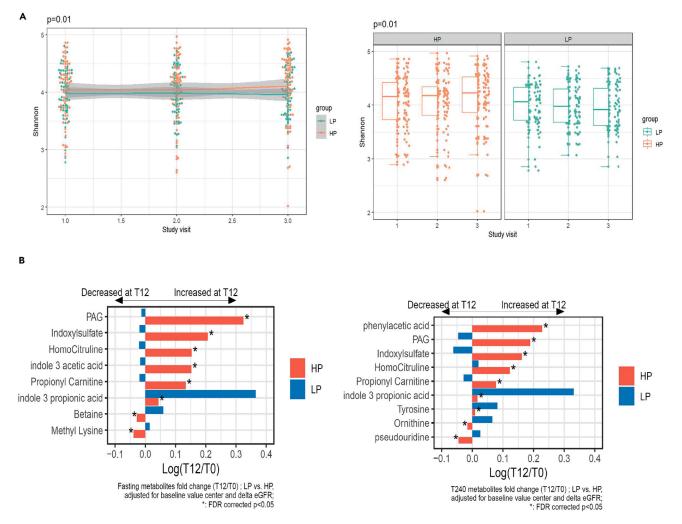


Figure 5. Effect of dietary intervention an alpha diversity

(A) Effect of dietary intervention an alpha diversity (Shannon index). A high protein diet increased Shannon index (p = 0.01).
(B) Fasting plasma metabolite levels and post-prandial metabolite levels 240 minutes after a mixed meal test. A high protein diet induces several changes in plasma metabolome both fasting (left panel) and 240 min post MMT (right panel). Metabolite fold changes were analyzed after log transformation in a linear model correcting for baseline value, center and delta estimated glomerular filtration rate (eGFR). All analyses were corrected for false discovery rate (FDR). PAG: Phenylacetylglutamine.

STAR***METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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- $\,\,\odot\,\,$ Study population and ethics approval
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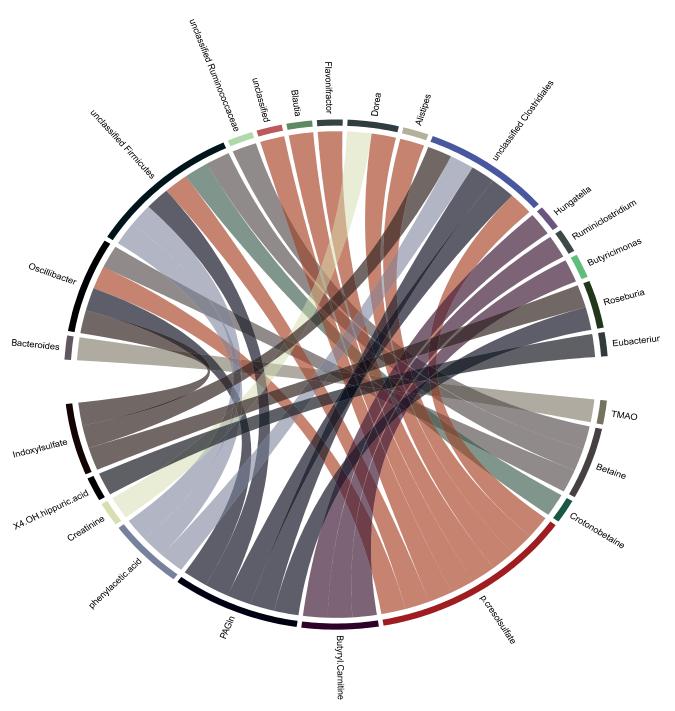


Figure 6. Associations between individual taxa and plasma metabolite levels

Displayed are only significant associations using linear mixed effect models and false discovery rate (FDR) correction.

- Mixed-meal test
- O DNA extraction, library preparation and gut microbiota sequencing analysis
- O Metabolomics
- O Targeted LC-MS/MS analysis of selected metabolites in human plasma
- $\odot\;$ Sample preparation for histidine, Imidazole Propionate and urocanate quantification
- QUANTIFICATION AND STATISTICAL ANALYSIS





SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107471.

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AUTHOR CONTRIBUTIONS

I.A., P.B.L., K.C., and M.N. coordinated the clinical trial and drafted the first version of the manuscript. S.A. and S.L.O. coordinated the dietary adherence. R.P.N., V.T., and M.D. performed the metagenamic analysis. I.N., S.L.H., P.O.B., K.B., and F.B. coordinated the metabolomics analyses and integration in the study. I.A. also performed metabolomics analysis with the help of a Fulbright Scholarhip (2021). E.S., R.A., F.V., S.B., E.B., J.T.A., L.A.H., A.G., E.L., S.P.M.B., H.H., A.K.G., and S.J.P.S. helped with drafting the manuscript and provided input in the trial design and analyses.

DECLARATION OF INTERESTS

M.N. is in the SAB of Caelus health; however, this is not relevant for the current paper.

S.L.H. reports being named as co-inventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics, being a paid consultant formerly for Procter & Gamble in the past, and currently with Zehna Therapeutics, and both receiving research funds from Procter & Gamble, Zehna Therapeutics, and Roche Diagnostics, and being eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Procter & Gamble, Zehna Therapeutics, and Cleveland HeartLab, a wholly owned subsidiary of Quest Diagnostics.

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STAR*METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human plasma metabolomics data	This study (PI Prof. Dr. M. Nieuwdorp)	N/A
Human fecal metagenomics data	This study (PI Prof. Dr. M. Nieuwdorp)	N/A
Chemicals, peptides, and recombinant proteins		
Histidine-D5,15N3	Cambridge Isotope Lab. Inc, Cambridge, UK	(Koh et al. 2018) ⁴⁰
imidazole propionate-13C3	Astra Zeneca, Cambridge, UK	(Koh et al. 2018) ⁴⁰
urocanate-13C3	Astra Zeneca, Cambridge, UK	(Koh et al. 2018) ⁴⁰
Critical commercial assays		
DNA extraction kit	QIAamp DNA Mini kit	N/A
Software and algorithms		
open software program R version 4.2.1	(R Core Team (2022). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL Https://Www.R-Project. Org/., ND)	https://www.R-project.org/
MEDUSA pipeline N/A		(Karlsson et al. 2014) ⁷²
Other		
Freestyle Libre Glucose Monitoring System	ABBOTT DIABETES CARE IN FRANCE Abbott France S.A.S. Abbott Diabetes Care 94528 Rungis Cedex France	version 1.0
Nutridrink Compact Protein	Nutricia Advanced Medical Nutrition, Strawberry compa Amsterdam, the Netherlands	
Food diary log "Mijn Eetmeter"	Stichting Voedingscentrum Nederland	N/A

RESOURCE AVAILABILITY

Lead contact

Further information should be directed to and will be fulfilled by the lead contact Prof. Dr. M. Nieuwdorp (m.nieuwdorp@amsterdamumc.nl).

Materials availability

This study did not generate new unique reagents or materials.

Data and code availability

- Data reported in this paper will be shared by the lead contact upon reasonable request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study population and ethics approval

This study recruited non-insulin dependent individuals with T2D. Participants were recruited through local newspapers, social network advertisements general practitioners, or during a visit to the center where they





were followed-up for their T2D. Inclusion criteria were: presence of T2D as defined by the American Diabetes Association, ⁶⁰ stable use of metformin for \geq 3 months, as metformin therapy is among the first lines of antidiabetic drugs and has a profound effect on gut microbiota, ^{61,62} BMI \geq 25 kg/m², age 40–70 years, Caucasian, Caribbean or African origin. Exclusion criteria were: use of insulin therapy, antibiotic usage within the last three months, uncontrolled diabetes (HbA1c>9% i.e., 75 mmol/mol), vegetarian diet, presence of chronic inflammatory disease, use of pre-pro-synbiotics, use of proton-pump-inhibitor, eGFR < 50 mL/min/1.73 m², active malignancy, unmotivated or unable to adhere to the diet. The baseline characteristics of participants are described in Table 1.

The study was approved by the local Institutional Review Board of both centers and was carried out simultaneously in Amsterdam UMC, location AMC and Paris, University Hospital Pitié-Salpêtrière, Sorbonne University. The study was conducted in accordance with the Declaration of Helsinki and registered at the clinical trial registry: NCT03732690 and NCT03732690. Written informed consent was obtained from all participants.

METHOD DETAILS

Study design

This study was a 12-week randomized controlled, non-blinded, isocaloric dietary intervention clinical trial (Figure 1). Participants were randomized to follow either a HP diet (HP) or a LP diet (LP) for a duration of 12 weeks and visited the study location fasted at three times (Week 0, Week 6 and Week 12). Before each visit, participants collected 24 h and fresh fecal samples and delivered them in a cool box. Feces samples were immediately frozen in -80° C.

Two weeks before each study visit, participants wore a continuous glucose monitor (Freestyle Libre) in order to obtain real-life data. At baseline and week 12, participants underwent a MMT to determine insulin resistance, which provides a more physiological response compared to oral-glucose tolerance test.^{63,64} Primary objective of the study was to investigate the effect of the diet on post-prandial glycemic excursion (AUC) after the MMT. Secondary objectives were to analyze the effects of the diets on (i) post-prandial glycemic excursion using continuous glucose monitors, on glycemic control and metabolic markers such as HbA1c and cholesterol levels, (ii) gut microbiota composition, alpha and beta diversity and (iii) serum gut-derived metabolites levels. All individuals had a moderate to normal renal function, which was defined as an eGFR > 50 mL/min/1.73 m², according to the MDRD formula.^{65,66}

Diet

The objective in the HP group was to reach 30% of total energy intake (En%) from protein. The objective in the LP diet (LP) was to limit protein to 10% of total energy intake (En%). These En% were used as target, as they are among the extreme of the reference intake of protein recommended.⁶⁷ In both groups, no caloric restriction was performed. Baseline energy intake requirements were estimated using resting energy expenditure measured by indirect calorimetry adjusted for physical activity level. To avoid the confounding effect of weight changes on the outcomes of this study, participants were instructed to not change their lifestyle throughout this study, with the exception of protein composition in their diet.

Before randomization and during the entire study period, participants had filled out a three-day food diary weekly (two weekdays and one weekend day, randomized every week). Personalized adaptations of their diet were then given by a trained dietician to reach the protein consumption objectives of the allocated group (HP or LP) using a mix of dietary guidance and specific food deliveries to their homes. Participants were provided with HP or LP snacks and meals, which filled approximately half of their total energy intake throughout the study. In the HP group protein supplements consisted mostly out of animal protein, well balanced between red,white meat and fish. The detailed composition of the food supplements is provided in the supplementary information (Table S1) For the other half of their food intake, they were instructed to favor/avoid certain foods using lists of high or LP containing foods and example menus. Throughout the study, participants had weekly phone contact with the dietician to ensure dietary compliance and to provide additional guidance if the protein objectives were not reached. In addition to dietician interviews, compliance to the diet was evaluated using 24-h dietary recalls, collected 3 days per week, so a total of 12*3=36 food diaries per subject, and by collecting 24-h urine samples before each study visit in order to determine the urea/creatinine ratio, a validated marker for protein intake.⁶⁸

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Anthropometric and clinical measurements

At baseline and week 12 (end of study), body composition was measured through Bioelectrical impedance analysis and resting energy expenditure through indirect.⁶⁹ Blood samples were collected after an overnight fast. Fasting serum glucose, triglycerides and HbA1c were measured using enzymatic methods. Examination of these anthropometric and biological outcomes were part of secondary outcomes to be evaluated.

Mixed-meal test

Participants underwent a 4-h MMT.⁶³ Briefly, participants visited the study center at baseline and week 12. Subjects were fasted for at least 8 h before the site visit and an intravenous catheter was placed in a distal arm vein. Baseline blood sampling was obtained and afterward participants immediately ingested a liquid meal solution (Nutridrink, Nutricia Advanced Medical Nutrition, Amsterdam, the Netherlands) containing 600 kcal (35% fat, 49% carbohydrates and 16% proteins) blood was sampled at fixed time point for the duration of 4 h, centrifuged and stored in the minus 80°C, until further analysis. Blood was drawn for metabolite analyses at baseline (fasted) and after 240 min post-prandial, after ingestion of the MMT. This procedure was done before the dietary protein intervention and after 12 weeks of following either an HP or an LP diet in order to determine (240 min post prandial) plasma metabolite changes modulated via 12 weeks of dietary protein intervention.

DNA extraction, library preparation and gut microbiota sequencing analysis

DNA extraction and library preparation was performed as previously published.^{70,71} Briefly, fecal samples were extracted in Lysing Matrix E tubes (MP Biomedicals) containing ASL buffer (Qiagen). Lysis was obtained after homogenization by vortexing for 2 min followed by two cycles of heating at 90 °C for 10 min with afterward three bursts of bead beating at 5.5 m s-1 for 60 s in a FastPrep-24 instrument (MP Biomedicals). After each beadbeating burst, samples were placed on ice for 5 min. Supernatants containing fecal DNA were collected after two cycles by centrifugation at 4 °C. Supernatants from the two centrifugation steps were pooled, and a 600µL aliquot from each sample was purified using the QIAamp DNA Mini kit (QIAGEN) in the QIAcube instrument (QIAGEN) using the procedure for human DNA analysis. Samples were eluted in 200 µL of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0). Libraries for shotgun metagenomic sequencing were prepared by a PCR-free method; library preparation and sequencing were performed at Novogene (Cambridge, UK) on a HiSeq instrument (Illumina) with 150-bp paired-end reads and 6 G data per sample. The MEDUSA pipeline was used to process shotgun metagenomics.⁷² Briefly, Total fecal genomic DNA was extracted from 100 to 150 mg of feces by repeated bead beating using a modification of the IHMS DNA extraction protocol Q.⁷⁰ libraries for shotgun metagenomic sequencing were prepared by a PCR-free method; library preparation and sequencing were performed at Novogene (Cambridge, UK) on an Illumina NovaSeg 6000 S4 flow cell with 150-bp paired-end reads and 6 G data per sample. Raw reads were processed with a pipeline implemented with NGless v.1.0.⁷³ Briefly, reads were first preprocessed by filtering basecalls with a phred score below 25 and reads with less than 45 bp of length. Then, contaminants were filtered by mapping the quality filtered reads against a database containing human, animal, fungus and plant genomes (minimum match size = 45 and minidentity percentage = 95). Filtered reads were mapped with bwa⁷⁴ against the IGC (Integrated Gene Catalog), a 9.9 million human gut microbial genes collection.¹⁵ Gene abundance table was computed with the NGless dist1 option where multiple mapped reads are distributed based on the coverage of singly mapped reads. The gene abundance table generated with NGless was then treated with MetaOminer V1.2⁷⁶ for rarefaction to 10⁷ reads and RPKM normalization. A second catalog of Co-Abundance Gene groups (CAG) that accompanied the IGC, those CAGs with more than 500 genes were considered as Metagenomic Species (MGS). The relative abundance for each MGS was established as the average abundance of the 50 most correlated genes. Classification at the species level for each MGS was stablished when at least 50% of the MGS matched the same NCBI reference genome at 95% identity and 90% of the length coverage. For superior taxonomic levels, the criteria were relaxed to 85% and 75% of identity to assign genus and phylum respectively. Finally, abundances of gut microbial derived metabolic modules (GMM) were determined from gene abundance tables according the classification proposed by Vieira-Silva.⁷

Metabolomics

Plasma metabolites were measured using stable-isotope-dilution LC-MS/MS as recently described^{39,78} Quantification of histidine, ImP and urocanate were similarly performed using stable isotope dilution LC-MS/MS, using heavy isotope labeled internal standards (Histidine-D5,15N3, Cambridge Isotope Lab. Inc, ImP-13C3 and urocanate-13C3, Astra Zeneca, Cambridge, UK).⁴⁰



Targeted LC-MS/MS analysis of selected metabolites in human plasma

Stable-isotope-dilution LC-MS/MS was used for quantification of metabolites as previously described with some modifications.⁷⁸ An aliquot (20 μ L) of plasma was mixed with ice-cold methanolic solution of internal standards (D4-tryptamine; D5-indole-3-acetic acid; D2-indole-3-propionic acid; D5-phenylacetylglut-amine; D2-5-OH- indole-3-acetic acid; D5-hippuric acid; 13C2-phenylacetic acid; D4-2-OH-benzoic acid; D4-4-OH-benzoic acid; D7-p-cresol sulfate; D4-indoxyl sulfate; D3-acisoga; D3-L-acetylcarnitine; D7-ADMA; 13C6-arginine; D9-betaine; D9- γ -butyrobetaine; D3-butyrylcarnitine; D3-carnitine; D4-citrulline; D9-choline; D3-creatinine; D9-crotonobetaine; 13C615N-leucine; 13C615N2-lysine; D3-octanoylcarnitine; D6-ornithine; 13C6-phenylalanine; D3-propionylcarnitine; D9-TMAO; D9-trimethyllysine; 13C915N-tyrosine; 13C515N-valine; D4-mannosyl-tryptophan and 13C15N2- pseudouridine) was added to plasma samples (80 μ L), followed by vortexing and centrifuging (21,000 × g; 4°C for 15 min). The clear supernatant was then transferred to glass vials with microinserts.

LC-MS/MS analysis was performed on a chromatographic system consisting of two Shimadzu LC-3AD 0 pumps (Nexera X2), a CTO 20AC oven operating at 30°C (for the reverse phase method) and 40°C (for the normal phase method), and a SIL-30 AC-MP autosampler in tandem with 8050 triple guadruple mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). The following ion source parameters were applied: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; interface temperature, 300°C; desolvation line temperature, 250°C; heat block temperature, 400°C; and drying gas flow, 10 L/min. A tandem of a Luna Silica column (150 mm × 2.0 mm; 5 µm) (Cat # 00f-4274-B0, Phenomenex, Torrance, CA) and a Kinetex C18 column (50 mm × 2.1 mm; 2.6 μm) (Cat # 00B-4462-AN, Phenomenex, Torrance, CA) was used for chromatographic separation under a non-linear gradient of 0.1% propionic acid in water (solvent A) and 0.1% acetic acid in methanol (solvent B). Electrospray ionization in positive mode with multiple reaction monitoring (MRM) was used with the following transitions: m/z $161.00 \rightarrow 144.05$ for tryptamine; m/z $164.90 \rightarrow 148.20$ for D4-tryptamine; m/z 176.00→130.10 for indole-3-acetic acid; m/z 181.20→134.25 for D5-indole-3-acetic acid; m/z 190.00 → 130.10 for indole-3-propionic acid; m/z 191.80 \rightarrow 0.05 for D2-indole-3-propionic acid; m/z 206.20 \rightarrow 118.10 for indole-3-lactic acid; m/z 265.2→130.15 for PAGIn; m/z 270.1→130.15 for D5-PAGIn; m/z 192.00→146.10 for 5-OH-indole-3-acetic acid; m/z 194.20→148.10 for D2-5-OH-indole-3- acetic acid; m/z 180.00 → 105.10 for hippuric acid; m/z 185.00 → 110.15 for D5-hippuric acid; m/z 195.80 → 121.10 for 2-OH-, 3-OH- and 4-OH-hippuric acid; m/z 185.00→126.10 for acisoga; m/z 188.10→126.10 for D3-acisoga; m/z 76.10→59.10 for TMAO; m/z 85.00→66.25 for D9-TMAO; m/z 104.00→60.05 for choline; m/z 113.10→ 69.20 D9-choline; m/z 118.10 \rightarrow 58.10 for betaine; 127.00 \rightarrow 66.10 for D9-betaine; m/z 162.00 \rightarrow 103.00 for carnitine; m/z 165.00 \rightarrow 103.05 for D3-carnitine; m/z 146.00 \rightarrow 87.05 for γ -butyrobetaine; m/z 155.00 \rightarrow 69.20 for D9- γ -butyrobetaine; m/z 144.00 \rightarrow 8.10 crotonobetaine; m/z 153.00 \rightarrow 66.15 for D9-crotonobetaine; m/z 133.00 \rightarrow 116.05 for ornithine; m/z 139.00 → 76.10 for D6-ornithine; m/z 147.00 → 130.10 for lysine; m/z 155.00 → 90.05 for u-lysine; m/z 161.00 \rightarrow 84.10 for methyl-lysine; m/z 175.00 \rightarrow 84.10 for dimethyl-lysine; m/z 189.00 \rightarrow 84.10 for trimethyl-lysine; m/z 198.10 \rightarrow 84.10 for D9-trimethyl-lysine; m/z 175.00 \rightarrow 60.10 for arginine; m/z 181.00 \rightarrow 74.15 for 13C6-arginine; m/z 176.10 \rightarrow 70.10 for citruline; m/z 180.00 \rightarrow 74.15 for D4-citruline; m/z 189.00 \rightarrow 70.05 for monomethyl-arginine; m/z 189.00 \rightarrow 84.15 for homoarginine; m/z 190.00 \rightarrow 84.05 for homocitruline; m/z 203.00 \rightarrow 70.05 for ADMA; m/z 210.00 \rightarrow 77.05 for D7-ADMA; m/z 203.00 \rightarrow 172.10 for SDMA; m/z 114.10 \rightarrow 44.05 for creatinine; m/z 116.90 \rightarrow 47.10 for D3-creatinine; m/z 165.90 \rightarrow 120.05 for phenylalanine; m/z 172.10 → 126.05 for 13C6-phenylalanine; m/z 182.10 → 136.00 for tyrosine; m/z 192.10 → 145.05 for u-tyrosine; m/z 118.10 \rightarrow 55.15 for valine; m/z 124.10 \rightarrow 77.05 for u-valine; m/z 132.00 \rightarrow 30.20 for leucine; m/z $139.00 \rightarrow 92.15$ for u-leucine; m/z $132.00 \rightarrow 69.00$ for isoleucine; m/z $204.00 \rightarrow 84.95$ for acetyl-carnitine; m/z 207.00→85.00 for D3-acetyl-carnitine; m/z 218.00→84.95 for propionyl-carnitine; m/z 220.90→85.00 for D3-propionyl- carnitine; m/z 232.00→85.00 for butyryl-carnitine; m/z 234.90→85.00 D3-butyryl-carnitine; m/z $367.00 \rightarrow 247.00$ for mannosyl-tryptophan; m/z $371.00 \rightarrow 251.00$ for D4-mannosyl-tryptophan; m/z $243.00 \rightarrow 153.00$ for pseudouridine and m/z 246.00 $\rightarrow 156.00$ for 13C15N2- pseudouridine. Electrospray ionization in negative mode with MRM was used with the following transitions: $m/z 135.00 \rightarrow 91.00$ for phenylacetic acid; m/z 137.00→92.00 for 13C2- phenylacetc acid; m/z 136.9→93.05 for 2-OH-, 3-OH and 4-OH-benzoic acid; m/z 141.50→97.10 for D4-2-OH and D4-4-OH-benzoic acid; m/z 181.10→163.15 for 4-OH- phenyllactic acid; m/z 186.70 \rightarrow 107.00 for p-cresol sulfate; m/z 193.90 \rightarrow 114.10 D7-p-cresol sulfate; m/z 211.90 \rightarrow 79.05 for indoxyl sulfate; m/z 215.50 \rightarrow 80.00 for D5-indoxyl sulfate and m/z 1730.10 \rightarrow 93.1 for phenol sulfate.

Sample preparation for histidine, Imidazole Propionate and urocanate quantification

For targeted measurement of histidine, ImP and urocanate, plasma samples were precipitated in 1.3 mL glass vials using 3 volumes of ice-cold acetonitrile containing internal standards (Histidine-D515N3,





Cambridge Isotope Lab. Inc, ImP-13C3 and urocanate-13C3, Astra Zeneca, Cambridge, UK). After vortexing and centrifugation, the supernatant was transferred to new glass vials and evaporated under a stream of nitrogen. The samples were then reconstituted in 5% HCI (37%) in 1-butanol and placed in oven at 70°C for 1 h allowing the n-butyl ester to be formed. After derivatization, the samples were evaporated and reconstituted in 100 μ L of water:acetonitrile [90:10]. The samples were then analyzed using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). The analytical system consisted of an Acquity UPLC I-class binary pump, sample manager and column manager coupled to a Xevo TQ-XS (Waters, Milford, MA, USA). The samples (2 μ L) were injected onto a C18 BEH column (2.1 × 50 mm with 1.7 μ m particles, Waters, Milford, MA, USA) and separated using a gradient consisting of water with 0.1% formic acid (A-phase) and acetonitrile with 0.1% formic acid (B-phase). The analytes were detected with MRM using the transitions 212/110 (histidine), 197/81 (ImP) and 195/93 (urocanate).

For the internal standards, the transitions 220/118, 200/82 and 198/95 respectively were used. Calibration curves of histidine, ImP and urocanate were made in methanol and treated the same way as the samples.

QUANTIFICATION AND STATISTICAL ANALYSIS

Based on studies and a hypothesized peak-difference in postprandial glucose excursion (13.0 mmol/L in HP group vs. 10.1 LP group; SD 2.8) following the MMT, we calculated that we needed 60 subjects per arm to detect a significant difference in this trial. This number was based on a significance level of 0.05 and 80% power and was calculated using an online power calculation (www.biomath.info/power). Delta changes before and after intervention were calculated and the effect of the intervention (HP vs. LP) on these changes was analyzed using linear mixed effect models generated with lme4 (v1.1.30) and lmerTest (v3.1.3) R packages and linear regression models correcting for baseline value, site center and using subject ID as random effect in the mixed-effect models, to account for baseline differences. For the linear regression model: Delta variable = group + baseline value variable+ center, for the linear mixed effect models delta_change = group+center | ID as random effect. In the metabolic module analysis additional adjustment for ethnicity was added. In addition to determine the significance of the diet in the abundance of the different GMM we compared the fits of the model adjusted for the diet with a simpler model without diet adjustment.

For metabolites related analyses further adjustment was performed on eGFR changes. All statistical analyses were done using the open software program R (version 4.2.1).⁷⁹ FreeStyle Libre data were processed using the CGDA package.⁸⁰ A p value <0.05 was considered statistically significant. Originally, the aim was to include 120 subject per center in order to have enough power to detect ethnic specific effects in sub-group analysis. However, due to the COVID pandemic, the inclusion rate was hampered and the analysis was restricted to the main objective without sub-group analysis.

Annex III: COVID-19 and its Severity in Bariatric Surgery-Operated Patients

COVID-19 and its Severity in Bariatric Surgery-Operated Patients

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Objective: Obesity is a major risk factor for severe forms of coronavirus disease (COVID-19), but little is known about the post–bariatric surgery (BS) setting. The prevalence of likely COVID-19 and its risk factors in patients followed up after BS was assessed.

Methods: A total of 738 patients who underwent BS and were followed up at a university medical center were surveyed. A retrospective comparison of characteristics at baseline, 1 year after BS, and at the time of lockdown was performed between patients with COVID-19–likely events (CL) based on a combination of reported symptoms and those for whom COVID-19 was unlikely.

Results: CL occurred in 62 (8.4%) patients, among whom 4 (6.4%) had a severe form requiring hospitalization and 1 (1.6%) died. The CL group had a higher proportion of persistent type 2 diabetes (T2D) at last followup (36.2% vs. 20.3%, P=0.01). BMI at the time of lockdown was lower in the CL group (30.2±5.1 vs. 32.8±6.5 kg/m²; P<0.01) with higher percent weight loss since BS in the CL group. Severe forms of COVID-19 requiring hospitalization were associated with persistent T2D at the last follow-up visit.

Conclusions: In BS patients, CL were associated with persistent T2D and lower BMI.

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Introduction

Obesity is a recognized major risk factor for severe forms of coronavirus disease (COVID-19) and related mortality, independently of obesity-associated comorbidities (1-4). Although several hypotheses have been suggested to explain why, much remains to be understood. Beyond the increased prevalence of type 2 diabetes (T2D), cardiovascular disease, alterations in respiratory function, and increased risk of pulmonary embolism, the obesity-related low-grade inflammation could be an additional mechanism (5).

Bariatric surgery (BS) is increasingly performed in persons with severe obesity, especially in France, which ranks third worldwide regarding the number of patients operated on (6). However, very little is known on COVID-19 in post-BS patients. On the one hand, beneficial effects induced by BS could decrease patient's risk for severe COVID-19. Indeed, BS leads to successful long-term weight loss, metabolic improvement

Study importance

What is already known?

- Obesity is a major risk factor for severe forms of coronavirus disease (COVID-19) and related mortality.
- Bariatric surgery leads to successful long-term weight loss and improvement of comorbidities that may decrease the negative outcomes of COVID-19.

What does this study add?

- The prevalence of COVID-19–likely events in bariatric surgery–operated patients is similar to what is observed in the general population.
- Remission of type 2 diabetes after bariatric surgery is associated with a lower risk for COVID-19.
- A higher surgery-induced weight loss and lower weight and BMI post surgery are associated with COVID-19–likely events.

How might these results change the direction of research or the focus of clinical practice?

- Patients after bariatric surgery, in the case of nonremission of type 2 diabetes, should be considered at higher risk for COVID-19.
- Further research is needed to clarify the links between weight loss, malnutrition, and COVID-19.

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See Commentary, pg. 19.

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(including T2D remission) (7), improvement in sleep apnea syndrome (8), and decreased low-grade systemic inflammation (9). On the other hand, BS can lead to malnutrition and to vitamin deficiencies such as vitamin D, of which a deficit was suggested to enhance the severity of COVID-19 (10). Though recent guidelines were proposed to help prioritizing which patients could undergo bariatric and metabolic surgery during the COVID-19 pandemic (11), whether patients who already had BS display a particular susceptibility to COVID-19 risk and severity needs urgent evaluation.

We conducted a retrospective observational study in a cohort of patients who had BS to estimate the prevalence of COVID-19 and evaluate factors associated with COVID-19 incidence and severity in this setting.

Methods

Study population

The study is based on our ongoing BS cohort, "BARICAN," of 937 patients followed up at the Nutrition Department of Pitié-Salpêtrière University Hospital (Paris, France). Patients were operated on in the Surgery Departments of Hôtel-Dieu, Ambroise Paré, and Pitié-Salpêtrière hospitals in Paris between 2004 and 2020. Ethical approval was obtained from the French Research Ethics Committee of CPP Ile de France-1 N°13533 and by the "Commission nationale de l'informatique et des libertés" No. 1222666. Informed written consent was obtained from all participants.

BS, baseline and follow-up, and clinical and biological data collection

Detailed patients' clinical, biological, and anthropometric characteristics were obtained before surgery (baseline) and 12 months after BS. T2D status was defined as proposed by the American Diabetes Association (12) at baseline, at 12 months, and at the last follow-up before the survey (i.e., last known T2D status). Body composition was assessed by whole-body fan-beam dual-energy x-ray absorptiometry scan (Hologic Inc., Waltham, MA, USA) (13). Blood samples were collected after a 12-hour overnight fast. Insulin resistance was assessed by the homeostatic model assessment of insulin resistance (14).

Data collection during lockdown

Lockdown in France extended from March 10 to May 11, 2020. All patients received a phone call between April 27, 2020, and May 27, 2020, and were asked to contribute to a survey by answering a set of standardized questions about COVID-19, medical events, and changes in lifestyle during lockdown. If the patient was unable to answer to the survey (e.g., hospitalization, death), information was gathered from relatives. A patient was considered lost to follow-up if no answer was obtained after three calls. COVID-19 events were considered likely (CL) in patients who had experienced an episode of anosmia/dysgueusia or the association of fever (self-measured temperature $\ge 37.8^{\circ}$ C) and one symptom among cough, dyspnea, nose discharge/swelling, or odynophagia since February 2020. The combination of such symptoms has been considered as most relevant for the CL status in recent reports (15,16). Patients hospitalized for COVID-19 or with a positive polymerase chain reaction test for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were also considered as CL. Self-measured

weight before the lockdown period was recorded. Patients' smoking and vitamin supplementation status were reported. Recent exposure defined by a close contact with a person with confirmed Sars-CoV-2 infection was noted.

Statistical analysis

Continuous variables were expressed as mean (SD). Categorical variables were expressed as absolute values and percentages. Continuous variables with a nonparametric distribution were log-transformed before analysis. Student's *t* tests for continuous variables and Pearson's χ^2 test or Fisher's exact test for categorical variables were used to compare characteristics of patients according to their COVID-19–likely status (likely [CL] or unlikely infection [CU]). For significant results, a multivariate logistic regression was performed, adjusted on the time elapsed since surgery and last known T2D status. Significance was set at *P*<0.05. Analyses were conducted using R studio software version 1.2.1335 (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Baseline and 12-month characteristics

From the 937 operated patients followed up, we could collect recent information for 738 (78.8%) of them. Patients were middle-aged and mostly female, and 44.0% had T2D before surgery (Table 1). The main procedures performed were Roux-en-Y gastric bypass (54.4%) and sleeve gastrectomy (45.0%). Only four (0.4%) had gastric banding. The mean time elapsed between surgery and the time of the call was 3.7 (2.7) years, and the proportion of patients within their first year of follow-up (i.e., weight-loss phase) did not differ between CL and CU groups (Table 2). Mean weight loss 1 year after surgery was close to 30% of preoperative weight, and the proportion of T2D was halved 1 year after surgery (Table 1).

Prevalence and factors associated with CL

After BS, 62 (8.4%) patients were categorized as CL. There was no difference regarding the type of surgery or baseline characteristics between CL and CU groups. However, time elapsed since surgery was significantly higher in CL (P=0.01). One year after BS, the CL group had a higher proportion of patients with persistent T2D and higher HbA_{1c} level (Table 1). Persistent T2D at the last follow-up visit before survey was also positively associated with CL (Table 2). BMI at the time of lockdown was lower in the CL versus CU group, with a higher percent weight loss and a lower prevalence of obesity (BMI>30). Importantly, a multivariate analysis showed these differences were independent of the last known T2D status and the time since surgery (Table 2). CL patients reported more often a recent COVID-19 exposure (P<0.01). There was no significant difference in the vitamin supplementation status between the two groups.

Characteristics of the patients with severe forms of COVID-19

Among the 62 CL cases, 4 (6.4%) required hospitalization and 1 (1.6%) death was reported. Mean age was 61.8 years. Importantly, the proportion of patients with persistent T2D was higher in patients requiring hospitalization than in those who did not (respectively, n=4 [100%] vs. n=17 [31.4%]; P=0.014) (Table 3).

	Ba	seline (before sur	gery)		12	months after surg	gery	
	All, <i>N</i> =738	COVID-19 unlikely, <i>n</i> = 676	COVID-19 likely, <i>n</i> =62	P value	All, N=553	COVID-19 unlikely, <i>n</i> = 501	COVID-19 likely, <i>n</i> = 52	P value
Age (y)	50.0 (12.3)	50.1 (12.3)	49.6 (12.9)	0.77	51.0 (12.3)	51.0 (12.2)	50.7 (12.5)	0.86
Sex (male), <i>n</i> (%)	160 (21.7)	148 (21.9)	12 (19.4)	0.76	114 (20.6)	105 (21.0)	9 (17.3)	0.66
Caucasian, <i>n</i> (%)	611 (82.8)	560 (82.8)	51 (82.3)	1.00	443 (80.1)	401 (80.0)	42 (80.8)	1.00
Weight (kg)	123.6 (21.4)	123.8 (21.5)	121.7 (20.6)	0.47	88.8 (19.6)	89.1 (19.7)	85.8 (17.5)	0.21
Height (cm)	166.7 (8.6)	166.6 (8.6)	167.4 (9.3)	0.51	166.5 (8.6)	166.5 (8.6)	167.0 (9.3)	0.72
BMI (kg/m ²)	44.7 (6.6)	44.8 (6.5)	44.2 (7.2)	0.57	32.2 (6.3)	32.2 (6.2)	31.4 (7.4)	0.44
Body fat (%)	48.1 (5.1)	48.0 (5.0)	48.5 (6.1)	0.57	38.7 (6.7)	38.7 (6.6)	38.3 (7.5)	0.73
Type of surgery, n (%)				1.00				1.00
Gastric banding	4 (0.5)	4 (0.6)	0 (0.0)		3 (0.5)	3 (0.6)	0 (0.0)	
RYGB	399 (54.4)	365 (54.4)	34 (54.8)		317 (57.4)	287 (57.4)	30 (57.7)	
Sleeve gastrectomy	330 (45.0)	302 (45.0)	28 (45.2)		232 (42.0)	210 (42.0)	22 (42.3)	
Weight loss (%)	-	-	-		28.4 (8.4)	28.3 (8.3)	29.0 (9.8)	0.64
Excess body weight loss (%)					68.6 (23.8)	68.1 (23.4)	72.6 (26.7)	0.25
Obstructive sleep apnea syndrome, <i>n</i> (%)	512 (73.2)	472 (73.8)	40 (67.8)	0.40	201 (40.0)	184 (40.5)	17 (34.7)	0.52
Hypertension, <i>n</i> (%)	367 (53.0)	338 (53.1)	29 (50.9)	0.85	220 (39.9)	198 (39.6)	22 (43.1)	0.73
Type 2 diabetes, n (%)	308 (44.0)	279 (43.6)	29 (48.3)	0.57	116 (21.0)	97 (19.4)	19 (36.5)	0.01
Glycemia (mmol/L)	6.2 (2.1)	6.2 (2.1)	6.7 (2.6)	0.14	5.1 (1.2)	5.0 (1.2)	5.2 (1.2)	0.58
HbA _{1c} (%)	5.7 (0.8)	5.7 (0.7)	6.0 (1.1)	0.10	5.7 (0.8)	5.7 (0.7)	6.0 (1.1)	0.04
HOMA-IR	5.3 (3.7)	5.3 (3.7)	5.7 (4.0)	0.43	1.9 (1.2)	1.9 (1.2)	2.0 (1.3)	0.57
IL6 (pg/mL)	6.2 (20.7)	6.2 (21.5)	6.1 (7.3)	0.22	5.0 (18.3)	4.8 (18.1)	7.9 (19.9)	0.09
Vitamin D (ng/mL)	22.0 (10.0)	22.0 (10.2)	22.6 (8.3)	0.65	31.1 (9.3)	31.0 (9.2)	32.0 (10.1)	0.49
Vitamin B12 (pmol/L)	321 (143)	322 (137)	306 (197)	0.17	283 (149)	284 (141)	273 (212)	0.23

TABLE 1 Characteristics of the cohort before and 12 months after bariatric surgery according to COVID-19-likely status

Results expressed as mean (SD) for continuous data and n (%) for categorical data. P values result from Student t test for continuous data and χ^2 or Fisher exact test for categorical data between the two groups.

HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment of insulin resistance; IL6, interleukin 6; RYGB, Roux-en-Y gastric bypass.

Discussion

We surveyed a cohort of over 700 patients with a history of obesity followed up after BS to assess the prevalence of CL in this population and describe associated characteristics. The prevalence of CL was 8.4%, among which 6.4% were severe forms requiring hospitalization. The main difference between CL and CU patients was a higher BS-induced weight loss as well as lower weight and BMI at the time of lockdown for CL. Patients likely to have had COVID-19 also had a more often persistent T2D after surgery.

This is the first study to report and describe COVID-19 cases after BS, limiting the possibility to compare with results from other countries. However, at the time of our survey, a global estimation was performed in the greater Paris region (Ile de France), the area of residence of the majority of our patients, and reported an estimated prevalence of COVID-19 cases of 9.9% (17). This appears in the same magnitude as what we describe herein, with a similar proportion of patients requiring hospitalization.

T2D is recognized as a major risk factor for severe forms of COVID-19 (18,19), and our study suggests that this remains true after BS. Patients

with persistent T2D after BS were at higher risk for CL. Importantly, persistent T2D was associated with severe outcome of COVID-19.

One of the main differences observed in our likely infected patients was a lower BMI at the time of the survey, which is consistent with a more important post-BS percent weight loss, and this was independent of the duration of follow-up after BS. Malnutrition is known to be associated with the risk for viral pneumonia since the 1918 influenza pandemic (20), and it is possible that BS-induced malnutrition may contribute to a higher risk for infection with Sars-CoV-2. Here, we did not observe a difference in vitamin supplementation status between the two groups who also had similar vitamin levels in their 1-year follow-up. However, we cannot rule out differences in vitamin levels at the time of the infection.

Some limitations need to be mentioned. The definition of CL used here derives from a combination of self-reported symptoms with a good diagnostic performance but lacks specificity (15). Another limitation is that the time elapsed between surgery and our survey was heterogeneous and significantly different between the two groups, but importantly, adjustment for time since surgery did not alter our findings. Finally, potential confounders such as socioeconomic status, size of family, and working conditions could not be considered in this study.

Brief Cutting Edge Report

COVID-19 AND OBESITY

	All, <i>N</i> =738	COVID-19 unlikely, <i>n</i> = 676	COVID-19 likely, <i>n</i> = 62	P ^a	Adjusted odds ratio (95% Cl) ^b	Adjusted P ^t
Time since surgery (y)	3.7 (2.7)	3.7 (2.7)	4.2 (2.3)	0.01	1.02 (0.92-1.12)	0.68
Surgery < 1 year ago, n (%)	81 (11.0)	78 (11.5)	3 (4.8)	0.16	0.70 (0.11-2.68)	0.65
Last known T2D status	141 (21.8)	120 (20.3)	21 (36.2)	0.01	2.17 (1.20-3.86)	< 0.01
Weight (kg)	90.7 (20.4)	91.2 (20.8)	85.0 (14.6)	< 0.01	0.98 (0.97-0.99)	0.05
BMI (kg/m ²)	32.6 (6.5)	32.8 (6.5)	30.2 (5.1)	< 0.01	0.92 (0.87-0.97)	< 0.01
Obesity (BMI > 30), <i>n</i> (%)	424 (61.7)	396 (63.1)	28 (47.5)	0.03	0.52 (0.30-0.92)	0.02
Weight loss (%)	26.3 (10.9)	26.0 (10.9)	29.3 (10.7)	0.03	1.03 (1.00-1.06)	0.04
Excess body weight loss (%)	63.2 (27.5)	62.4 (27.7)	71.6 (24.9)	0.01	1.01 (1.00-1.03)	0.02
Vitamin supplements, <i>n</i> (%)	581 (81.0)	535 (81.4)	46 (76.7)	0.47	0.70 (0.36-1.44)	0.30
Corticosteroid treatment at last visit, n (%)	16 (2.5)	15 (2.6)	1 (1.8)	1.00	0.66 (0.04-3.43)	0.70
Confirmed Sars-CoV-2 exposure, n (%)	135 (18.9)	108 (16.5)	27 (45.8)	< 0.01	5.59 (3.04-10.3)	< 0.01
Smoker, <i>n</i> (%)	74 (10.5)	66 (10.2)	8 (13.6)	0.55	1.64 (0.68-3.54)	0.23

TABLE 2 Characteristics of the cohort at the time of lockdown according to COVID-19-likely status

Results expressed as mean (SD) for continuous data and n (%) for categorical data.

^aP values result from Student t test for continuous data and χ^2 or Fisher exact test for categorical data between the two groups.

^bLogistic regression adjusted for time since surgery and last known T2D status.

TABLE 3 Characteristics of COVID-19 hospitalized patients

patients after BS in the times of COVID-19 and the potential links between malnutrition and the risk for COVID-19.**O**

	COVID-19 patients requiring hospitalization, n=4
Age (y)	61.8 (4.08)
Sex (male), <i>n</i> (%)	1 (25.0)
Type of surgery, <i>n</i> (%)	
RYGB	2 (50.0)
Sleeve	2 (50.0)
Characteristics at 12 months after surgery	,
Obstructive sleep apnea syndrome, <i>n</i> (%)	2 (50.0)
Hypertension, <i>n</i> (%)	4 (100)
Type 2 diabetes, <i>n</i> (%)	4 (100)
BMI (kg/m²)	32.7 (4.60)
Body fat (%)	36.4 (12.3)
Characteristics at the time of lockdown	
Time since surgery (y)	5.05 (1.96)
Type 2 diabetes at last follow-up, <i>n</i> (%)	4 (100)
Weight (kg)	90.5 (7.78)
BMI (kg/m²)	30.5 (2.63)
Weight loss (%)	23.2 (0.27)
Excess body weight loss (%)	63.6 (9.73)
Death, <i>n</i> (%)	1 (25.0)

Results expressed as mean (SD) for continuous data and n (%) for categorical data.

Conclusion

Patients followed up after BS displayed rates of CL that appear in line with those of the general population. Persistent T2D and lower BMI after BS are associated with the risk and the severity of COVID-19. Further work is needed to assess in more detail lifestyle changes of

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Summary

The gut microbiome is recognised as an important player in the development and maintenance of obesity. Most studies in this field have focused on the faecal microbiome (FM) due to its accessibility. Changes in its composition and function have been identified in obesity. However, the upper part of the small intestine is a major site for nutrient sensing and absorption, as well as an important site for the control of food intake and glucose homeostasis, among others. Few studies have examined the microbiome of the human upper small intestine (USIM).

My PhD hypothesis is that the USIM is an important player in metabolic deterioration in human obesity and is significantly distinct from other microbial niches (such as faeces and saliva).

We conducted a clinical research project on 30 age- and sex-matched participants with (n=15) and without (n=15) severe obesity. Duodenojejunal fluid (DJF) was obtained by aspiration during endoscopy. Phenotyping included clinical variables related to metabolic status, lifestyle and psychosocial variables using validated questionnaires. We performed metagenomics of the oral microbiome (OM), USIM and FM, as well as metabolomic analysis of the USIM and FM. We performed a detailed analysis of multi-omics datasets in different niches and of the integration of lifestyle, clinical and omics data.

The results show i) a clearly different composition of USIM compared to FM; ii) associations between USIM and lifestyle as well as clinical and biological phenotypes. Interestingly, these associations appear to have a larger effect size than the associations between these variables and FM; iii) a differential signature in the USIM between the group of severely obese participants and the control group with, notably, the identification of two altered taxa between the groups. Candidate bacterial species associated with biological phenotypes were identified.

Overall, the results suggest significant differences in USIM between the obese and non-obese groups, as well as relevant associations with the participants' clinical phenotypes and lifestyles. This research paves the way for the study of two specifically altered taxa between our participant groups using ex-vivo and in-vivo models and could lead to the development of innovative treatments for metabolic diseases.

<u>Résumé</u>

Le microbiote intestinal est reconnu comme un acteur important dans le développement et le maintien de l'obésité. La plupart des études dans ce domaine se sont concentrées sur le microbiote fécal (FM) en raison de son accessibilité. Des modifications de sa composition et de ses fonctions ont été identifiées dans l'obésité. Cependant, la partie supérieure de l'intestin grêle est un site majeur pour la détection et l'absorption des nutriments, ainsi qu'un site important pour le contrôle de la prise alimentaire et de l'homéostasie glucidique, entre autres. Pourtant, peu d'études ont examiné le microbiome de l'intestin grêle proximal (USIM) chez l'humain.

Mon hypothèse de thèse est que l'USIM est un acteur important de la détérioration métabolique dans l'obésité humaine et qu'il est significativement distinct des autres niches microbiennes (telles que les fèces et la salive).

Nous avons mené un projet de recherche clinique sur 30 participants appariés selon l'âge et le sexe, atteints (N=15) ou non (N=15) d'obésité sévère. Le liquide duodénojéjunal (DJF) a été obtenu par aspiration au cours d'une fibroscopie. Le phénotypage comprenait des variables cliniques liées au statut métabolique, au mode de vie et aux facteurs psychosociaux à l'aide de questionnaires validés. Les analyses métagénomiques du microbiome oral (OM), de l'USIM et du FM, ainsi que les données métabolomiques de l'USIM et du FM ont été intégrées aux données cliniques et du mode de vie.

Les résultats montrent i) une composition clairement différente de l'USIM par rapport au FM; ii) des associations entre l'USIM et le mode de vie ainsi que les phénotypes cliniques. Il est intéressant de noter que ces associations semblent avoir une taille d'effet plus importante que les associations entre ces variables et la FM ; iii) une signature différentielle dans l'USIM entre le groupe de participants souffrant d'obésité sévère et le groupe de contrôle avec, notamment, l'identification de deux taxons modifiés entre les groupes. Ces deux espèces bactériennes candidates sont associées aux phénotypes biologiques.

En conclusion, nos résultats suggèrent des différences significatives dans l'USIM entre les groupes avec ou sans obésité, ainsi que des associations pertinentes avec les phénotypes cliniques et les modes de vie des participants. Cette recherche ouvre la voie à l'étude de deux taxons spécifiquement altérés entre nos groupes de participants en utilisant des modèles ex-vivo et murins et pourrait conduire au développement de traitements innovants pour les maladies métaboliques.