# Microbial modulation of metabolic diseases

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*Cover illustration: The host-microbiota 2.0 by Marco Serravalle*

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To my Family

*Memento audere semper*. G. D'Annunzio

# Microbial modulation of metabolic diseases

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## ABSTRACT

The gut microbiota, the ensemble of microorganisms living in the gastrointestinal tract, and the host have a mutualist relationship. Alterations of this delicate equilibrium can lead to changes in microbiota composition and/or function leading to the onset of metabolic diseases (e.g., type 2 diabetes and non-alcoholic fatty liver diseases). The current knowledge of host-microbiota interaction, in health and disease, is limited. Here, by using a translational science approach, we were able to identify some of the mechanisms underlying the influence of the microbiota on impaired glucose and lipid metabolism. Specifically:

In **Paper I**, I explored the microbiota-host interaction and its effect on glucose metabolism. Particularly, by performing colonization of germ-free mice, I studied the effect on glucose metabolism over time. I investigated the different molecular mechanisms underlying the impaired metabolic profile induced by the colonization over time. These findings provide fundamental information on how to conduct studies on microbiota and metabolic diseases.

In **Paper II**, I identified a novel microbially-produced molecule, imidazole propionate, which is increased in the portal vein of subjects with type 2 diabetes. I demonstrated causality of this molecule in impaired glucose metabolism by administering it in both *in-vivo* and *in-vitro* models. Moreover, I identified molecular targets of imidazole propionate in the insulin signaling cascade, specifically on the insulin

receptor substrate proteins, and showed that this effect is mediated by activation of the mTOR complex.

In **Paper III**, I investigated whether the gut microbiota composition and function is altered in subjects with non-alcoholic fatty liver disease. In presence of steatosis, I observed a shift in microbiota composition characterized by increased abundance of bacteria from the oral cavity, ethanol-producing bacteria, and a reduction in butyrate producing bacteria. On a functional level, I observed an enrichment in functions related to metabolic functions and production of lipopolysaccharides in subjects with steatosis.

In conclusion, these findings show that the microbiota is an environmental factor that modulates metabolic diseases. Understanding the mechanisms underlying microbial impacts on host metabolism will aid in discovery of novel targets for the treatment of metabolic diseases in humans.

Keywords: gut microbiota, glucose metabolism, type 2 diabetes, imidazole propionate, non-alcoholic fatty liver disease

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# SAMMANFATTNING PÅ SVENSKA

Tarmfloran kallas de mikroorganismen som lever i tarmen. De lever i symbios med oss och i friskt tillstånd är tarmflorans sammansättning och vår fysiologi i balans. Om den här balansen rubbas kan förändringar i tarmflorans sammansättning och funktion bidraga till metabol sjukdom, till exempel typ 2 diabetes och icke alkoholrelaterad fettlever. Kunskapen om hur människa och tarmflora interagerar, i friskt och sjukt tillstånd, är i dagsläget begränsad. Därför använder jag här en translationell strategi för att identifiera mekanismer för hur tarmfloran påverkar en förändrad glukos och lipid metabolism.

I Artikel I undersöker jag interaktionen mellan tarmfloran och värdorganismen, samt dess påverkan på glukosmetabolismen. Genom att kolonisera bakteriefria möss studerar jag hur koloniseringen påverkar glukosmetabolismen över tid. Jag undersökte de tidpunktsspecifika mekanismer som utgör koloniseringens effekt på glukosmetabolismen. Våra resultat ger grundläggande information till hur studier om hur tarmfloran och dess metabola effekter bör utföras.

I Artikel II identifierar jag en ny mikrobiellt producerad molekyl, imidazolepropionat, som återfinns högre nivåer i portådern i personer med typ 2 diabetes. Jag visar den här molekylens direkta effekt på glukosmetabolismen genom att administrera den i *in vivo* och *in vitro* modeller. Jag identifierar även den molekylära mekanismen för imidazolepropionat i insulinsignalering, specifikt dess effekt på insulinreceptorsubstratet samt att effekten medieras av mTOR-komplexet.

I Artikel III undersöker jag ifall tarmflorans sammansättning och funktion är förändrad i individer med icke alkoholrelaterad fettlever. Vid steatos observerar jag en förändring i tarmflorans komposition som karakteriseras av en ökad andel bakterier från munhålan, etanolproducerande bakterier samt en reduktion av butyratproducerande bakterier. Funktionellt observerar jag en ökning i metabola funktioner som är relaterad till produktion av lipopolysackarider i individer med steatos.

Sammanfattningsvis visar dessa resultat att tarmfloran är en faktor som påverkar metabola sjukdomar. Att förstå de underliggande mekanismerna av tarmflorans effekter på värdorganismens fysiologi kommer hjälpa oss i jakten på nya behandlingsstrategier av metabola sjukdomar.

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# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. *Molinaro A, Caesar R, Holm LM, Tremaroli V, Cani PD, Bäckhed F.* Host–microbiota interaction induces bi-phasic inflammation and glucose intolerance in mice.

*Molecular Metabolism 2017 Nov;6(11):1371-1380. doi: 10.1016/j.*

II. *Koh A, Molinaro A, Ståhlman M, Khan MT, Schmidt C, Mannerås-Holm L, Wu H, Carreras A, Jeong H, Olofsson L, Bergh PO, Gerdes V, Hartstra A, de Brauw M, Perkins R, Nieuwdorp M, Bergström G, Bäckhed F.* Microbially produced imidazole propionate impairs insulin signaling through mTORC1.

*Manuscript.*

III. *Molinaro A, Wu H, Schoenauer US, Datz C, Bergström G, Marschall HU, Tilg H, Tremaroli V, Bäckhed F.* Steatosis is associated with altered microbiome in humans.

*Manuscript.*

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| Abx | Antibiotics |
| Akt | Protein kinase B |
| ALT | Alanine amino transferase |
| AUC | Area under the curve |
| BA | Bile acid |
| BCAA | Branched-chain amino acid |
| BMI | Body mass index |
| BSH | Bile salt hydrolase |
| CONV-D | Conventionalized derived |
| CONVR, CONV-R | Conventionally raised |
| CONV-RD | Conventionally re-derived |
| CVD | Cardio-vascular diseases |
| Fasn | Fatty acid synthase |
| FXR | Farnesoid X receptor |
| G6pase | Glucose 6-phosphatase |
| GF | Germ-free |
| GGT | Gamma Glutamyl Transferase |
| HbA1c | Glycated hemoglobin |
| HDL | High density lipoprotein |
| HEK293 | Human embryonic kidney cells 293 |

| | |
|---|---|
| hutU | Urocanate hydratase |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate protein |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| KO | KEGG ontology |
| LDL | Low density lipoproein |
| LPS | Liposaccharides |
| MAPK | Mitogen-activated protein kinase |
| mTORC1 | Mammalian/mechanistic target of rapamycin complex 1 |
| NAFLD | Non-alcoholic fatty liver disease |
| PCoA | Principal coordinate analysis |
| Pepck | Phosphoenolpyruvate carboxykinase |
| PPIs | Proton pump inhibitors |
| RagA/B | Ras-related binding A/B |
| Rps6kb1 | Ribosomal protein S6 kinase beta-1 |
| siRNA | Small interfering RNA |
| SREBP | Sterol regulatory element-binding protein |
| TMAO | Trimethylamine N-oxide |
| T2D | Type 2 diabetes |
| UrdAs | Urocanate reductases |
| WAT | White adipose tissue |

# 1 INTRODUCTION

This thesis investigates the role of the gut microbiota in metabolic diseases. Metabolic diseases, such as type 2 diabetes and non-alcoholic fatty liver disease (NAFLD), are a major problem for modern society and health care systems. The scientific community has made intense efforts to understand the pathogenic mechanism(s) behind these diseases, which are multifactorial. Evidence suggests that the gut microbiota may act as pivotal player in the pathogenesis of metabolic disease.

Humans harbor numerous bacteria. Most of those bacteria reside in the intestinal tract and are defined as the "gut microbiota". Mutual beneficial interaction during evolution has permitted bacteria to colonize the human gastrointestinal tract. However, unhealthy dietary habits, lifestyle, or medications can perturb this fragile equilibrium and lead to pathological interactions between bacteria and the host. Perturbation of the physiological host-microbiota interaction can affect the host's ability to process macronutrients from diet, such as glucose and lipids, and contributes to the onset of metabolic diseases.

In this thesis, I will discuss how the gut microbiota influences the host physiology in metabolic diseases thus contributing to the pathogenesis of type2 diabetes and NAFLD.

## 1.1 METABOLIC DISEASES

Metabolic diseases are metabolic disorders characterized by an alteration of a specific metabolic processes [1]. They can be inherited or acquired. Inherited or congenital metabolic diseases are due to genetic enzyme defects, which can affect specific metabolic pathways and organs of the body. Hundreds of different inherited metabolic diseases have been described (e.g., Gaucher, Farber disease, Niemann-Pick disease, phenylketonuria, tyrosinemia). They are rare and in most cases, there are no curative treatments available [2].

Besides those rare inherited metabolic diseases, the most common metabolic diseases are acquired (e.g., type 2 diabetes, cardiovascular disease, and NAFLD) and they constitute the focus of this thesis [3, 4]. Metabolic diseases are associated with increased morbidity and mortality [5]. Risk factors for metabolic diseases can be clustered in a so called metabolic syndrome, which is a syndrome characterized by high blood pressure, high blood sugar, high serum triglycerides, and low high-density lipoprotein levels associated with adiposity [3]. The incidence of metabolic syndrome has increased in recent decades, and it now affects the 25% of the adult population in the USA.

Pathophysiological mechanisms of metabolic diseases are still not fully understood. However, several hypotheses have been proposed over time, including insulin resistance, low-grade chronic inflammation, and oxidative stress [4, 6].

## 1.2 THE GLOBAL BURDEN OF METABOLIC DISEASES

Metabolic diseases have a multifactorial pathogenesis [7]. Obesity is the main risk factor for such diseases. The World Health Organization defines obesity as abnormal and excessive fat accumulation, measured as body mass index (BMI) $\geq$30, that could affect health [8]. The prevalence of obesity has increased in the last decades and, to date, it affects more than 34% of the adults in USA. Moreover, another 34% of the population is overweight, thus prone to become obese, indicating that more than half of the population is at risk to develop metabolic diseases [9, 10, 11, 12]. The obesity epidemic is not limited to adults or Western countries. Currently, it affects 17% of children and adolescents in USA [13, 14] and recent data from 195 countries show that the prevalence of obesity is around 12% in adults worldwide, meaning that 107.7 million children and 603.7 million adults are obese and, thus, at risk of developing metabolic disease [15]. However, it should be noted that people from Asian compared to western countries develop metabolic diseases at even lower BMI. This could be due to a different BMI-body fat distribution, dietary habits, genetics, or the microbiota [16, 17].

In 2015, 4 million deaths were due to obesity-related diseases (7.1% of the deaths from any cause). In particular, cardiovascular diseases accounted for 2.7 million deaths in obese subjects (70% of total death in obese subjects), while diabetes is the second leading cause of obesity-related deaths (0.6 million) [15]. Moreover, in 2015 30.3 million US obese subjects (9.4% of the population) had diabetes and 84.1 million had pre-diabetes [18]. The diabetes prevalence is slightly lower in Europe compared with the US (7.3%), but dramatic differences exist in prevalence among other populations (3.8% in Africa, 10.7% in Middle East and North Africa, 11.5% in North America and the Caribbean, 9.6% in South and Central America, 9.1% in Southeast Asia, and 8.8% in Western Pacific) [19].

Considering the diabetes prevalence and that it represents the 7th leading cause of death in the USA, it is easy to understand that diabetes also has a profound impact on the health care system. In the USA, the costs of type 2 diabetes in 2017 were $327 billion. Among those $90 billion are due to reduced productivity [18].

The prevalence of NAFLD in USA and Europe is similar, 24% and 23%, respectively [20, 21]. NAFLD is even more prevalent in other countries (31% in South America, 32% in Middle East and 27% in Asia) [20], but less common in Africa (14%) [20]. Among populations with risk factors such as type 2 diabetes, the prevalence of NAFLD is even higher (57%) [22, 23]. However, it should be noted that all the epidemiological data available on NAFLD are influenced by the method used for the diagnosis (i.e., serum transaminases, MRI, ultrasound, or liver biopsy) [21]. In the USA, the annual direct medical costs of NAFLD are about $103 billion, while in European countries (cumulative data from Germany, France, Italy, and United Kingdom) it is about €35 billion [24].

Unfortunately, the predictions for the future are not encouraging. Obesity is projected to increase worldwide, and in western countries more than half of the population will be obese by 2030 [25]. The p for metabolic diseases are even worse. The incidence of type 2 diabetes will increase by 165%, expecting 29 million cases in 2050 [26]. Similarly, its prevalence will increase from 14% to 33%, meaning that 1 in 3 persons in the USA will be affected by diabetes [26].

Data on NAFLD are not encouraging either. The prevalence of NAFLD is estimated to increase by 21% until 2030, raising from 83.1 to 100.9 million cases with a predicted prevalence of 33.5% among the general population, mirroring the diabetes prevalence [24, 27].

Bearing this in mind, focusing on metabolic diseases and their major risk factors is very important for the scientific community. Efforts to develop preventive strategies as well as effective treatments are required. However, understanding the complex pathophysiology of metabolic diseases is fundamental for developing any possible intervention. The research conducted in this thesis will add some more pieces to the puzzle of the pathophysiology of metabolic diseases.

## 1.3 GUT MICROBIOTA AS AN ORGAN

The human body is colonized with more than 100 trillion microbes [28]. The number of microbes living in/on our body exceeds the number of human cells, meaning that we are mainly composed by microbial cells [29, 30]. The term microbiota indicates the collection of microbes living in a specific environment, such as the skin, the mouth, the urogenital tract or the intestine [31]. The majority of microbes living in our body are resident in our gut. The human gut microbiota has a specific composition, which is clearly different from environmental communities (e.g., the soils or the water) or from other regions of the body (e.g., skin or the urogenital tract) [32]. However, the gut microbiota is not only composed of bacteria but also of viruses, archaea, and unicellular eukaryotic organisms [33, 34, 35]. The majority of the studies published up to now have focused on the bacterial species in the gut, but recently interest is also growing on the non-bacterial components of the microbiota [36]. In this thesis, I will focus on the composition, function, and influence of gut bacterial communities on host pathophysiology.

Humans are colonized by bacteria at birth, which are first acquired during passage through the vaginal tract in vaginal delivery or by the environment in caesarian delivery [37]. During time, humans acquire an adult microbiota. Length of breast or formula feeding and, later in life, different diet patterns

influence the development and composition of the adult microbiota until senescence [38, 39, 40, 41].

Bacterial abundance and composition varies along the intestinal tract. Indeed, each gastrointestinal segment has a distinct microbiota and this is due to anatomical and physiological conditions of each different segment. Changes in nutrient availability, mucous thickness, pH, and oxygen pressure are the major factors affecting microbiota composition and abundance [37, 42, 43, 44]. The majority of the gut microbiota is composed of strict anaerobes, followed by facultative anaerobes and aerobes [Human Microbiome Project Consortium, 45]. The number of bacteria in the stomach and duodenum range from $10^1$-$10^3$/g of content. It increases to $10^4$-$10^7$ bacteria/g of content in the jejunum and ileum, and reaches its peak to $10^{11}$-$10^{12}$ bacteria/g in the colon [31, 46, 47]. The gut microbiota has been estimated to have an approximate mass of 1 to 2 kg in adults [48].

When it comes to taxonomical classification, based on molecular phylogeny, bacteria can be classified in broad lineage groups known as phyla [Human Microbiome Project Consortium, 45, 49]. Phyla can then be sub-classified into class, order, sub-order, family, genus, and species, progressively narrowing down the genetic characteristics of each bacteria. Firmicutes and Bacteroidetes represent the 90% of the bacterial phyla resident in the gut, and the remaining 10% are distributed into various bacterial phyla (e.g. Actinobacteria, Proteobacteria, Verrucomicrobia, Tenericutes, Fusobacteria. etc.) [50, 51].

In terms of bacterial composition along the gastrointestinal tract, data from the mucosa associated microbiota show that *Lactobacillus* and *Streptococcus* are the most abbundant bacterial genera in the stomach-duodenum, while *Bacteroides, Enterobacteria, Enterococcus, Clostridium, Lactobacillus* and *Veilonella* are the most abundant genera in the ileum. In the colon, genera belonging to *Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Enterococcus, Ruminococcus, Peptostreptococcus, Propionibacterium, Lactobacillus, Escherichia, and Streptococcus* are the most highly represented [52, 53, 54].

In healthy adults, genetics, immunological response, diet, and use of pharmaceuticals (e.g. anti/pre/pro-biotics) are major factors influencing

microbiota composition and determining its variability among individuals [55, 56, 57, 58, 59]. Despite these potential confounding factors, a common preserved microbial signature, on a phylum level, can be found in health or disease [45]. However, at a genus and species level, inter-individual differences are observed, suggesting that there is a specific bacteria-host interaction at a phylum level [45].

Humans and gut microbiota have co-evolved into a mutualistic relationship. Bacteria live and replicate in the human intestinal tract providing to the host several metabolic and biochemical functions, such as digestion of otherwise indigestible nutrients, metabolism of bile acids and xenobiotics, and production of vitamins [60, 61]. The microbiota also plays a role in protecting the host against pathogens by creating a solid network among species, regulating the immunomodulation of the host immune system, and modulating the intestinal barrier and motility [62, 63, 64]. The microbiota has endocrine function by secreting several molecules that can act as hormone-like molecules such as: short chain fatty acids (SCFA: acetate, butyrate, propionate), neurotransmitters (serotonin, dopamine, noradrenaline and γ-aminobutyric acid), precursors of neuroactive compounds (tryptophan and kynurenine, *L*-dopa), secondary bile acids, trimethylamine, cortisol and gastrointestinal hormones (ghrelin, leptin, glucagon-like peptide-1, and peptide YY [65].

At the genomic level, the collection of microbial genes (i.e., the microbiome) outnumbers the genes in the human genome by a factor of at least 500 [49]. This vast amount of bacterial genetic material significantly expands host metabolic capacity [66, 67]. However, many of these pathways and their implication in health and disease remain unknown.

From a biochemical point of view, the gut microbiota can produce a much larger and heterogeneous number of biochemical reactions than any other endocrine organ in the body. A conventional endocrine organ can produce one or few hormones, while the gut microbiota has the ability to produce several different hormone-like molecules [65]. Considering all the microbiota characteristics, functions, and abilities to interact with other organs in the body, the gut microbiota might be defined as an “virtual endocrine organ” [65].

## 1.4 GUT MICROBIOTA AND HOST PHYSIOLOGY

Data on microbiota-host interaction mostly come from experiments comparing germ free (GF) animals and animals with a normally acquired gut microbiota (i.e., conventionally raised, CONV-R) [68]. GF animals are born and raised in a sterile environment where everything, from air to food, is sterile. The microbiota has a profound effect on host physiology in several aspects of the host development.

### 1.4.1 EFFECT ON INTESTINAL PHYSIOLOGY

One hallmark of GF mice is the presence of an increased cecal size due to lack of bacteria able to ferment dietary fibers. Accumulation of fibers induces an osmotic pressure that retains water in the cecal lumen, causing its enlargement [69]. In the absence of bacteria, the total intestinal mass and surface area are reduced [Gordon, 70]. GF mice display several intestinal histological alterations. Intestinal villi are thinner and longer due to the absence of pathogens and allow more nutrient absorption [71]. These histological findings are associated with a slower gut transit as a response to relative malnourishment caused by the absence of microbial products of metabolism [72]. Microbiota colonization of GF mice can reverse these alterations by remodeling the villi structure, making them larger and with increased angiogenesis for sufficient tissue oxygenation [73].

On a functional level, intestinal permeability is affected by the presence of bacteria in the gut [74, 75, 76]. GF mice also display a reduced gut transit time and absorption of dietary nutrients as compared to CONV-R animals. These differences as based on the reduced levels of short chain fatty acids (SCFA) in GF mice that lack bacteria able to ferment fibers [72, 77]. SCFA are not only the preferred energy source for colonocytes, but they can also act as signal molecules affecting several host functions [61].

The gut microbiota also regulates the maturation of the enteric nervous system. GF mice have an immature enteric nervous system (ENS) compared to CONV-R animals but it becomes similar to that of CONV-R upon

colonization with a normal gut microbiota. This phenotype is due to a gut microbiota-dependent serotonin release [78].

### 1.4.2 EFFECT ON IMMUNE SYSTEM PHYSIOLOGY

Maturation and functionality of the immune system is deeply regulated by the microbiota [79]. Bacteria-host interaction in the intestine is fundamental for proper development immune tolerance and non-self-recognition [80].

The intestinal mucus layer, which covers the epithelial cells, has a protective function against bacterial invasion, has a reduced thickness and a different structure in GF mice compared to CONV-R [44, 81].

GF mice display several immunological anomalies compared with CONV-R animals [79, 82]. They have fewer and smaller Peyer patches, lamina propria germinal centers, and lymph nodes in the intestine. Moreover, in the absence of bacteria, secretion of immunoglobulin A from plasma cells in Peyer patches and antimicrobial peptides from Paneth cells is drastically reduced [83]. Finally, the number of lymphocytes in the GF intestine is reduced and they are functionally less cytotoxic compared with CONV-R mice [79, 84].

### 1.4.3 EFFECT ON HOST METABOLISM

An experiment performed 50 years ago compared the response to starvation in CONV-R and GF mice and demonstrated that CONV-R survived significantly better than GF mice, despite a similar body weight loss over time [85]. This was likely due to decreased ability to switch between different types of energy sources in GF mice relative to their CONV-R counterparts (i.e., sugar, fat, proteins) [85]. Indeed, the gut microbiota is necessary to the host to extract energy from food [86], but it is also essential to integrate several metabolic processes involved in host metabolism [64]. Bacteria help humans in several metabolic functions such as to digest indigestible food particles (e.g. fibers and starch), metabolize xenobiotics,

produce vitamins, and metabolize bile acids [87, 88]. How these activities influence host metabolism will be discussed in the next paragraphs.

The gut microbiota affects many other host physiological aspects, which are widely reviewed by *Wostmann* and *Schroeder et al*., but I will not discuss them here for space issues [89, 90]

Taken together, these studies show that the microbiota has a profound effect on host physiology and is fundamental for host development and interaction with the surrounding environment. However, in addition to its role in normal development and physiologic function, the gut microbiota also plays a pivotal role in the pathophysiology of metabolic disease, as described in more details in the following paragraphs.

## 1.5 GUT MICROBIOTA AND OBESITY

Several studies over the past decade have suggested an important role of the gut microbiota in the onset of obesity and metabolic disease. Data from experimental models have clearly shown that microbiota has a major role in obesity [91, 92, 93, 94, 95]. GF mice have lower body weight, a smaller adipose tissue size, and less inflammation than CONV-R mice, likely due to ability of microbiota to improve host energy extraction and utilization from a fiber-rich diet [86, 96]. Colonization of GF mice with a normal cecal microbiota from CONV-R mice restores the adiposity and weight within 2 weeks [86, 97]. Moreover, when GF mice are challenged with a high fat/high sucrose diet, as a model of diet-induced obesity, they are protected against the development of obesity and metabolic complications [91, 94, 98], likely due to other mechanisms than solely digestion of dietary fiber. These include activation of AMPK, angiopoietin-like protein 4, and reduced inflammation [91, 96, 99].

Data on the microbiota composition in genetically obese mice have shown increased levels of Firmicutes and reduced levels of Bacteroidetes, as compared to lean mice [100]. Similar alterations were observed in early human studies on gut microbiota that compared the microbiota composition in obese and lean individuals [101]. However, after this initial evidence,

conflicting results on the ratio between Firmicutes and Bacteroidetes in obesity have been reported [102, 103, 104, 105, 106]. These data should be interpreted with care because the studies have, to a large extent, been underpowered [107]. However, one common finding is that obesity is associated with reduced microbial diversity [107, 108]. Furthermore, data from observational studies provide only a single time-point of the gut microbiota profile in obesity and cannot exclude that obesity changes the gut microbiota rather than that the microbiota cause obesity. Is the altered gut microbiota composition in obesity just a reflection of an unhealthy lifestyle/diet or is it actually the cause of the metabolic disorder?

Data from colonization experiments of GF mice with a microbiota from genetically obese mice or from human twins that are discordant for obesity showed that obesity is transmissible through gut microbiota [92, 109]. In agreement with these studies, a case report demonstrated weight gain in a woman following fecal microbiota transplantation (FMT) from an healthy overweight donor as treatment for *Clostridium difficile* infection [110]. These data support the hypothesis that the gut microbiota is a pathogenic factor in the onset of obesity.

On the other hand, some reports that showed few bacteria protecting against obesity. The abundance of *Akkermansia muciniphila*, a mucin-degrading bacterium, which resides in the mucus layer, inversely correlates with obesity and some metabolic diseases [105, 111, 112, 113, 114]. Similarly, data on gut microbiota from twins and their parents showed that *Christensenella minuta* is a heritable bacteria that protects against obesity and its abundance is affected by the host genetics [55].

Furthermore, some studies showed that gut microbiota contributes to the pathogenesis of undernourishment. Colonization of GF mice with gut microbiota from undernourished individuals transmits some of the features of undernourishment. These data suggest that gut microbiota also plays a causative role in undernourishment [115, 116].

Taken together, this evidence suggests that gut microbiota plays a pivotal role in the modulation of body weight and adiposity in both mice and humans. However, we are still far from fully understanding of precisely how the gut microbiota exerts these effects on human metabolism.

## 1.6 GUT MICROBIOTA, GLUCOSE METABOLISM AND TYPE 2 DIABETES

Gut microbiota has been implicated in glucose metabolism both in mice and humans [117]. GF mice display improved glucose metabolism compared with CONV-R mice, whereas colonization with a normal microbiota impairs glucose metabolism compared with the GF state [86, 91, 97, 98]. Consistently, ablation of the gut microbiota with antibiotics in CONV-R mice improves glucose metabolism [74, 118, 119], demonstrating that the microbiota contributes to glucose intolerance in mice.

Observational data on the microbiome in humans with type 2 diabetes have provided some insights on how the microbiome may contribute to type 2 diabetes. Two independent studies of a European and a Chinese population showed a common microbiota shift in the presence of diabetes [120, 121]. Despite the regional, ethnic, and dietary differences of these two cohorts, type 2 diabetes was associated with high levels of *Lactobacillus* and low levels of butyrate producing bacteria *Roseburia* and *Faecalibacterium prausnitzii*, showing the potential importance of the microbiota, over other confounding factors, in affecting glucose metabolism and thus type 2 diabetes in humans [120]. The presence of an altered microbiota composition and function in subjects with type 2 diabetes has been extensively confirmed by other independent studies [122, 123, 124].

Interestingly, data on pre-diabetic subjects (a clinical condition characterized by abnormal glucose levels which may precede diabetes onset) show the presence of a microbial shift in this group as well. This shift is characterized by decreased abundance of the genus *Clostridium* and of the mucin-degrading bacterium *A. muciniphila* [125]. Data are consistent with the observation that *Clostridium* species are indirectly associated with blood glucose levels in subjects with type 2 diabetes [120]. The gut microbiota shift observed in subjects with pre-diabetes has also been described in subjects with chronic diseases characterized by low-grade inflammation (e.g., type 2 diabetes, inflammatory bowel syndrome, colorectal adenomas) [108, 126, 127]. These findings might indicate that gut microbial alterations in pre-diabetes could be the marker of a low-grade inflammation that later on will lead to type 2 diabetes [125].

Data from interventional studies provide some insights in the causative role of microbiota in impaired glucose metabolism and type 2 diabetes. In humans with the metabolic syndrome, microbiota manipulation with antibiotics or with fecal microbiota transplantation affects insulin sensitivity [128, 129, 130]. Metformin is widely used for the treatment for type 2 diabetes but its mechanism of action is poorly understood [131]. Interestingly, recent data showed changes in the gut microbiota is metformin treated patients and the beneficial effects of metformin, at least in part, may be attributed to the altered microbiome [132, 133].

In conclusion, several studies showed that gut microbiota is an important environmental factor that affects glucose metabolism and type 2 diabetes. However, the mechanism underneath this microbiota contribution on glucose metabolism in physiological and pathological conditions still needs investigation.

## 1.7 GUT MICROBIOTA AND NAFLD

NAFLD is one of the features of metabolic syndrome and obesity [134]. NAFLD encompasses a wide range of liver diseases characterized by increased hepatic lipid content (more than 5% of the hepatocytes) in absence of other known factors of liver damage, including ethanol intake. It ranges from simple steatosis, characterized only by fat accumulation in the liver, to steato-hepatitis (NASH) characterized by fat accumulation and inflammation in the liver. NASH can then evolve to fibrosis, cirrhosis and ultimately liver cancer [135]. It is estimated that one fourth of the adult population is affected by NAFLD in western countries which has become one of the major indications for liver transplantation in the USA [136, 137].

The gut microbiota is an environmental factor that affects fat storage in the liver and has been shown to be involved in NAFLD pathogenesis [86, 138]. Data from experimental models and human observational studies have helped us to start understanding the mechanisms behind it.

The hypothesis that microbiota can modulate fat accumulation in the liver comes from studies comparing GF and CONV-R mice. On a normal chow

diet (low fat, low sugar), GF mice have less liver fat content than CONV-R mice [86]. After colonization of GF mice with a normal microbiota, liver fat content is restored, by inducing hepatic expression of several genes involved in the *de novo* lipogenesis, a process that produces lipids from glucose excess in the diet [86]. Moreover, GF mice, challenged with high fat or high sugar diet, are resistant to diet-induced liver fat accumulation that instead is observed in CONV-R animals challenged with the same diet [91, 94, 98], although the specific contribution of dietary fat and sucrose need to be clarified. Consistently, gut microbiota ablation with antibiotics protects mice from diet induced fat accumulation in the liver, when challenged with high fat diet [139]. Taken together these data indicate that in mice, microbiota is involved in several features of NAFLD.

In humans, several studies have aimed to identify specific bacterial strains that are associated with NAFLD in humans. Unfortunately, findings were not consistent although they all were able to observe shifts in microbiota composition or function in the presence of NAFLD [140, 141, 142, 143, 144, 145, 146, 147, 148]. The reason for inconsistent findings in NAFLD and microbiota may be due to diet/ethnical/genetic factors or to bias in the selection and stratification of the cohorts, such as use of non-antibiotic medications that might affect microbiota composition [149]. To date, few data are available on the metabolic function of bacteria involved in NAFLD. Moreover, data from interventional studies in humans with NAFLD provide support to the hypothesis that an altered microbiota composition plays a pivotal role also for NAFLD. Modulating gut microbiota with prebiotics reduces liver steatosis and *de novo* lipogenesis, confirming in humans a link between microbes and hepatic lipid metabolism [150, 151, 152, 153].

However, as in type 2 diabetes, despite observational studies showing altered microbiota composition and function, the mechanisms behind the microbiota's contribution to NAFLD are still not fully understood.

Colonization of GF mice with a microbiota from mice or humans with NAFLD can transmit some features of NAFLD to mice, indicating a causative role of bacteria in the onset of NAFLD [154, 155]. Moreover, also NASH seems to be a microbiota driven disease. Co-housing of wild type mice with mice carrying a genetic mutation for the development of NASH can transmit the NASH phenotype to the wild type animals. The NASH

transmission is due to the transfer of microbiota between the two mice genotypes due to cohousing [156].

Taken together, the gut microbiota is involved in the pathogenesis of NAFLD, although a consistent microbiota signature remains to be identified. However, the mechanisms underlying the microbiota contribution to NALFD still need to identified and characterized.

## 1.8 METABOLITES AND MICROBIOTA: BEYOND ASSOCIATION STUDIES ON GUT MICROBIOTA

In the past decade, several data have shown a shift in microbiota composition and function in subjects with metabolic diseases. Observational studies showed that an altered microbiota composition was associated not only with metabolic diseases (such as type 2 diabetes, obesity, atherosclerosis, NAFLD) but even intestinal (e.g., inflammatory bowel disease, irritable bowel syndrome, colon cancer) or immunological diseases (e.g., type 1 diabetes, allergy, asthma) [100, 101, 120, 127, 148, 157, 158, 159, 160, 161, 162, 163, 164]. However, those studies have so far only demonstrated associations between an altered microbial composition and a specific disease, without providing a mechanistic explanation of the findings. Recent evidence provided information on how the microbiota communicates with target organs of the host through microbial produced molecules [89]. These molecules can be structural components of the bacteria, such as lipopolysaccharides (LPS), or bacterial metabolites. Microbial produced molecules affect target organs directly, by reaching them through the blood stream, or indirectly, by an intermediate signaling to the enteric nervous system or by inducing hormones' secretion in the intestine [89].

Several microbial metabolites mediated mechanisms have been proposed in the pathogenies of metabolic diseases:

*LPS mediated inflammation.* LPS is responsible for a low-grade inflammatory status in metabolic tissues (e.g., adipose tissue, liver) that will

then affect several aspects of host metabolism including glucose metabolism and lipid storage in the liver [74, 97, 119, 165, 166, 167].

*SCFA mediated signaling.* SCFA are bacterial fermentation metabolites generated from degradation of dietary fibers and are mainly represented by succinate, butyrate, propionate and acetate [61]. Their effects on host physiology are performed directly or by bindings to the G-protein-coupled receptor (GPCR) GPR41 (also known as FFAR3) or GRP43 (or FFAR2) [168]. SCFA are involved several metabolic processes, such as gluconeogenesis, insulin secretion, food intake, gut motility, and GLP-1 secretion [61, 169, 170, 171, 172, 173, 174]. In humans, among different SCFA, a promising player in metabolic diseases is represented by butyrate. Gut microbiota of subjects with type 2 diabetes is characterized by a reduced number of butyrate-producing bacteria [120]. Experimental data showed that butyrate administration leads to a significant improvement of metabolic features and insulin sensitivity in mice [175, 176, 177, 178]. Thus, one may speculate that it might be useful to supplement butyrate in humans with type 2 diabetes to improve their glycemic control. However, data on the effect of butyrate supplementation in humans are neither encouraging nor consistent with the experimental findings [179]. This could be due to that oral supplementation may not reach the colon or that other unknown molecules produced by butyrate producers' bacteria are important. However, the reduced number of butyrate-producing bacteria in subjects with type 2 diabetes might just be a consequence of the increase of other potential harmful bacteria [121]. However, it is still unclear if SCFA serum concentrations in humans are high enough to act as proper hormones [180].

*Bile acids mediated signaling.* The primary bile acids, produced by the liver, are converted in the intestine into secondary bile acids by the gut microbiota. Alteration of the bile acids pool has been observed in subjects with type 2 diabetes and NAFLD [181, 182]. Bile acids have been implicated in the regulation of glucose metabolism due to its affinity for several nuclear receptors, especially for the nuclear receptor farnesoid X receptor (FXR) and the membrane-type receptor for bile acids (M-BAR, or more commonly TGR5) [183]. Bile acid dependent FXR or TGR5 activation/inhibition in different metabolic tissues have different effects on metabolism. Thus,

microbiota regulation of bile acids could be responsible for the fine regulation of FXR and TGR5 function in different target tissues [88].

*Bacterial ethanol production in NAFLD.* NAFLD is characterized by the absence of alcohol intake. However, alcoholic and non-alcoholic liver diseases share some common histological features [184, 185]. It has been shown that, both animal models and humans, with NAFLD have higher levels of ethanol in the circulating blood [186, 187]. This finding could be due to the production of ethanol from other nutrients provided with the diet by an altered microbiota in NAFLD [188]. Consistently, subjects with NAFLD have increased expression of several genes involved in alcohol metabolism [189]. Thus, the terminology non-alcoholic liver disease (NAFLD) might need to be modified in the next future in bacterial-mediated alcoholic fatty liver disease (BAFLD) or bacterial-induced fatty liver disease (BIFLD). It should be also noticed that alcohol itself can affect intestinal permeability, determining a "leaky gut' allowing several other potentially dangerous molecules produced by the microbiota to reach the liver and the blood circulation form the intestinal lumen [190, 191].

*Microbial phenylacetic acid production in NAFLD.* Beside ethanol, it has recently been shown that phenylacetic acid is a microbially produced metabolite that is increased in the serum of women with NAFLD. When administrated *in vitro* and *in vivo* in experimental models it can induce some features of NAFLD, suggesting its causative role in the onset of NAFLD [155].

Beside these known metabolites, there are hundreds of unknown microbially produced metabolites that can affect host (patho)physiology. Some reports from experimental models showed that the plasma from CONV-R mice is highly enriched of metabolites compared to GF or antibiotic treated ones [192, 193]. Specifically, more than 400 metabolites were significantly altered by the presence of microbiota. These findings may be due to a direct effect of microbiota on metabolite levels or indirectly by affecting host intestinal physiology and thus host metabolites utilization [192]. Accordingly, many research groups focus on metabolomics methods to identify metabolites that are differentially abundant in patients and healthy controls to be used as biomarkers or drug targets.

To date, the role in health and disease of the vast majority of bacterial produced/regulated metabolites is still unknown, and thus a major hurdle in microbiota research in metabolic diseases is to translate findings from association studies into a pathophysiologic mechanism of the diseases.

Manipulating the gut microbiota in an untargeted way by removing it or replacing it with antibiotics or fecal microbiota transplantation, respectively, has provided a proof of concept that microbiota modulation could be a strategy for the treatment for metabolic diseases [128, 130]. However, from an untargeted approach to the treatment of metabolic diseases there is the needs to move to a more targeted one. Thus, identifying microbially produced metabolites responsible for the metabolic effect of microbiota on the host in metabolic diseases could be a strategy to identify potential molecules to target for a microbiota-oriented treatment.

## 1.9 INSULIN SIGNALING AT GLANCE

On January 1st 1922, in Canada, the history of diabetes treatment in humans begun. Drs. Banting and Best used insulin for the first time to treat a 14-year-old boy with severe diabetes. The treatment saved the young patient's life, reducing dramatically levels of glucose in the blood and urine. For this finding Dr. Banting was awarded with the Nobel Prize in 1923. This sensational milestone in human medicine was preceded by several decades of studies on diabetes, starting with the discovery of Langerhans' islands by Dr. Langerhans in 1869 followed by decades of studies on a potential islets' secreted hormone (called "insulin" from insula, a Latin word for island) [194]. The first animal model, used in the field of diabetes, was a diabetic dog that was treated with pancreatic extract by Dr. Popescu in Romania in 1921 [195].

During research for more than a century much knowledge has been gained on both type 1 and 2 diabetes as well as on insulin resistance and identification of the insulin signaling cascade. The first theories on insulin signaling were postulated in 1949 and included the possibility that insulin interacted with cell membrane facilitating sugar uptake. At that time, there

was no idea for a possible ligand-receptor cascade reaction due to insulin. Only in the 1970s, several independent groups showed with radiolabelling techniques that insulin could bind to a receptor on the cell membrane surface. From these initial discoveries, in just half a century, large steps have been done in identifying the different proteins involved in the cascade of insulin signaling [196]. However, there is still a lot that has to be investigated to fully understand insulin signaling.

Insulin is produced and secreted by the β cells in the pancreatic islet. It is the most potent anabolic hormone, promoting the synthesis and storage of lipids, proteins and carbohydrates. Although all cell types are responsive to insulin, insulin-sensitive tissues are the liver, the muscles and the adipose tissue [197]. Insulin regulates glucose homeostasis by promoting glucose uptake in muscle and adipose tissue, while suppressing hepatic gluconeogenesis. Insulin is also important for lipid homeostasis, stimulating lipogenesis in fat and liver, and inhibiting lipolysis in fat and muscle. Altered insulin response is the main characteristic of an altered metabolic condition called "insulin resistance" which may lead to type 2 diabetes. Diet, genetics and environmental factor has been implicated in insulin resistance pathogenesis, however, up to date the exact underlying mechanism is still unknown. Defects in insulin signaling have been shown to be fundamental for the onset of insulin resistance [197].

During health, as response to a meal, the pancreatic β-cell releases insulin, which is a peptide that acts as a hormone with several effects on body physiology [198]. The overall effect is to exploit the nutrients from the diet including increased glucose uptake by increased abundance of glucose transporters on the cell surface and reduced endogenous hepatic glucose production. Target tissues (e.g., muscles, liver, adipose tissue, brain) express the insulin receptor (IR) [199], which consists of 2 types of subunits: one subunit α and two subunits β. Different isoforms exists (A, B, and mixed ones), due to alternative exons 11 splicing with different affinity to insulin or insulin growth factor 1 (IGF-1) and different tissue specific expression [200]. The interaction between insulin and IR is the first step on insulin signaling: the β-subunits initiate a cascade of trans-phosphorylation between β-subunits that increases the ability of the IR to phosphorylate substrate proteins, such as the insulin receptor substrate (IRS) proteins [201, 202].

IRS include 6 different proteins (1-6), which act as scaffolds to organize and mediate signaling complexes to the cell membrane [203, 204]. Due to its particular structure (presence of pleckstrin homology domain and of phosphotyrosine binding domains), IRS proteins are able to be recruited and activated at the cell membrane [205, 206]. Activated IR can phosphorylate IRS in multiple tyrosine residues that form binding sites for intracellular molecules that contain an Src-homology 2 (SH2)domains (Figure 1) [207].

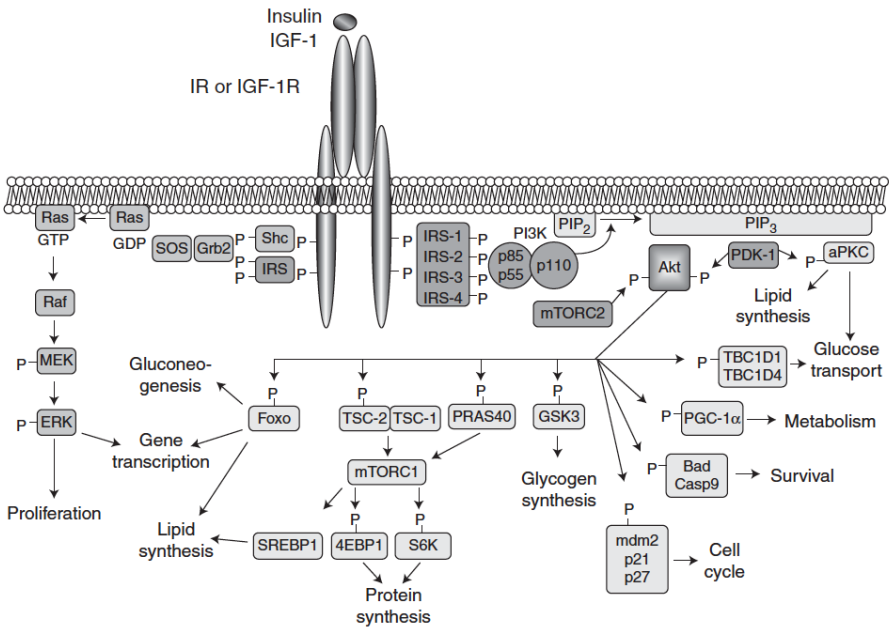


***Figure 1: Insulin- and IGF-1-signaling pathways****. The figure is taken from Boucher J et al. Cold Spring Harb Perspect Biol. 2014 Jan 1;6(1).*

IRS-1 and IRS-2 are the primary mediators of insulin-dependent glucose utilization in most tissues [208]. After activation, IRS proteins interact with PI3-kinases in the (PI3K)-Akt pathway, where PI3K consists of a regulatory and a catalytic subunit [209]. Upon activation (i.e., tyrosine-phosphorylated), IRS proteins activate PI3K by direct binding of the two SH2 domains to the regulatory subunits [205]. Next, activated PI3K catalytic subunit phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) and produces phosphatidylinositol (3,4,5)-triphosphate (PIP3). PI3K-IRSs

complex is also supported by regulatory elements: p85α/β, p55α/β/γ and p50α. Moreover, p110α, β, and δ help to increase PI3K catalytic subunit stability [199].

PIP3 interacts with 3-phosphoinositide-dependent protein kinase 1 (PDK-1) leading to the recruitment of protein-kinase B (Akt) to the plasma membrane for its activation by phosphorylation a threonine residue 308 [210, 211]. However, Akt full activation requires also a second phosphorylation at Serin-473 mediated by the mammalian target of rapamycin complex 2 (mTORC2 complex) [212]. mTORC2 is a protein complex that regulates cellular proliferation as well as the cytoskeleton and cell remodelling. mTORC2 complex is formed by several proteins mTOR, a serine/threonine protein kinase in the PI3K-related kinase (PIKK) family, the rapamycin-insensitive companion of mTOR (RICTOR), target of rapamycin complex subunit LST8 (GBK), mammalian stress-activated protein kinase interacting protein 1 (mSIN1), as well as Protor 1/2, DEPTOR, and TTI1 and TEL2 [213].

Activated Akt phosphorylates and inhibits TBC1 domain family member 4 (TBC1D4/AS160), a GTPase protein activating several Rab proteins due to change from its GDP to GTP bound state [214, 215, 216]. Activation of Rab protein allows the vesicle containing glucose transporter type 4 (GLUT4) to migrate and fuse with the cell membrane, allowing glucose to be actively transported inside the cell lumen through GLUT4 [217]. GLUT4 is one of the main glucose transporters on the cell membrane. It is stored inside the cell in transport vesicles and, upon insulin stimulation, it is rapidly translocated to cell membrane [218].

It should be noticed that activated Akt phosphorylates several other downstream targets, which amplify the signal and the complexity of the insulin signaling. The Forkhead box O (Foxo) family is one of the Akt target and controls the expression of genes involved in lipogenesis and gluconeogenesis [219]. Akt also regulates gluconeogenesis and fatty acid oxidation through peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1a), glycogen synthase thought the regulation of glycogen synthase kinase 3 (GSK-3) [220, 221]. Degradation of the Tuberous sclerosis complex protein 2 (TSC-2), due to Akt activation,

induces the activation of the mammalian target of rapamycin complex 1 (mTORC1) [213].

Akt dependent phosphorylation and subsequent inhibition of the inhibiting effect of the proline-rich Akt substrate 40 KDa (PRAS40) on mTORC1 complex also activates mTORC1. mTORC1 is composed by several proteins: mTOR a serine/threonine protein kinase in the PI3K-related kinase (PIKK) family, the regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8), Proline-rich Akt1 substrate 1 (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR) [213]. mTORC1 complex is a sensor of impaired nutrient, energy, redox status and regulates cell protein, lipids, and nucleotides synthesis. Feeding/fasting, insulin, rapamycin, growth factors, phosphatidic acid, certain amino acids and their derivatives (e.g., l-leucine, arginine and β-hydroxy β-methylbutyric acid), mechanical stimuli, and oxidative stress can induce the activation of the mTORC1 complex [213].

Several negative feedback loops on insulin signaling have been described. These safety loops aim to control insulin signaling and avoid its inappropriate activation. mTORC1 has a negative feedback loop on insulin signaling. Activation of mTORC1 induces the phosphorylation and inhibits 4Ebinding protein 1 (4E-BP1), which then activates ribosomal protein S6 kinases S6K1 [222]. S6K1 inhibits the insulin signal transduction by affecting IRS phosphorylation (i.e., increasing serine and reducing tyrosine phosphorylation). Reduced IRS tyrosine phosphorylation reduces IRS activity and increased IRS serine phosphorylation induces the proteasome degradation of IRS, so blocking insulin signal propagation (Figure 2) [223, 224].

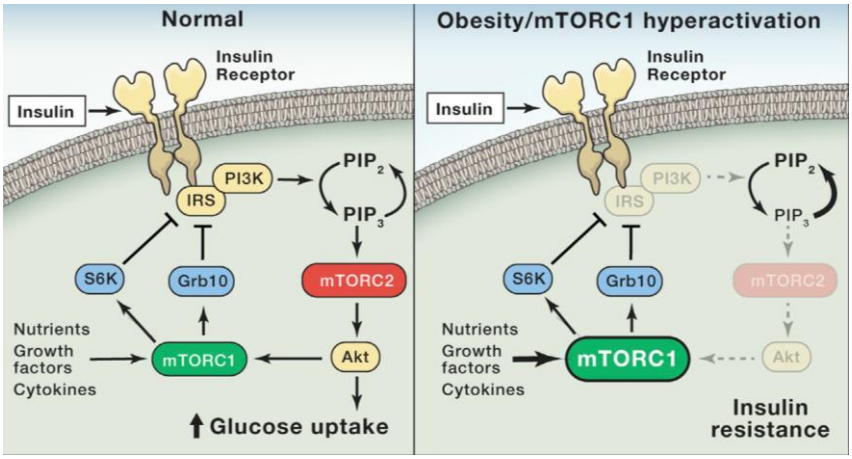


***Figure 2: mTORC1, mTORC2 and insulin sensitivity in normal physiological condition and obesity***. *The figure is taken from Saxton RA and Sabatini DM Cell. 2017 Mar 9;168(6):960-976.*

# 2 SUMMARY OF THE FIELD OF INTEREST

Interactions between bacteria and the human host start at birth and continue thought the whole life. Gut microbiota is an environmental factor that modulates metabolism in humans and is implicated in the pathogenesis of metabolic diseases, such as type 2 diabetes and NAFLD.

Defining specific bacteria responsible for the onset of metabolic diseases and understanding the kinetics of host-microbiota interaction is important to decrypt the mechanisms behind the microbiota impact on metabolic diseases.

A challenge in the field is to move from descriptive associations between microbiota and metabolic diseases to experimental data demonstrating the causative role of the altered microbiota on the onset of metabolic diseases.

Identifying microbially produced metabolites affecting the host pathophysiology would enable to demonstrate the microbial contribution to metabolic diseases and identify new treatment strategies.

# 3 AIMS

The general aim of this thesis is to investigate the role of the gut microbiota in metabolic diseases and specifically to identify the microbiota contribution on altered glucose metabolism and abnormal fat accumulation in the liver (i.e., steatosis), which are the steps contributing to the onset of type 2 diabetes and NAFLD.

The specific aims are:

1. Paper I: to evaluate in a time fashion way the impact of microbiota colonization on host glucose metabolism.

2. Paper II: to evaluate the mechanism behind the microbiota effect on host glucose metabolism by identifying microbiota produced molecules that affect target metabolic tissues.

3. Paper III: to evaluate the microbiota composition in subjects with steatosis and to identify a microbiota altered function in the presence of ectopic fat accumulation in the liver.

# 4 MAIN RESULTS AND DISCUSSION

## 4.1 PAPER I

Colonization of GF mice is a widely used tool to study the effect of microbiota colonization on host metabolism. The aim of Paper I is to study the kinetics of the gut microbiota colonization on host metabolism during colonization of GF mice and, specifically, provide a temporal resolution for how the microbiota affects different metabolic processes in metabolic organs, such as the liver and the adipose tissue.

### 4.1.1 COLONIZATION OF GF MICE PRODUCES A BI-PHASIC GLUCOSE INTOLERANCE

By gavaging the cecum content of age- and sex-matched CONV-R, we colonized GF recipient mice (resulting in conventionalized mice; CONV-D). We followed them for 28 days evaluating the microbiota effect on glucose metabolism and adiposity through time at specific time points (1, 3, 7, 14, 28 days) (figure 3).

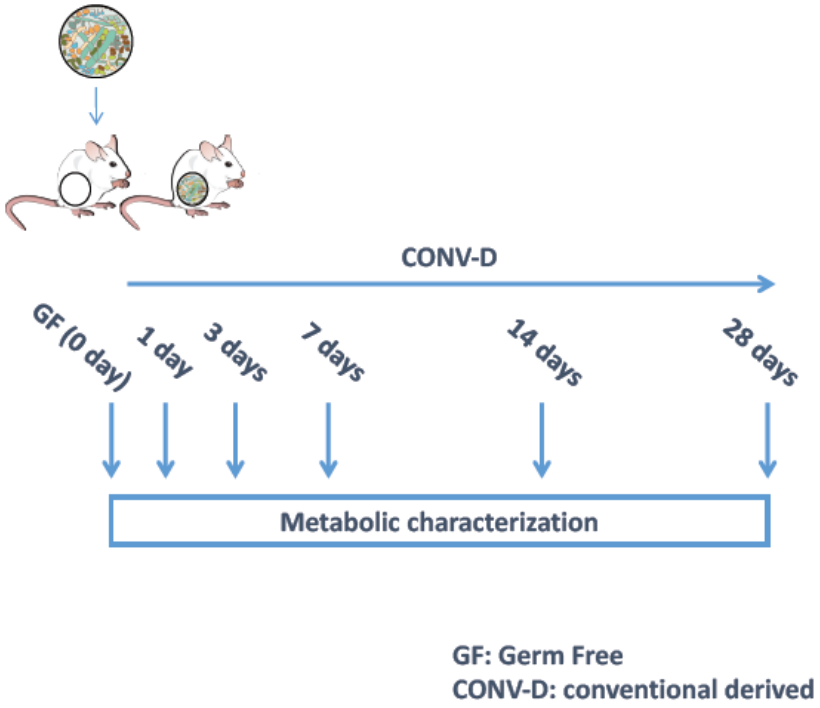


***Figure 3: experimental set-up for colonization experiment in GF mice.***

By performing intraperitoneal glucose and insulin tolerance tests, we observed that the gut microbiota impairs glucose metabolism in a bi-phasic fashion during colonization. An *early* phase of impairment occurring within the first 3 days of colonization and a second, *delayed*, phase occurring 14-28 days after colonization (Figure 4).

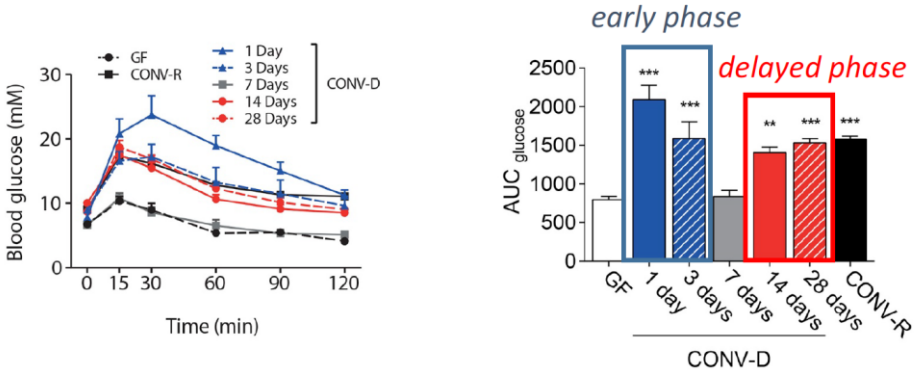


***Figure 4: Intraperitoneal glucose tolerance test and calculated Area Under the Curve (AUC) during colonization of GF mice***.

### 4.1.2 COLONIZATION OF GF MICE IS CHARACTERIZED BY A BI-PHASIC INFLAMMATORY RESPONSE

Next, we investigated the mechanisms contributing to these findings. The *early* phase was associated with a systemic inflammatory response, which was independent of adiposity, as shown by peak of LPS and cytokines secretion. This inflammatory response mostly affected the liver, as shown by the staining for F4/80 (a pan-macrophages marker) and the expression of pro/anti-inflammatory genes *(i.e., Emr1, Saa3, Tnf-, Il6, Il10, Mgl1)* (Figure 5).

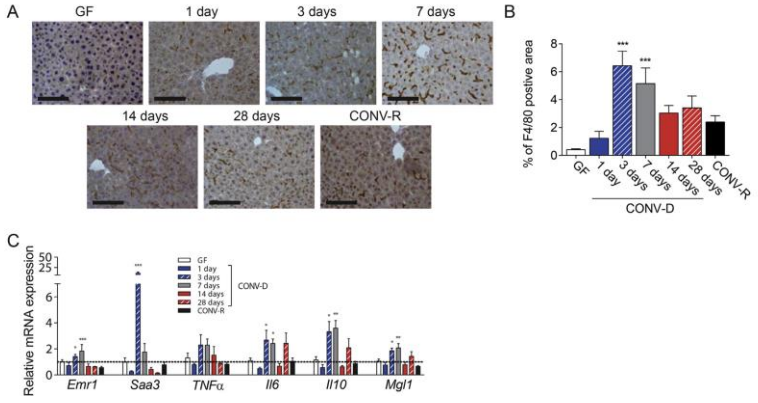


***Figure 5. Colonization of GF mice induces increased inflammatory response in the liver during the early phase in the liver.*** *Representative figures (A) and quantification of F4/80 positive area (B) in the liver, (scale bars 100 mm). C. Relative mRNA expression in the liver for Emr1, Saa3, TNFa, Il6, Il10, and Mgl1 in germ-free (GF), conventionalized (CONV-D) and conventionally raised (CONV-R) mice.*

On the other hand, the *delayed* phase of glucose impairment was associated with adipose tissue expansion and inflammation, as shown by increased number of crown-like structures (a marker of adipose tissue inflammation), increased infiltration of pro-inflammatory macrophages, and expression of pro-anti-inflammatory genes in adipose tissue (eWAT; Figure 6). We hypothesized that the *delayed* phase is due to increased adiposity and adipose tissue inflammation and thus of relevance for studying type 2 diabetes.

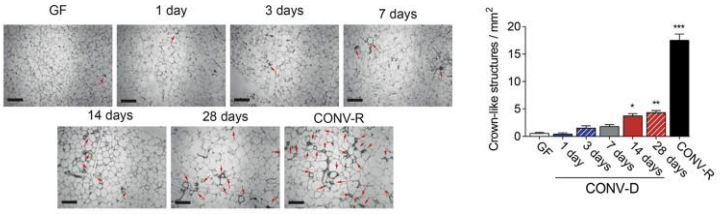


***Figure 6: Colonization of GF mice induces increased inflammatory response in the EWAT during the delayed phase***. *EWAT crown-like structure positive area (scale bars 100 mm), in GF, CONV-D, and CONV-R mice*

### 4.1.3 RE-COLONIZATION OF ANTIBIOTIC TREATED MICE IS CHARACTERIZED ONLY BY A DELAYED PHASE OF GLUCOSE IMPAIRMENT

GF mice display several immunological defects due to the absence of interaction with bacterial antigens [82]. Thus, we wanted to test if the metabolic phenotypes observed during the colonization of GF mice were due to the immaturity of their immune system. Therefore, we performed a re-colonization experiment on antibiotics (Abx) treated mice (CONV-RD). We first ablated microbiota by 10-day treatment with high doses of four different antibiotics and then re-colonized mice by gavaging the cecum content of age- and sex-matched CONV-R (Figure 7).

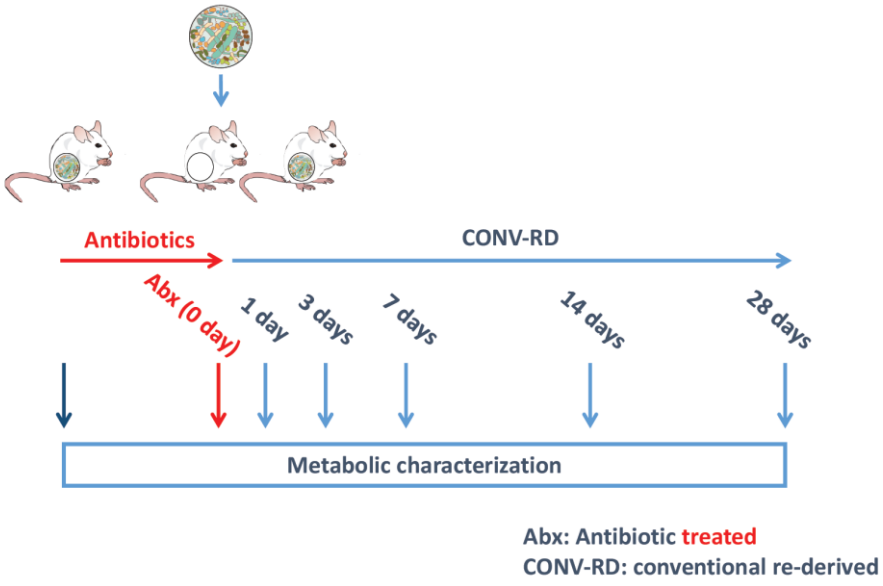


***Figure 7. Experimental set-up for re-colonization experiment in GF mice***

In CONV-RD mice, in intraperitoneal glucose tolerance test, we observed only a *delayed* phase of glucose impairment that was associated with increased adiposity (Figure 8), suggesting that the initial phase was due to the immature and naïve gut in GF mice.

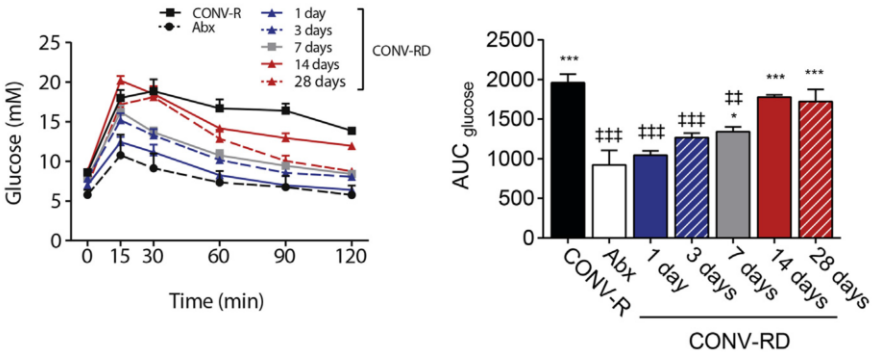


***Figure 8: Intraperitoneal glucose tolerance test and calculated Area Under the Curve (AUC) during re-colonization of CONV-RD mice.***

Taken together our findings indicate that the *early* phase of glucose impairment during colonization of GF mice is due to an uncontrolled inflammatory response of the GF naïve immune system towards gut antigens. The *delayed* phase is instead due to increased adiposity and independent of the immune system integrity.

### 4.1.4 CONCLUSIONS PAPER I

Microbiota colonization of GF mice affects adiposity and glucose metabolism and it is widely used as tool to study microbiota effect on the host [97, 225]. However, the kinetics of these effects and their molecular mechanisms are unknown. Here, we show that the establishment of the microbial community in mice modulates glucose tolerance during time in a bi-phasic way. Our data also provides some insights on the mechanisms behind the two different phases of glucose impairment during colonization. The *early* phase is likely due to an immature gut, and thus not occurring in antibiotic treated CONV-R mice, resulting in a transiently increased intestinal permeability of pro-inflammatory molecules (such as LPS) eventually leading to impaired glucose tolerance. The *delayed* phase is induced by increased adiposity, mainly due to increased adiposity and EWAT inflammation and thus more relevant for studying type 2 diabetes.

Modulation of microbiota early in life has been shown to be fundamental for the onset of metabolic diseases later in life [226]. Human bacterial colonization starts at birth. It is a complex process that undergoes several phases of maturation. Thus, I hypothesize that selection of bacteria that may cause disease early in life, due to for example perturbation of the intestinal microbiota (i.e. with antibiotics), can lead to the development of metabolic diseases later in life [226].

This paper provides some important information on the time point selection for using GF mice colonization as a tool to study gut microbiota effect on the host.

This manuscript is attached as appendix I.

## 4.2 PAPER II

The exact mechanism behind gut microbiota modulation of host metabolism is still under investigation. One of the hypotheses is that gut microbiota converts environmental signals (e.g., macronutrients from the diet) into signaling metabolites, which allow the microbiota to communicate with host metabolic tissues [89]. Finding new microbially produced molecules responsible for the metabolic effect of the microbiota is a challenge for the field.

In this project, we aimed to identify microbially produced metabolites that can modulate glucose metabolism in humans and mice. In particular, we focused on amino acid-derived signaling molecules. On the other hand, other microbiota-derived metabolites, such as branched-chain amino acids (BCAA) and amino acid-derived uremic toxins, have been shown to be harmful for the host physiology [109, 227, 228, 229].

### 4.2.1 IMIDAZOLE PROPIONATE IS ASSOCIATED WITH TYPE 2 DIABETES IN HUMANS

Due to the anatomical structure of the enterohepatic circulation, microbially produced metabolites from the gut reach the liver through the portal vein and then enter the systemic circulation. We collected blood samples from portal and peripheral vein in a cohort of 15 obese subjects (n=5 with type 2 diabetes and n=10 without diabetes) undergoing bariatric surgery (discovery cohort). To identify amino acid-derived microbial metabolites that may contribute to insulin resistance and type 2 diabetes, we performed untargeted metabolomics on plasma samples from portal blood. Only four amino acid-derived metabolites (dopamine sulfate, glutamate, imidazole propionate, and N-acetylputrescine) were significantly higher in the portal plasma of subjects with type 2 diabetes than in those without diabetes (Figure 9A). In order to select the microbially produced metabolites, we tested which of those 4 metabolites were regulated by microbiota by evaluating their levels in the portal and peripheral vein in GF, CONVR, and antibiotics' treated mice. We only identified imidazole propionate (ImP) as a microbially produced amino acid-derived metabolite associated with type 2 diabetes (Figure 9B).

To validate our initial findings, we evaluated ImP levels in the peripheral blood of a larger validation cohort of 649 individuals with normal glucose tolerance or different grade of altered glucose metabolism. After correcting for potential confounding factors (e.g., BMI, sex, and age), we observed significantly higher levels of ImP across the groups ($P < 0.0001$) (Figure 9C).

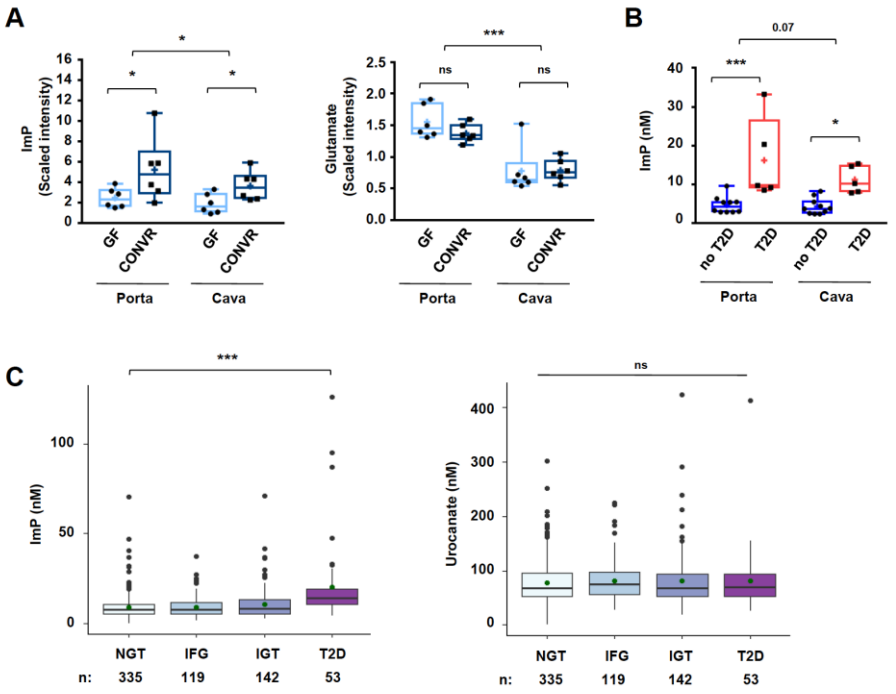


***Figure 9. Imidazole propionate (ImP) is increased in individuals with type 2 diabetes****. A, Relative levels of ImP and glutamate in portal and vena cava plasma of GF and CONVR mice (n = 6 per group). B, Portal and peripheral plasma levels of ImP in obese BMI-matched subjects without type 2 diabetes (no T2D, n = 10) or with T2D (n = 5). C, Peripheral plasma levels of ImP or urocanate in humans with normal glucose tolerance (NGT), impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and treatment-naive T2D. Data presented as box plots show minimum, 25% quartile, median, 75% quartile, maximum, and mean (marked as +). *P < 0.05, ***P < 0.001; ns, not significant. P values were determined by two-way ANOVA with repeated measurements (A, B), Kruskal-Wallis test followed by Dunnett's test (C).*

ImP is produced from histidine by bacteria requiring reduction of a double bond from the precursor urocanate. While both human and bacterial cells can produce glutamate from histidine, only bacteria can convert urocanate (a histidine metabolite the ImP precursor) to ImP, by using a specific bacterial enzyme, the urocanate reductase (urdA) (Figure 10).

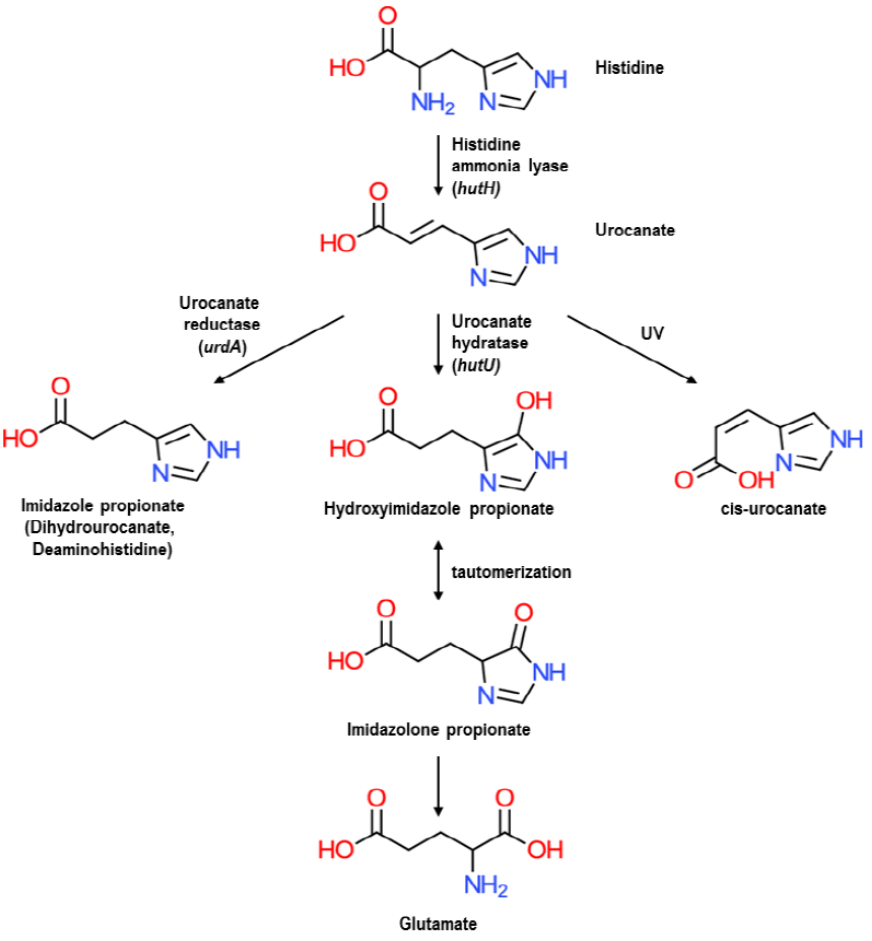


***Figure 10. Histidine utilization pathway.***

By using an *in vitro* gut simulator and batch cultures (see method part for detailed description of the method), we could demonstrate that ImP is a

histidine derived metabolite produced by microbial communities that are present in subjects with type 2 diabetes (Figure 11A-C).

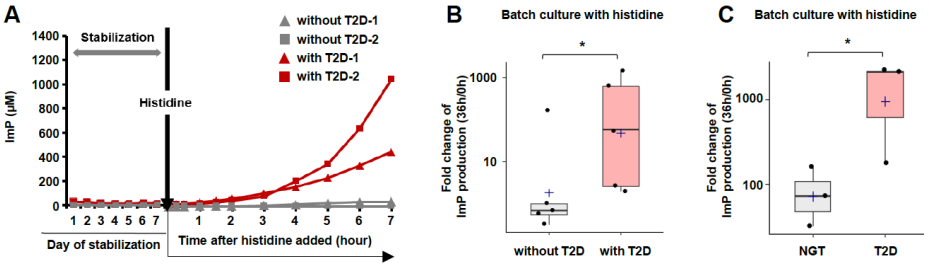


***Figure 11. Imidazole propionate is microbially produced***. *A, Production of imidazole propionate (ImP) from 10 mM histidine in an in vitro gut simulator inoculated (in separate experiments) with feces from two subjects without type 2 diabetes (T2D) or from two subjects with T2D. B, End-point measurements of ImP production from 10 mM histidine in batch culture experiments using feces from obese BMI-matched subjects without T2D (n = 5) or with T2D (n = 5). C, End-point measurements of ImP production from 10 mM histidine in batch culture experiments using feces from subjects with normal glucose tolerance (NGT) (n = 3) or with treatment-naive T2D (n = 3).*

### 4.2.2 IMIDAZOLE PROPIONATE IMPAIRS GLUCOSE TOLERANCE AND INSULIN SIGNALING

To test whether ImP treatment could affect glucose metabolism, we injected vehicle or ImP intraperitoneally into GF or CONV-R mice for 3 days. We observed that ImP treatment induces glucose intolerance in both GF and CONV-R mice (Figure 12). Moreover, by implanting slow-releasing pellets containing either placebo or ImP under the skin of CONV-R mice, we showed that also long-term treatment (i.e., 14 days) with ImP impairs glucose tolerance.

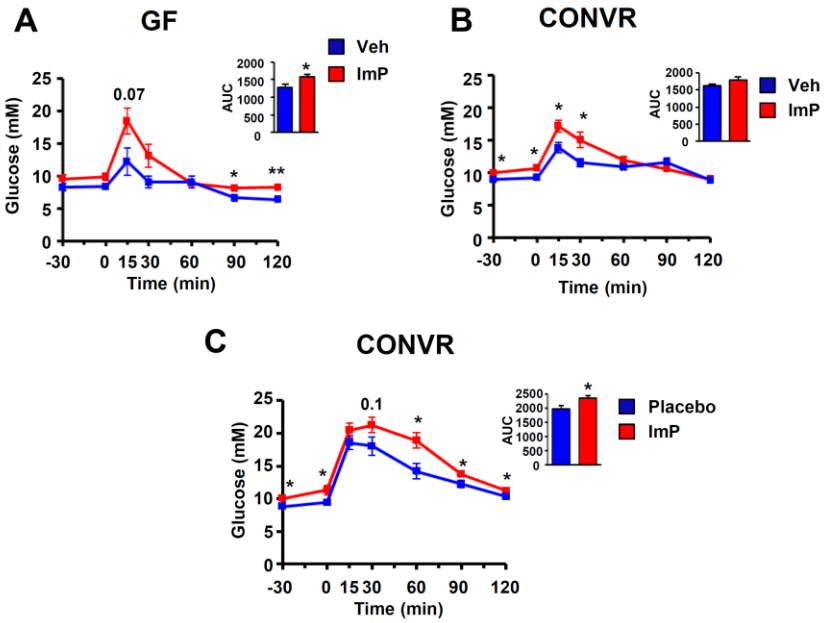


***Figure 12. Imidazole propionate impairs glucose tolerance in mice.*** *A, intraperitoneal glucose tolerance test in GF mice after intraperitoneal injection of vehicle (Veh, 1% DMSO in water; n = 4) or ImP (500 µg; n = 5) twice per day for 3 days. Intraperitoneal glucose tolerance test in CONR mice after intraperitoneal injection of vehicle (n = 4) or ImP (100 µg; n = 4) twice per day for 3 days. C, Intraperitoneal glucose tolerance test in CONVR mice implanted with pellets containing placebo (n = 5) or ImP (n = 6) for 14 days (350 µg/day).*

Thus, we identified a novel metabolite ImP that is increased in humans with type 2 diabetes and produced from histidine by microbiota of subjects with type 2 diabetes. We also showed that ImP induces impaired glucose tolerance once administrated in mice.

Next, by performing Western blot for key proteins involved in the insulin signaling pathway, we aimed to identify a specific ImP target in the insulin signaling pathway (see Figure 1). ImP treatment in GF mice affected insulin signaling in the liver by reducing IRS1 and IRS2 protein concentrations (no significant changes in hepatic Irs1 and Irs2 mRNA levels). In CONV-R mice, ImP treatment needs at least 14 days to affect IRS2 levels in liver

without affecting IRS1. We observed similar effects, either on IRS1 or IRS2, in muscle and white adipose tissue (WAT) both in GF and CONV-R mice (Figure 13A-C).

To further investigate the role of ImP on insulin signaling, we used primary hepatocytes treated with ImP. Both a short- and a long-term ImP treatment (8 and 20 h) affected insulin signaling at the IRS1 and IRS2 levels (Figure 13D-F). Short-term treatment blunted IRS1 tyrosine 612 phosphorylation measured by Western blot (Figure 15J). IRS1 tyrosine 612 phosphorylation is a necessary step for all the downstream steps of insulin signaling. Long-term treatment, instead, reduced IRS1 and IRS2 levels and inhibited insulin-stimulated Akt phosphorylation (Figure 13F). By using a proteasome inhibitor (MG132) together with ImP treatment, we also showed that IRS1 and IRS2 reduction are depending on the ImP proteasome activation (Figure 13G). Taken together, these data show that ImP affects insulin signaling at IRS level (specifically by inhibiting tyrosine phosphorylation or reducing protein levels).

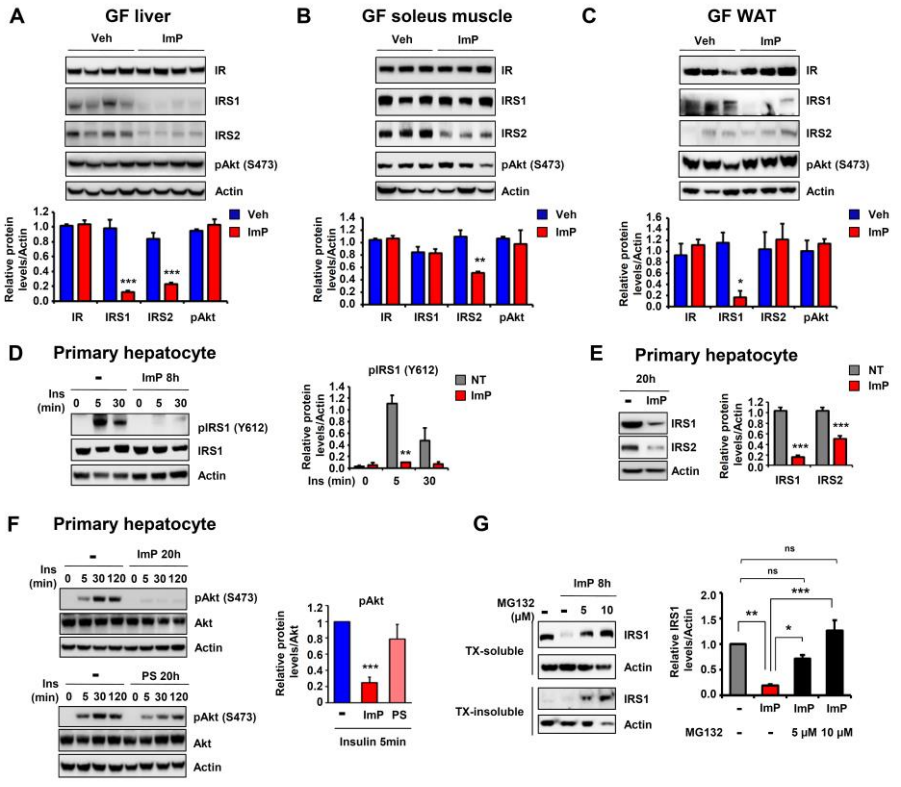


***Figure 13. Imidazole propionate impairs insulin signaling in mice.*** *Western blot (and quantification) of A, liver lysates, B, soleus muscle lysates and C, white adipose tissue (WAT) lysates from GF mice showing effect of 3-day treatment with vehicle or ImP (500 μg twice per day) on insulin signaling components. D, Effect of ImP (100 μM for 8 h) on insulin-stimulated IRS1 tyrosine phosphorylation in primary hepatocytes (n = 3). E, Effect of long-term ImP treatment (100μM for 20 h) on IRS protein levels in primary hepatocytes (n = 7). F, Effect of ImP or the tyrosine-derived microbial metabolite phenol sulfate (PS) (both 100 μM for 20 h) on Akt activation simulated by insulin (5 nM) in primary hepatocytes (n = 4). G, Effect of the proteasome inhibitor MG132 on IRS reduction induced by ImP (100 μM) in serum-starved HEK293 cells (n = 3); cells were co-treated with MG132 and ImP for 8 h. TX, triton. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. Unpaired two-tailed Student's t-tests (A-F). One-way ANOVA followed by Tukey's multiple comparisons test (G).*

### 4.2.3 IMIDAZOLE PROPIONATE ACTIVATES MTORC1

mTORC1 complex activation has a negative feedback on insulin signaling by inhibiting IRS activity through its tyrosine phosphorylation and by inducing IRS proteasome degradation by IRS serine phosphorylation [213]. To demonstrate a possible involvement of ImP on mTORC1 activation, we treated primary hepatocytes with ImP with or without rapamycin (inhibitor of mTORC1) (Figure 14A). By performing Western blotting for S6K1 phosphorylation (a marker of mTORC1 activation), we showed that ImP increased mTORC1 activation and IRS1 and IRS2 degradation and that this process is blocked by mTORC1 inhibition by rapamycin (Figure 14A). Consistently with our *in vitro* data, we observed *in vivo* that, after 3 days of treatment, ImP increased S6K1 phosphorylation in liver, soleus muscle, and WAT of CONV-R mice. These findings indicate that ImP activates mTORC1 complex (Figure 14B).

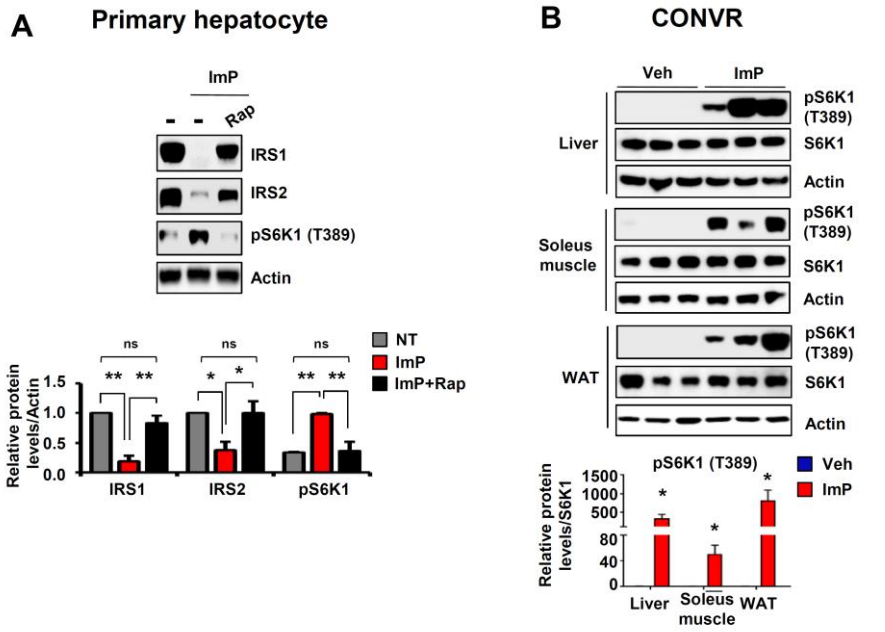


***Figure 14. Imidazole propionate activates mTORC1.*** *A, Rapamycin (Rap, 20 nM) inhibits the effect of imidazole propionate (ImP) (100 μM for 20 h) on IRS and pS6K1 in primary hepatocytes (n = 3). B, Immunoblot of liver, soleus muscle, and WAT lysates from CONV-R mice showing that 3-day treatment with vehicle (Veh) or ImP (100 μg twice per day) increases phosphorylation of S6K1 (n = 3). Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant. One-way ANOVA followed by Tukey's test (A) and unpaired two-tailed Student's t-tests (B).*

### 4.2.4 IMIDAZOLE PROPIONATE-INDUCED MTORC1 ACTIVATION IS DEPENDENT ON P62 PHOSPHORYLATION

Phosphorylation of the adaptor protein p62 at T269 and S272 has recently been shown to be critical for amino acid induction of mTORC1 activation [230]. By Western blot analysis, we showed that ImP treatment induced p62 phosphorylation both *in vitro* and *in vivo* (liver and primary hepatocytes

from CONV-R mice) (Figure 15A-B). *In vitro*, this response was not affected by treatment with inhibitors of the mTORC1 complex (Rapamycin or Torin 1) (Figure 15B), indicating that mTORC1 is downstream of p62 phosphorylation. Furthermore, by using two different siRNA for p62, we tested the effect of knockdown p62 in presence of ImP. By performing Western blot, we were able to observe that, after knocking down p62, ImP-p62-induced S6K1 and IRS1 S636/S639 phosphorylation is dramatically reduced (Figure 15C). Taken together these data suggest that p62 is an essential key protein in ImP-induced mTORC1 activation.

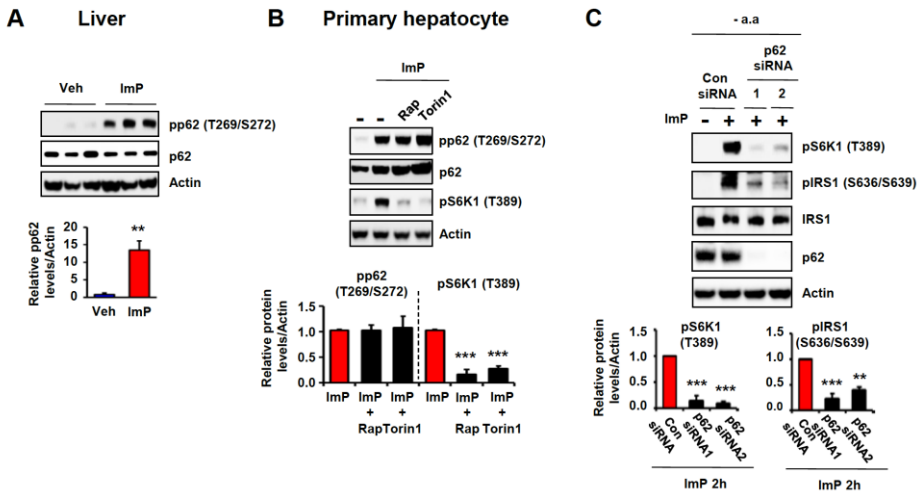


***Figure 15. Imidazole propionate induces p62 phosphorylation upstream of mTORC1.*** *A, Western blot of liver lysates from CONVR mice showing that 3-day treatment with vehicle (Veh) or imidazole propionate (ImP) (100 µg twice per day) increases phosphorylation of p62 (n = 3). B, Effect of ImP (100 µM), rapamycin (Rap, 20 nM) or Torin1 (250 nM) for 12 h on p62 phosphorylation (T269 and S272) in primary hepatocytes (n = 3). C, Effect of p62 knockdown on ImP-induced signaling. HEK293 cells were transfected with control siRNA (Con siRNA) or non-overlapping siRNAs against p62 (p62 siRNA1 or siRNA2) and incubated with ImP (100 µM) in the absence of amino acids (-a.a) for 2 h (n = 3). Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. Unpaired two-tailed Student's t-tests.*

### 4.2.5 ALTERNATIVE P38 PROMOTES IMIDAZOLE PROPIONATE-INDUCED P62/MTORC1 ACTIVATION

p38 mitogen-activated protein kinase (MAPK) has 4 isoforms in mammalian cells, which can be divided into two subsets depending on their substrate specificity: p38α/p38β and p38γ/p38δ (also called alternative p38 MAPK) [231]. Only p38δ can mediate the amino acid-induced activation of p62/mTORC1 [230]. By using siRNA for knockdown of each p38 isoforms in HEK293 cells we showed with Western blot that knocking down of p38α, p38β and p38δ did not affect p62/mTORC1 or IRS1 activation (Figure 16A-B). Rather, p38γ knockdown induced phosphorylation of p62, S6K1, and IRS1 (Figure 18B-C).

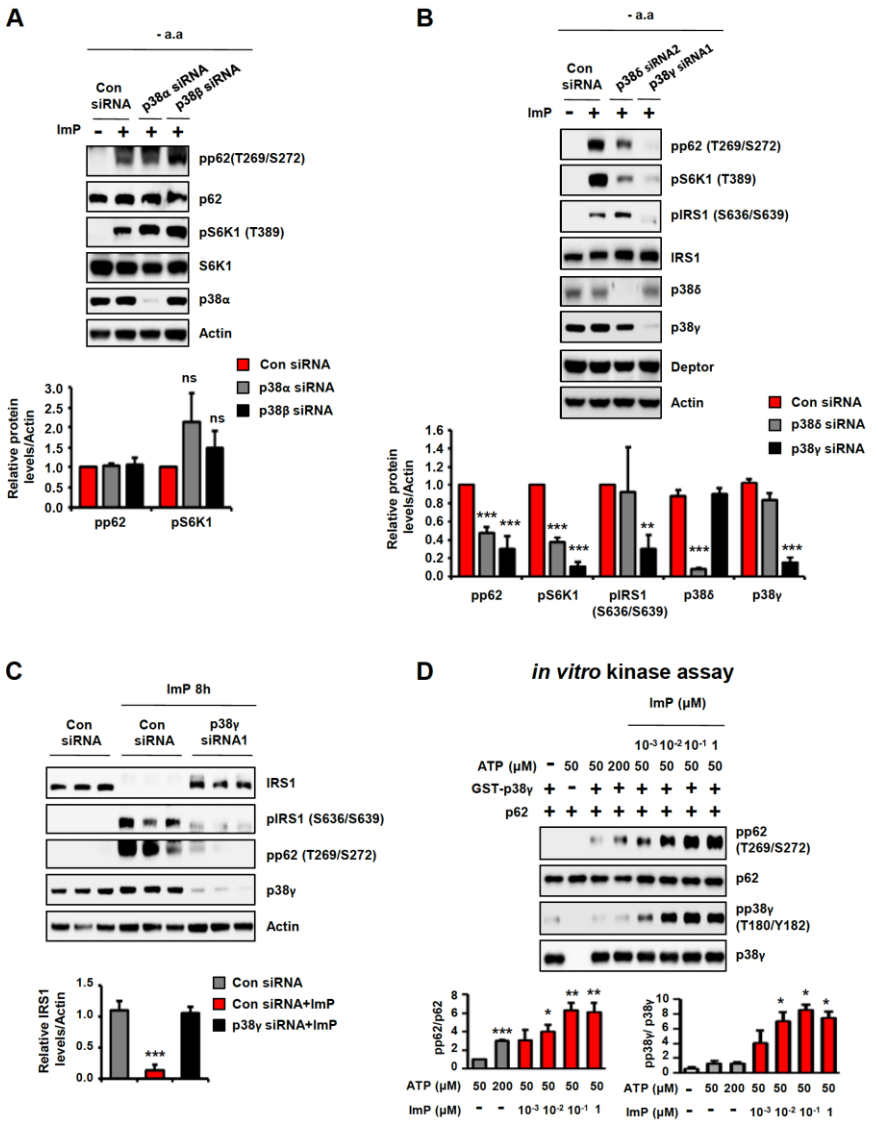


***Figure 16. p38γ is responsible for imidazole propionate sensing.*** *A, Effect of conventional p38 knockdown on imidazole propionate (ImP)-induced signaling (n = 3). HEK293 cells were transfected with control siRNA (Con siRNA), p38α siRNA, or p38β siRNA and incubated with ImP (100 μM) in the absence of amino acids (- a.a) for 1 h (n = 3). B, Effect of alternative p38 knockdown on ImP-induced signaling (n = 3). HEK293 cells were transfected with control siRNA (Con siRNA),*

*p38δ siRNA2, or p38γ siRNA1 and incubated with ImP (100 μM) in the absence of amino acids for 1 h. C, Effect of p38γ depletion on ImP-induced IRS reduction in serum-starved HEK293 cells (n = 6). D, in vitro kinase assay (n = 3). p38γ and p62 were preincubated and the kinase reaction was started by adding ATP in the absence or presence of ImP. Data are mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant. One-way ANOVA followed by Dunnett's test (A,B). Unpaired two-tailed Student's t-tests (C,D).*

Next, to evaluate potential interactions between p38γ, p62, and ImP, we performed an *in vitro* kinase assay (see appendix 4 for detailed description of the method), by combining together p38γ and p62 proteins with ATP in the absence or presence of ImP. By performing Western blot analysis before and after ATP induced kinetic reaction, we showed that p62 phosphorylation (at threonine 269 and serine 272) was induced by p38γ in an ATP concentration dependent fashion (50 to 200 μM). ATP itself did not induce p38γ auto phosphorylation (Figure 16C).

To summarize our findings, ImP affects insulin signaling at the level of IRS through the activation of p38γ MAPK, which promotes p62 phosphorylation and, subsequently, activation of mTORC1 (Figure 17).

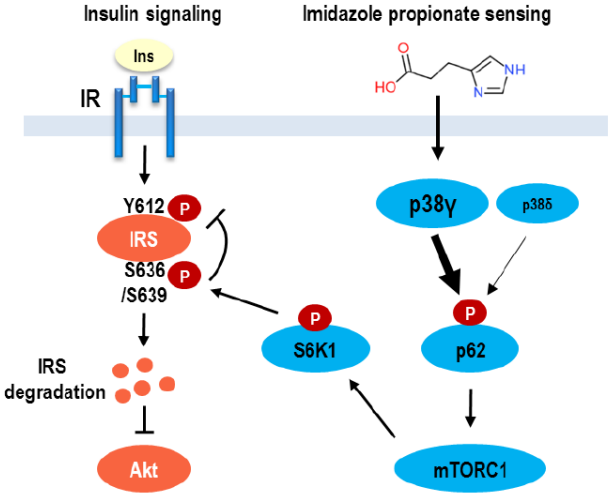


***Figure 17. Representative schema of Imidazole propionate (ImP) pathway.***

### 4.2.6 IMIDAZOLE PROPIONATE ASSOCIATES WITH P62/S6K1 PHOSPHORYLATION IN HUMAN LIVER

We showed that ImP levels were significantly higher in the portal plasma of subjects with than in those without type 2 diabetes. To understand the pathophysiological role of this finding, we analysed, by Western blot in liver samples, different key proteins in the insulin signaling pathway in the discovery cohort (n = 5 diabetics and 7 non-diabetics, see paragraph 4.2.1). Correlation analysis showed that portal levels of ImP positively correlated with p62 and S6K1 phosphorylation. These data support our *in vivo* findings that ImP impairs insulin signaling through the p62/mTORC1 pathway (Figure 18).

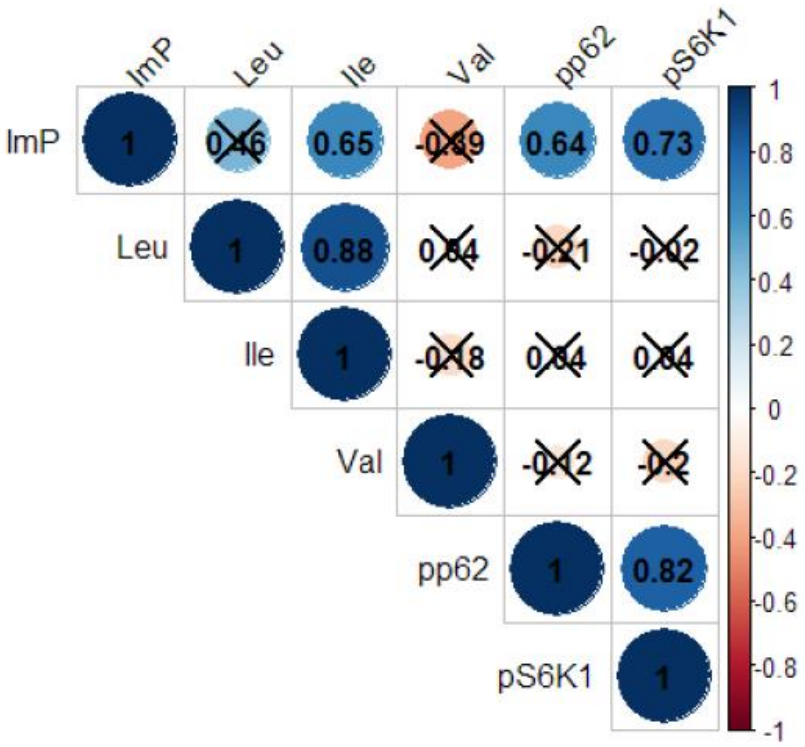


***Figure 18. Imidazole propionate correlates with p62/S6K1 phosphorylation in human liver.*** *Pearson's correlations between portal levels of ImP or BCAAs (Leu, Ile, or Val) and phosphorylation of p62 or S6K1 in the human liver. Nonsignificant correlations (P value > 0.05) are indicated by crosses.*

Taken together, our findings indicate that the microbially produced metabolite, ImP, contributes to the pathogenesis of type 2 diabetes by affecting p38γ/p62/mTORC1 pathway.

### 4.2.7 CONCLUSIONS PAPER II

In this paper, we identified imidazole propionate as a microbially produced histidine-derived metabolite that is increased in subjects with type 2 diabetes. To demonstrate causality in type 2 diabetes, we showed that ImP treatment impairs glucose tolerance in mice. Furthermore, we identified the molecular target of ImP on the insulin signaling, e.i. IRS through activation of the p38γ/p62/mTORC1 pathway.
So far, this is the first study that is able to provide a complete and exhaustive mechanism on the role of bacteria in glucose impairment from bacteria via a unique metabolite to a specific molecular target.
Histidine from the diet *per se* is good for health. Human cells and a healthy microbiota can convert histidine to glutamate, which has been shown to improve glucose metabolism [232]. However, an unhealthy microbiota (i.e., the one associated with type 2 diabetes) has bacteria able to convert histidine in urocanate and then in ImP. As we showed in this paper, ImP is detrimental for health by generating insulin resistance.
This paper provides new insights in the causative role of microbiota on type 2 diabetes. It adds even more complexity to the big picture of diet-microbiota interactions in health and disease. The same nutrient (e.g. histidine) can have complete different metabolic effects on the host depending on the composition of the microbiota and thus the end product of its metabolism.

This manuscript is attached as appendix II.

It should be notice that our data showed serum levels of ImP not only higher in subjects with type 2 diabetes but also with similar concentrations in the portal and peripheral blood. This indicates that ImP might have also systemic effects and not only limited to the liver. Thus, after showing that ImP is involved in the onset of type 2 diabetes we next investigate if ImP also is involved in the pathogenesis of other features of the metabolic syndrome such as cardiovascular diseases and NAFLD.

### 4.2.8 IMIDAZOLE PROPIONATE IS INVOLVED IN CARDIOVASCULAR DISEASES IN HUMANS

### 4.2.9 IMIDAZOLE PROPIONATE IS NOT INVOLVED IN THE PATHOGESIS OF NAFLD

## 4.3 PAPER III

One third of the general population is affected by NAFLD, which is a metabolic disease characterized by ectopic fat accumulation in the liver. Several reports have shown that an altered microbiota composition is associated with the whole spectrum of NAFLD in humans [138, 142, 147, 148]. However, the shift in microbiota function in NAFLD is poorly documented in humans.

The aim of this paper was to use a metagenomics approach to identify microbial species and functions responsible for the onset of NAFLD in humans. Among the different features of NAFLD, we specifically focus on simple steatosis in order to decrypt the role of bacteria on the initial stage of NAFLD.

### 4.3.1 STEATOSIS HAS A SPECIFIC INTESTINAL MICROBIOTA TAXONOMY

The composition and genetic content of the gut microbiome were characterized by whole genome analyses of fecal samples of a discovery cohort of subjects with biopsy-proven steatosis (n=18) without any sign of liver inflammation (NAS score <3, macro vesicular steatosis >10%) and healthy controls, matched for age and gender (n=18). To avoid any possible bias, we did not include subjects who used metformin, antibiotics and proton pump inhibitors due to its known effect on gut microbiota composition (Jackson et al., 2016; Wu et al., 2017). We used the MEDUSA pipeline, which is a tool for metagenomic data analysis that allow simultaneous taxonomic and gene annotation reads to non-redundant catalogs of microbial genes and genomes [235]. We observed a significant shift of the overall gut microbiota composition (Figure 23A-B) in presence of steatosis that accounted for about 8% of the total compositional variation. In agreement with previous reports in patients with cirrhosis, (Jie et al., 2017; Qin et al., 2014). we observed an enrichment of bacteria usually resident in the oral cavity or the vagina in the gut microbiota of subjects with NAFLD.

To better characterize gut microbiota composition in subjects with steatosis, we performed a distance-based redundancy analysis (dbRDA), which evaluates if the variation of the microbiota can be explained by other variables rather than presence of steatosis. We observed that alanine aminotransferase was the only clinical variable contributing significantly to the variation of gut microbiota composition. (Figure 23C).

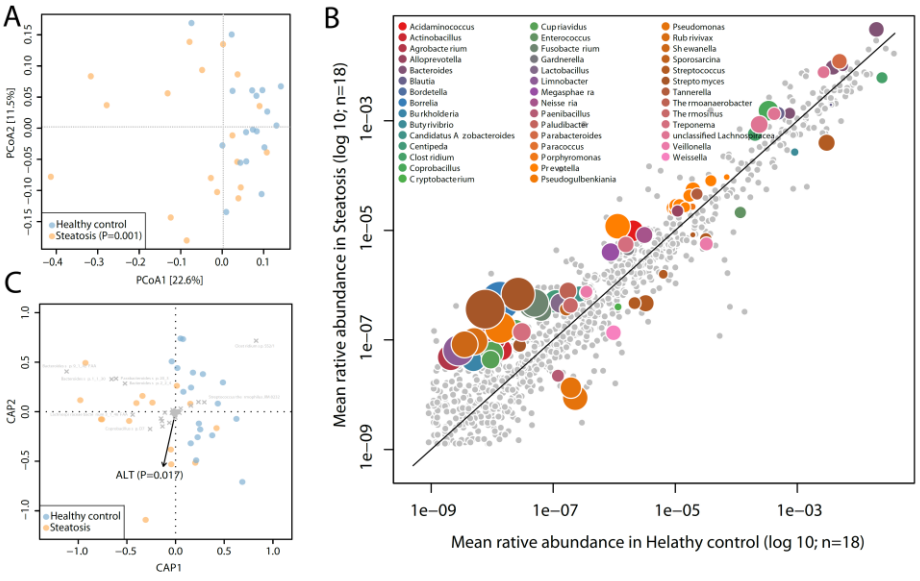


***Figure 23. Gut microbiota composition in patients with steatosis in the discovery cohort.*** *A, Principal coordinates analysis (PCoA) plot based on Bray-Curtis dissimilarity index calculated for all bacterial genomes detected in the fecal metagenomics samples ($P_{group}$=0.001; Adonis test with 1000 permutations). The values reported in brackets next to the axes indicate the amount of percent compositional variability explained by the first two principal coordinates (PCoA1 and PCoA2); B, Bubble plots showing the abundance of microbial genomes in patients with steatosis and healthy controls. Each dot represents a microbial genome; colored dots indicate genomes with significantly different abundance in steatosis compared to healthy controls (colors indicate taxonomic affiliation at genus level); C, Distance-based redundancy analysis (dbRDA) based on Bray-Curtis dissimilarity index calculated for the genomes with significantly different abundance in steatosis compared to healthy controls ($P_{group}$=0.001; PBMI=0.28; the genomes included in this analysis are shown in panel B and listed in Table S4). ALT, alanine transferase; CAP, constrained analysis of principal coordinates.*

To confirm our findings, we recruited a validation study cohort composed of subjects with or without steatosis, whose diagnosis was based on ultrasound examination (n=33 subjects per each group). We found a significant, although less pronounced, shift in the overall gut microbiota composition in presence of steatosis, which accounted for about 3% of the total compositional variation.

To identify common signature of the bacterial genome in NAFLD, we combined the findings of the two cohorts. Eleven genomes ware similarly altered in both NAFLD cohorts . Whereas only the genome of the butyrate producer *Clostridium sp*. SS2/1 was consistently reduced, three groups of microbes were found to be enriched in subjects with steatosis: *Bacteroides* (n=2), *Prevotella* (n=2), oral bacteria associated with periodontitis (n=3), and bacteria associated with ethanol production (n=3).

### 4.3.2 STEATOSIS IS LINKED TO A SHIFT IN GUT MICROBIOTA FUNCTIONAL POTENTIAL

Next, we investigated whether the functional potential of the gut microbiota is altered in the presence of steatosis. To identify microbial gene functions present in the metagenomics samples, we annotated high-quality reads to microbial genes using the KEGG Orthology (KO) database (Kanehisa and Goto, 2000). We performed a principal coordinate analysis (PCoA), which is a method that help us to explore and to visualize similarities or dissimilarities of the relative abundance of all annotated KOs for the metagenomes. Comparison of fold changes for all the differentially abundant KOs showed that the majority of microbial functions changed in the same direction in both cohorts. Sixty-one KOs were altered in both cohorts, of which 57 functions were altered in both cohorts and consistently in the same direction. These showed strong correlation in particular with parameters of liver damage and BMI. Steatosis was linked to a shift in the abundance of several microbial metabolic pathways in both cohorts: biosynthesis of LPS and functions related to cell growth (i.e. genes involved in sporulation) were the most enriched and depleted pathways, respectively, in both the discovery and validation cohorts (Figure 24).

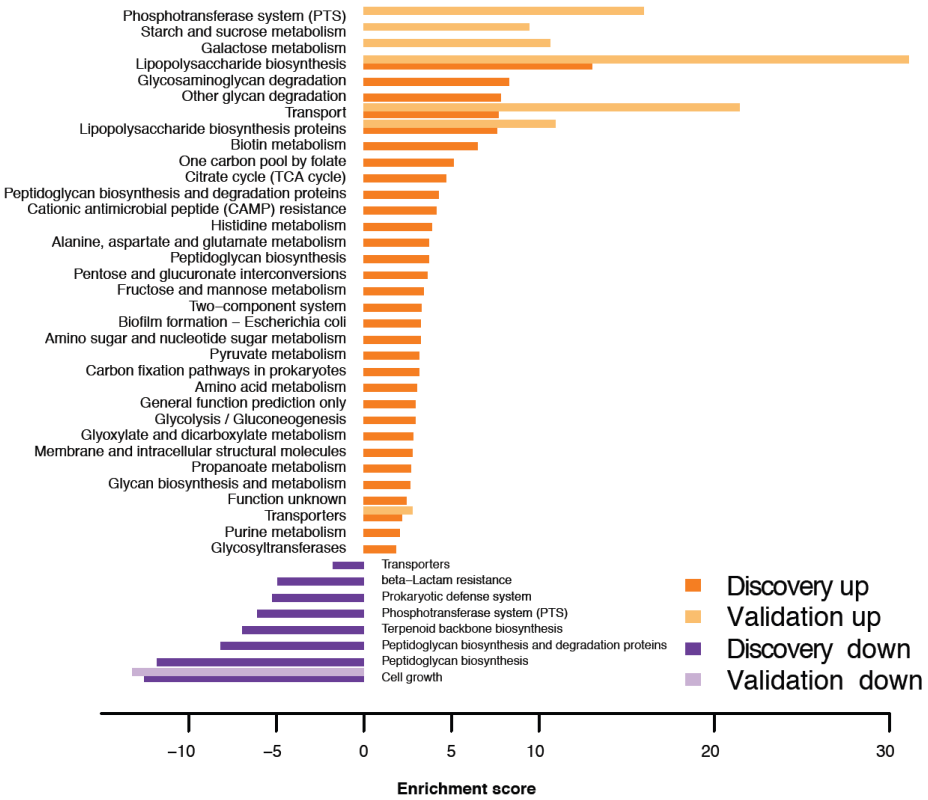


***Figure 24. Functional shifts in the gut microbiome associated with steatosis.*** *Pathway enrichment analysis for the differentially abundant gene functions in the discovery and validation cohorts.*

Thus, in patients with early-stage NAFLD, we observed a shift in gut microbiota composition and function that supports the hypothesis that microbial ethanol production, LPS-driven inflammation, and reduced butyrate production may all contribute to the onset of liver fat accumulation (i.e. steatosis) in humans.

### 4.3.3 CONCLUSIONS PAPER III

In this project, by using a metagenomics approach, we characterized gut microbiota composition and genetic in two independent cohorts of subjects with steatosis.

In presence of steatosis, we found some common variation in the abundance of several bacteria:

1. A reduction in butyrate producer Clostridium sp. SS2/. This observation is a consistent feature observed in subjects with cardio-metabolic diseases such as type 2 diabetes, cardiovascular diseases and NAFLD [120, 236, 237]. However, to date the mechanism behind this observation is still unknown; there are conflicting findings in experimental models and humans on butyrate supplementation [129, 130, 177, 179, 238]. Thus, it could reflect just an altered metabolic profile associated with steatosis.
2. An increased abundance of *Prevotella*, *Bacteroides* and *Parabacteroides*, which is consistent with previous observation in subjects with NAFLD [156, 188, 239].
3. An increased abundance of several bacteria (e.g.,*Prevotella bivia* JCVIHMP010, *Prevotella bergensis* DSM 17361, *Prevotella multiformis* DSM 16608, *Prevotella denticola* CRIS 18C-A and *Fusobacterium nucleatum* subsp. vincentii 4_1_13) belonging to oral bacteria linked to periodontal disease. It has been shown how bacteria involved in periodontitis can induce NAFLD in experimental models [146, 240, 241]. Moreover, translocation of bacteria from the oral cavity to the intestine due to pharmacological alteration in gastric secretion can induce NAFLD in mice. Of note, subjects with proton pump inhibitors therapy were not included in our study, and therefore our observations are not confounded by the use of this medication. One limitation of our study is the lack of data on gastric pH secretion that could have helped to understand the pathophysiology behind our observations.
4. An increased abundance of bacteria able to produce ethanol (e.g., the Spirochaete *Treponema primitia* ZAS-2, T*reponema azotonutricium* ZAS-9 and *Thermoanaerobacter wiegelii* Rt8.B1). Our findings are in agreement with previous results showing an increased microbial ethanol production in NAFLD in humans and experimental models [143, 186,

187]. Ethanol production could be one of the potential mechanisms of a gut microbiota contribution to the pathogenesis of steatosis.

In subjects with steatosis, the analysis of the genetic content of the microbiome showed an increased capacity to metabolize simple sugars and to produce pro-inflammatory molecules such as peptidoglycan and LPS. LPS is a known microbial produced molecule that can induce NAFLD [242].

Taken together, the shifts in gut microbiota composition and function that we observed in this study support the hypothesis of several microbial contributions to the complex pathogenesis of NAFLD. Ethanol production, LPS-driven inflammation, and reduced butyrate production may all contribute. These bacterial metabolic pathways could constitute an important preventive and therapeutic target to counteract disease development and progression in humans.

This study has strengths and limitations. Strength of our study is that we were able to corroborate the findings of the discovery cohort in a validation cohort. We tried to minimized confounders such as use of metformin and PPI, which are known drugs affecting microbiota composition [133, 243]. A limitation of this study is the small size of the study populations and differences in demographic and metabolic parameters (e.g., diet, BMI, blood lipids, and glucose metabolism) between the discovery and validation cohorts that could affect gut microbiota composition. Moreover, we could not validate all the findings of the discovery cohort in the validation cohort. This is due to the difference of the methods used to diagnose steatosis in the two cohorts. In the discovery cohort, we used the histological gold standard method while in the validation cohort, steatosis was diagnosed by liver ultrasound that fails to discriminate between simple steatosis and NASH. According to published data this approach might result in the unexpected inclusion of around 20% of individuals with NASH and were described to have a more heterogeneous fecal microbiota [244]. Another important limitation of the study is the observational character without experiments aiming to show a mechanistic role of bacteria in the onset of NAFLD. We will perform new experiments in the future (see future directions chapter).

This manuscript is attached as appendix II.

# 5 METODOLOGICAL CONSIDERATIONS

Here I will reflect on the experimental methods I used in this thesis and possible pros and cons related to them.

For detailed methods description go to the method section for paper I, II, and III (appendix I, II, and III respectively).

## 5.1 THE MOUSE MODEL TO STUDY MICROBIOTA

The mouse is one of the experimental models used to study metabolic diseases [245, 246]. It has some advantages, such as small body size, a short lifespan, and to option of genetical manipulation [245]. Mouse models are very similar to humans in terms of anatomy and biology [247, 248]. Indeed, humans and mice share 95% similarity in the DNA composition meaning that, with some exception, it is suitable to perform metabolic studies [249].

However, animal models have some disadvantages as well. Not all the findings made in animal models of diseases have been successfully translated into humans. This is likely due to failure of the animal models to accurately mimic the human disease condition or due to different pathophysiological mechanisms driving the disease onset in humans and mice [250]. Moreover, several factors can influence the results such as gender, age, laboratory environment (e.g., water, food, temperature, noise light/night cycle), stress conditions (e.g., type of housing, bedding), and occult bacterial or viral infections [251]. Thus, for each particular research field, animal models should be carefully selected and results interpreted according to potential bias caused by the model itself.

Regarding animal models used for microbiota-host interaction, several studies showed that the mouse is a reliable model [225, 252]. However, there are some important differences between mice and humans in microbiota

composition. Despite the fact that in humans and in mice the major phyla in the gut microbiota are Firmicutes and Bacteroidetes, on a genera levels there are substantial differences between murine and human microbiota. Approximately 85% of mouse gut microbiota bacterial genera are not present in human [50, 100, 101, 253, 254]. Some genera are highly abundant in human but not in mouse gut microbiota (*Prevotella*, *Faecalibacterium* and *Ruminococcus*) and *vice versa* (*Lactobacillus*, *Alistipes* and *Turicibacter*) [255]. On the other hand, some other genera (*Clostridium*, *Bacteroides* and *Blautia*) have a similar abundance in both humans and mice, showing a common core of bacteria preserved between species [255]. It should also be noticed that even in health, a human inter-individual variability has been observed in microbiota composition [67, 256, 257]. Similarly, variability in mice microbiota has been observed in different facilities across the world [258, 259, 260]. Moreover, different animal genetic background and housing can affect microbiota composition and thus its effect on the host, meaning that researchers have to control all these factors [261].

Another important difference between human and murine studies is that human studies usually use single or repeated stool sampling, whereas mice studies usually use cecum sampling. This might affect results reproducibility due the different composition and abundance of bacteria between feces and cecum samples [255, 262].

The experimental model most used in microbiota studies is represented by GF mice [68, 252]. GF mice are born and raised in sterile isolators where air, food and water are sterile [263]. Comparing and contrasting GF mice with their CONV-R counterparts is an effective tool to study the effect of host-microbiota interaction on host metabolism. However, GF mice are an extreme model and for several metabolic features different from normal CONV-R animals, such as altered bile acids profile, less adiposity, enlarged colon, altered epithelia structure, altered mucous secretion, altered immune system, and reduced vascular intestinal structure [73, 81, 86, 264, 265]. Thus, data on GF mice should be carefully interpreted and might be corroborated with data from animal models of microbiota ablation by antibiotics.

On the other hand, GF mice might be considered as a useful tool to evaluate the effect of a specific microbiota on host physiology, performing

colonization experiments and studying causal relationships between the microbiota and the metabolic phenotypes. In this particular scenario, GF mice are used as a "sterile bacterial broth" that we inoculate with a specific microbiota to study its effect on the host physiology [225]. This approach is visible in those studies where researchers compare the metabolic effect of colonization of GF mice with human gut microbiota from healthy subjects with that of GF mice colonized with gut microbiota from subjects with metabolic diseases (e.g., diabetes, obesity, metabolic syndrome) [133, 158, 266]. This methodological approach has, however, some limitations. Despite the use of specific anaerobic buffers for colonization procedures, which allow anaerobes bacteria to survive during the process, manipulation of the gut microbiota can affect the efficiency of bacterial mice colonization. Moreover, since human and mice microbiota are similar but not identical, some human bacteria may not be able to colonize mice due to host genetic and environmental factors. Indeed, GF mice colonized with mouse feces have a higher representation of Clostridiales compared to GF mice colonized with human feces (humanized), where there is a more pronounced representation of Verrucomicrobiales, Burkoholderiales, and Erysipelo-trichales [267]. The lower abundance of Clostridiales in the humanized mice is not due to a low representation of this taxon in the inoculum. In fact, it is due to the ecology of the human bacterial community during colonization process. Moreover, it should be noticed that colonization of GF mice with a human microbiota gives a less pronounced immunological effect than that with a mouse microbiota. However, colonization of GF mice with a human microbiota induces the same metabolic genes expression in the host as the colonization with a mouse microbiota [268].

There are some substantial differences between antibiotics treated mice and GF mice (see paper I). Thus, the choice between these two animal models to study microbiota-host interaction should be carefully evaluate according to the aim of the study.

## 5.2 *IN VIVO* MODELS OF METABOLIC DISEASES

In paper I and II, a mouse model was used to study the effect of microbiota on glucose metabolism. Here I would like to reflect on the pros and cons of using a mouse model to study glucose metabolism and, based on that, explain the methodological choices of this thesis.

Mice are widely used as model to study metabolic pathways, especially glucose metabolism [245, 246, 269]. In the different experimental part of this thesis, two different mouse strains were used, C57Bl/6J and Swiss Webster. The C57Bl/6 is one of the most used inbred strains in biology. This means that it is a stable and homogenous strain, which provides more reproducible experiments. C57Bl/6 are small in size (around 25 grams at 10 weeks of age), rather easy to bread and low susceptible to cancer and easily responsive to diet-induced obesity and its complications (www.taconic.com, www.criver.com). All these characteristics made C57Bl/6 mice a popular model for studying metabolic diseases [269]. In contrast, the Swiss Webster mouse model is an outbred strain. They have a high genetic variability, which makes them suitable for metabolic studies where the genetic background is not important and thus experimental findings are more translatable to human (patho)physiology. Swiss Websters are large in size (around 40 grams at 10 weeks of age) and spontaneously develop mild obesity features on chow diet [97].

In paper II, primary hepatocytes from C57Bl/6 mice were obtained. This was done in order to increase the reproducibility of the data and avoid any effect due to the high genetic variability of the Swiss Websters. Instead, in the colonization experiments in paper I, Swiss Webster mice were used because of their ability to have larger number of litters, which makes it easier to plan large experiments with several age-matched mice. Moreover, Swiss Webster mice develop, on chow diet, mild obesity and thus can maximize the metabolic effect of microbiota on host physiology in terms of adiposity and glucose metabolism. The specific characteristics of Swiss Webster mice make them suitable to perform experiments using chow diet, and thus avoiding possible confounding factor coming from a western style diet, such as pro-inflammatory fatty acids [270].

Furthermore, several factors should be considered as potential confounders when interpreting results of metabolic studies in mice:

- Mouse strains may affect glucose tolerance and insulin sensitivity on both chow and in high fat diet [271]. Glucose and insulin fasting levels can vary in mice of the same strain depending on the vendor from which they are purchased [260, 272]. So, to standardize results, the same mouse strain was always used throughout each paper and always purchased mice from the same vendor if mice needed to be acquired form commercial vendors.
- Mice husbandry can affect results. Thus, the same kind of husbandry and cages was used throughout all the experiments [273]. When mice were purchased from a vendor, mice were always allowed to acclimatize in our facility for at least two weeks before performing any experiment to reduce potential bias.
- Age and sex can affect glucose tolerance and insulin sensitivity in mice [274]. To overcome this issue, all the experiments were performed in male 12-15 weeks of age mice in order to use mature individuals and avoid bias due to hormones' fluctuation [275, 276, 277].
- Duration of fasting is another factor that can influence glucose metabolism [278]. There is a rather small variation in glucose levels in mice during fasting, depending on the liver ability to slowly deplete its glycogen content to maintain normal glucose levels. Some researchers let the mice fasting overnight (14–18 hours) to obtain more stable fasting glucose levels and deplete glycogen levels [279, 280]. However, this procedure can induce a metabolic stress due to the prolonged duration of fasting and can be thus limited to specific settings, such as studying hepatic gluconeogenesis. To avoid the metabolic stress and due to ethical restriction in our animal facility, all experiments were performed with a 4h fasting time. This short period of fasting time might increase a variability in the glucose tolerance and insulin sensitivity but, despite this limitation, significant differences were observed between experimental and control groups both in paper I and II.
- Changes in the circadian rhythms have been shown to affect gut microbiota and glucose metabolism [281, 282]. Moreover, glucose levels fluctuate during the day according to time of sampling [282]. To

overcome this issue, all the experiments have been performed at the same time of the day with the same procedure.

Measurement of fasting glucose and insulin levels, as well as glucose (GTT) and insulin (ITT) tolerance test, are common methods used to assess glucose metabolism in mice [271, 278].GTT is the most widely used test for assessing glucose homeostasis in mice. It provides a general impression on the whole-body glucose metabolism without proving any specific mechanism underneath. Instead, ITT provides more information on specific tissues' ability to respond to the insulin stimuli. During the ITT, insulin is usually metabolized in less than 10 minutes. The glucose reduction after insulin administration is mainly due to liver and muscle metabolism. In the interpretation of the results of the GTT or ITT, several factors should be taken into account:

- The dose of glucose or insulin administered for GTT or ITT experiments is a source of variability. There is not a consensus among published paper on the amount of those compound to use in the metabolic studies; some studies used either 1 or 2 g/kg glucose or 0.5 or 0.75mU/kg insulin [278, 283]. As shown in figure 25 the concentration of glucose used for the IPGGT can influence mice glucose levels during the test. Thus, it is crucial to perform optimization tests for each strain of mice. In paper I and II, two different doses of glucose were used. This is due to the fact that two different strains of mice were used (i.e., C57Bl/6 and Swiss Websters). When using Swiss Webster mice, it was necessary to increase the amount of glucose and insulin for tolerance test, because it is known that Swiss Westers on chow diet are fatter and more glucose intolerant and insulin resistant than C57Bl/6.

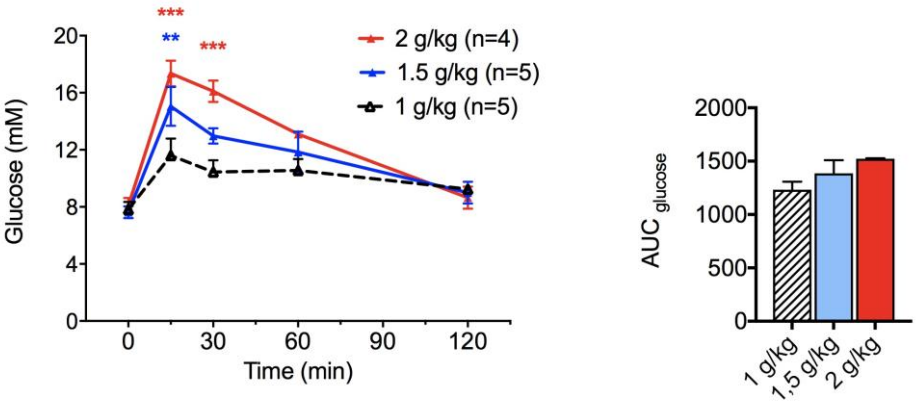


***Figure 25. Glucose levels and area under the curve (AUC) during intraperitoneal glucose tolerance test performed with different doses of glucose/kg in C57Bl/6****. Two-way ANOVA, **p<0.01; ***p<0.001. Unpublished data.*

- GTT and ITT provide a lot of high-quality data. However, the hyperinsulinemic–euglycemic clamp is the 'gold standard' for the study of whole-body insulin sensitivity *in vivo* [283, 284]. By removing (clamping) the endogenous glucose and insulin, it allows us to determine insulin sensitivity of the whole body. However, despite being the gold standard method, this technique has some disadvantages, such as needs of invasive surgery and high costs. In both paper I and II, the altered glucose metabolism was already observed and consistent during GTT or ITT thus, hyperinsulinemic–euglycemic clamp would not add more information. Moreover, in paper III, the focus was on the effect of ImP on liver glucose metabolism. By performing a hyperinsulinemic–euglycemic clamp, it is impossible to study liver insulin response, due to the clamping procedure that exclude hepatic glucose production during insulin stimulation. To overcome this issue, an *in vivo* insulin stimulation was performed by directly injecting insulin in the mouse blood stream and evaluating liver response to insulin in terms of phosphorylation of key enzyme involved in the insulin pathway.

## 5.3 *IN VITRO* MODELS OF METABOLIC DISEASES

*In vitro* studies are an important and widely used tool in the research on metabolic diseases [285, 286]. They allow hypothesis testing in a simpler and more controlled model. The most commonly used cell lines are immortalized cells obtained from cancers in humans or mice. For example, HepG2 liver cells were derived in 1983 from the liver cancer tissue of a 15-year-old Caucasian male. The advantage of using immortalized cells lines is that they are easy to proliferate, culture and treat with different compounds. However, the disadvantage is that, being able to survive potentially unlimited time, immortalized cell lines have acquired mutations in key genes involved in metabolic pathways, such as insulin signaling [287, 288]. Moreover, in many occasions, they lose key tissue specific characteristics, such molecules secretion or enzymatic activity [289]. Thus, using immortalized cell lines to study metabolic pathways might be misleading and researchers need to validate their findings in *in vivo* models or in primary cells [290].

To overcome the problem with immortalized cells, researchers can use primary cells. Primary cells are isolated directly from mouse/human tissues. These cells are more fragile and difficult to culture compared with cell lines. Primary cells need more nutrient-rich media compared with immortalized cell lines to survive, have a limited proliferation ability and cannot survive for more than few days/weeks. Moreover, to validate the results multiple isolations of primary cells from different subjects/animals are required, which increases time and costs to perform each experiment. However, primary cells preserve most of the characteristics of the original tissue, do not present mutations and are able to perform most of the tissue specific functions. Although primary cells are more difficult to handle, data obtained are more relevant and reflective of the *in vivo* environment.

For all these reasons, in paper II, both immortalized and primary cells lines were used. By doing so, data obtained from the immortalized cell lines were then confirmed using a more physiological system in primary cells. However, we proceeded even further and confirmed our hypothesis in an *in vivo* model using mice.

## 5.4 *IN VITRO* GUT SIMULATOR

In paper II, a simulated human intestinal redox model (SHRIM) was used [133](Figure 26). This method has recently been developed in our laboratory and helped us to explore the effect of a specific compound (in paper II, histidine) on a stabilized gut microbial community *in vitro*.

Briefly, SHRIM is a two-chamber fermenter with an anaerobic luminal chamber and an oxygen feeder. The luminal chamber is continuously stirred and kept at 37 °C to mimic intestinal motility. The oxygen feeder allows keeping a low pressure of oxygen in order to allow anaerobes bacteria to grow in the broth media. The growth media is composed of arabinogalactan, pectin, xylose, starch, glucose, yeast extract, peptone, mucin type II, and cysteine to allow the whole culturable bacteria community to grow and proliferate. Feces from human subjects were inoculated in the SHRIM growth medium and allow to stabilize during 1 week, before starting all the experiments. To add more complexity to the system and to mimic even more the physiology of digestive process *in vivo*, the growth media was acidified to around pH 2 with HCl buffer and subsequently neutralized with simulated pancreatic juice to a pH 6.9. The simulated pancreatic juice contained NaHCO3, ox gall bile salts and pancreatin.

SHIRM is a useful tool to study *in vitro* microbiota function and response to a specific stressor/compound (as in Paper II, histidine supplementation in presence or absence of a type 2 diabetes microbiota). It has several advantages as it allows to study the whole cultivable microbiota instead of few bacterial species, mimics several intestinal physiological factors such as motility, pH, oxygen pressure, pancreatic digestion, and controls availability of nutrients.

However, there are several factors that cannot be reproduced in this kind of *in vitro* gut model yet, such as intestinal epithelia motility and immune system interaction with bacteria. Findings with *in vitro* gut simulator should be validated, if possible, with *in vivo* models. Thus, for example in paper II, SHIRM findings were then integrated with some *in vivo* data.

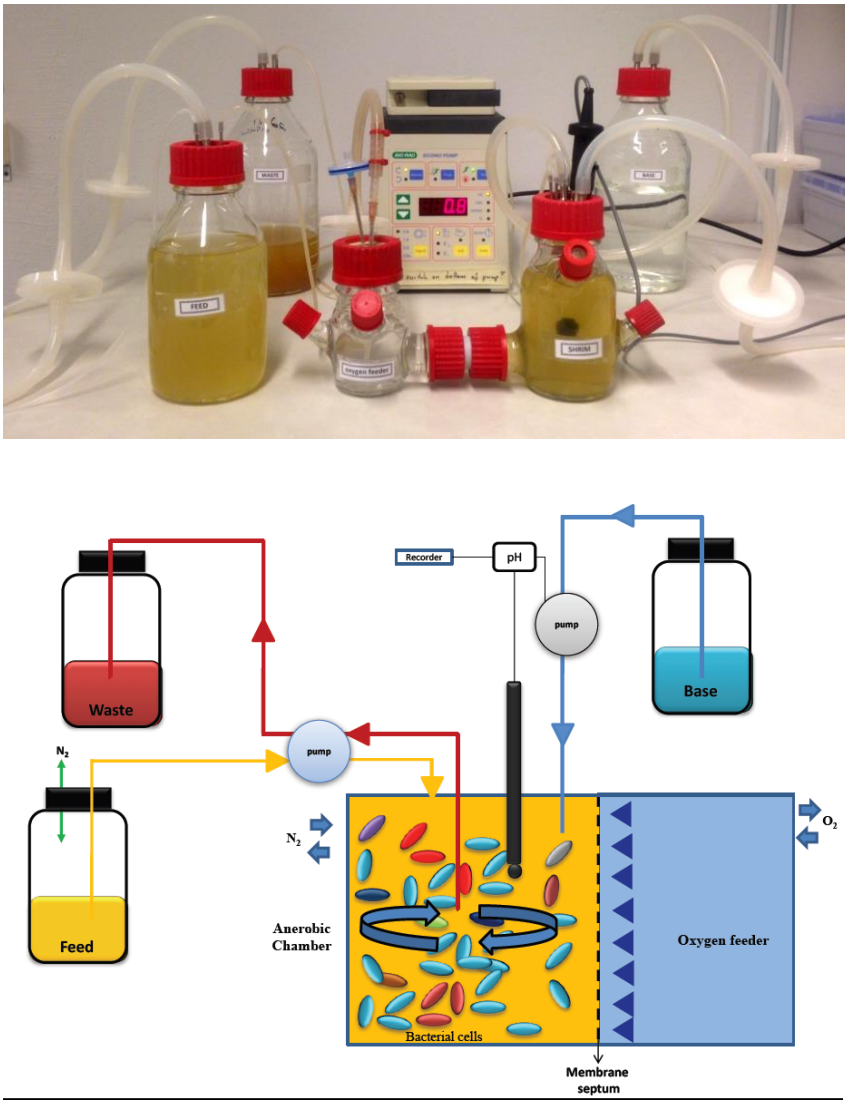


***Figure 26. Picture and schematic representation of a simulated human intestinal redox model (SHRIM).*** *Courtesy of Dr Mohamed Tanweer Kahn.*

# 5.5 METAGENOMIC STUDY OF MICROBIOTA

There are several methods to study the composition of the gut microbiota in humans, ranging from culture-based methods to culture-independent molecular techniques (Figure 27) [225].

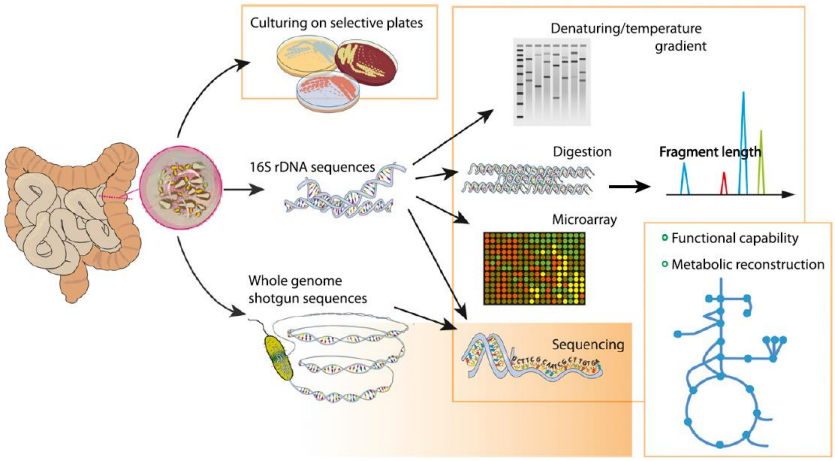


***Figure 27. The methods for the study of the gut microbiota include culturing on rich and selective growth media, as well as culture-independent techniques based on the characterization of the microbial 16S rRNA gene or the direct sequencing of the microbial genomes (also known as metagenomics).*** *The figure is taken from Karlsson et al Diabetes 62:3341–3349, 2013.*

Historically microbial communities have been characterized by culturing on selective growth media. However, the human gut microbiota is predominantly composed by strictly anaerobic bacteria that are difficult to culture and it is estimated that the culturable fraction of the human gut microbiota amounts to 20–50% of the species present in the communities [291, 292]. Therefore, culture-based methods provide a poor resolution of overall community composition and diversity, and nowadays these methods are limited to the study of pathogens and specific bacterial species.

Molecular methods have been developed to evaluate overall microbial composition and diversity and, in particular for Bacteria and Archaea, these

methods mostly concern the detection of the 16S ribosomal gene (16S rRNA gene), which contains conserved regions used to design conserved primers, as well as sequences with variability that are used to distinguish different taxonomic groups (also known as taxa). Common methods for the profiling of the 16S rRNA gene include temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), human intestinal tract chip (HITChip), and sequencing of the 16S rRNA gene [293]. TGGE, DGGE and T-RFLP allow a rapid analysis of gut microbiota composition but are semi-quantitative and normally do not provide taxonomic information. The HITChip instead, gives the possibility to detect 1,140 unique rRNA sequences with taxonomic information at species level, is quantitative, but can provide information only on known species [293].

Sequencing of the 16S rRNA gene has become more and more common due to the decrease in sequencing costs and to the possibility to obtain taxonomic information and relative high throughput [294, 295, 296]. In the bioinformatic analysis, raw sequences are clustered into Operational Taxonomic Units (OTUs) often using a cutoff of 97% identity, roughly corresponding to species-level, and the obtained OTUs can either be determined based on a reference databased or de novo, which allows the detection also of possible unknown taxa [297, 298, 299, 300]. However, this method, like the other 16S rRNA gene profiling methods mentioned above, is based on PCR amplification of the 16S rRNA gene and therefore suffers from bias in amplification efficiency for sequences belonging to different taxonomic groups [301].

16S rRNA gene analyses reveal which microbes are present in a community but do not provide information on bacterial functional potential. PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) is a bioinformatics tool that predicts which bacterial genes are present in a community based on the profiles of 16S rRNA gene relative abundances [302]. It is a useful and largely used tool; however, data on bacterial functions are only inferred and thus need to be validated with other methods, such as microbial profiling of the whole community DNA, *i.e.*, metagenomics analysis based on whole-genome shotgun (WGS) sequencing. WGS sequencing is a molecular technique that helps us to determines which microbes are present in a community as well as what they

are capable of doing (i.e., potential functional content), as it sequences the whole genomic content of a sample (i.e., metagenome) [303, 304, 305]. Metagenomics analysis is a useful tool but it has some disadvantages such as costs that heavily affected by sequencing depth and it cannot provide any information on real-time functional activities of the bacteria such as metatranscriptomics technique. Nevertheless, metagenomics analysis presently is the best available method for gut microbiome studies. However, technical challenges on samples handling should be taken into account when comes to perform WGS in humans such as long time between delivery of the samples and freezing, temperature of storage and methods used for bacterial genome extraction and amplification [293].

In paper III a metagenomics approach was used to evaluate microbiota compositional and functional shifts in the presence of NAFLD. However, data from paper III on microbiota composition/function in presence of steatosis findings are purely descriptive. Associational data on composition or function of bacteria with a specific disease (such as diabetes and NAFLD) are useful to discover a specific microbiota signature in disease condition [89]. Thus, we might end up in "the chicken or the egg dilemma" in the microbiota field: has the altered gut microbiota a causative role in metabolic diseases, such as type 2 diabetes and NAFLD, or is it just a consequence of the disease itself? In the future, data from longitudinal studies on populations at risk for metabolic diseases will help to elucidate the effect/function of a specific microbiota shift on a certain disease.

## 5.6 LARGE VS SMALL COHORT DATASET AND MICROBIOTA ANALYSIS

One of the big challenges for observational studies is the limited reproducibility of associative findings, especially when using small sample sizes. For example, for the search of novel common variant in genetics huge datasets are often enrolled [306]. The samples size is an important issue also in microbiota research, especially due to the high inter-individual variability of microbiota community composition [256, 257].

Data from the MetaHIT consortium, investigating metagenomes from 124 healthy European individuals, showed that nine microbial genera were share in more than 90% of the individuals [307]. Moreover, data from longitudinal studies on healthy individuals sampled several times have showed conserved microbiota composition of an individual over time [32, 67, 256, 308]. These findings might indicate that even studies with small sample size can be useful to detect microbiota shifts when considering conserved taxa and potential functions. However, in studying microbiota, this is not applicable to each human pathophysiological condition of interest. As an extreme example, microbiota shift after bariatric surgery is so dramatic that even with a small sample size it is possible to detect significant taxa and potential bacterial function alterations [266]. On the other hand, combining the finding of several studies comparing microbiota composition in obese vs lean subjects (10 different studies, 2786 subject in total, 31.8% obese) showed that the microbiota shift in obesity was relatively weak and affected by large interpersonal variation and insufficient sample sizes. Thus, it has been estimated that in obesity, to be able to detect a 5% microbiota variation (on a phylum level) determined by Shannon diversity (a statistical test that measure the microbiota community diversity) or in Bacteroidetes/Firmicutes ratio, you might need a samples size of 140 or 6,300 samples, respectively [107]. However, detailed phenotyping and exclusion of possible bias (consumption of drugs affecting microbiota composition, heterogeneous population in terms of confounding factors such as BMI, age, gender, and diet) affect sample size and reduce the number of subjects potentially enrolled in the study, but on the other hand they can strengthen the findings. In paper III we used a small discovery cohort of subjects with well-defined simple steatosis because we tried to maximize phenotyping and thus, we removed as many as possible known factors that might affect microbiota composition and function (use of antibiotics, metformin, proton pump inhibitors) [132, 133, 243, 309, 310]. The use of a bigger replication cohort, in our case, helped us to confirm and validate our findings and strengthened our results.

One limitation of studying big human cohorts is the type of sample to collect. Some types of samples are accessible only during special procedures such as abdominal surgery, as was done in my case for portal vein blood sampling, which is time consuming and risky. Thus, in paper II we used a small

discovery cohort of only 15 subjects with portal vein samples obtained during bariatric surgery where we were able to identify ImP as a microbial metabolite that was increased in subjects with diabetes. Despite a possible sample size bias, data from *in vitro* and *in vivo* experiments on ImP treatment confirmed our hypothesis that imidazole propionate is a microbial molecule that affects glucose metabolism. To confirm these finding we used a large confirmation cohort (649 subjects with/out diabetes) where we were not able to obtain portal vein blood; however, in peripheral blood we were able to confirm that imidazole propionate is increased in presence of diabetes.

# 6 ETHICAL CONSIDERATION

All animal and human experiments were performed under the local ethical committee approval. All the animal experiments have been planned and performed using the principle of the 3R, reduce, replace and redefine.

# 7 CONCLUSIONS

Taken together, in this thesis I showed how microbiota as an important environmental factor is able to modulate the pathophysiology of metabolic diseases, in particular type 2 diabetes and NAFLD.

Specifically, I was able to show that:

- Microbiota is an environmental factor that affects host glucose metabolism and fat storage in the liver.
- Host-microbiota interaction during colonization induces a bi-phasic inflammation and glucose intolerance in mice. The two phases of glucose impairment are depending on two different host-microbiota interaction mechanisms.

- Imidazole propionate is a microbially produced histidine-derived metabolite that is specifically produced by microbiota of subjects with type 2 diabetes.
- Imidazole propionate has a causality role in type 2 diabetes (impairs glucose tolerance by affecting insulin signaling at the level of IRS/ p38γ MAPK/ p62 /mTORC1).

- Steatosis is characterized by a shift in microbiota composition by increased abundance of bacteria from the oral cavity, which have been linked to periodontal disease, and with ethanol-producing bacteria.
- Steatosis is enriched in bacteria functions related to metabolic functions as well as production of LPS.

Overall, this thesis' findings show that the gut microbiota has to be considered as a potential target for the treatment of metabolic diseases in humans.

# 8 FUTURE PERSPECTIVES

In this thesis project, I have explored the role of microbiota in metabolic diseases with a special focus on glucose metabolism and liver fat accumulation. Although findings help to understand a bit more the complex host-microbiota interaction in metabolic diseases, there is still a lot to discover and clarify.

In paper I, I explored how the colonization of GF mice affects glucose metabolism and adiposity in the host, on a time course, defining critical time points to study metabolic pathways and microbiota in mice. I showed that the *early* phase of colonization is associated with an uncontrolled immunological reaction of an "un-trained, naive" immune system to microbiota antigens that leads to an impairment of glucose metabolism. To assess the role of a "trained" immune system in this process we would need to produce bone marrow chimeras in GF mice being reconstituted with bone marrow harvested from CONV-R mice. This could provide more information on the role of the immune system-microbiota interaction in metabolic diseases possibly. Several researchers use GF mice colonization (CONV-D mice) as a tool to study how microbiota affect host physiology by comparing analysis of feces in healthy subjects and those with diseases of interest. However, not all human bacterial microbiota species are present in the mouse gut and vice-versa. Thus, it is not always easy to translate murine data into human physiology. The next step is to perform similar experiments as in paper I using human feces or selected strains to assess the potential of human microbiota.

In paper II, I identified a microbially produced histidine metabolite, imidazole propionate (ImP),that can induce insulin resistance. In the light of searching for new potential treatments for insulin resistance in humans, ImP production and metabolism could be a new interesting target. Different approaches might be used to perform the microbiota-oriented therapy:

- In an untargeted way by replacing the whole diabetogenic microbiota able to produce ImP with fecal microbiota transplantation using a microbiota that is not able to produce ImP; from a healthy donor.

- In a targeted way, by developing anti-pre- or pro-biotics able to target specific bacterial strain(s) able to produce ImP. Potentially, we could develop chemical inhibitors for bacterial ImP production as new potential therapy for insulin resistance.
- Stratifying subjects with pre- or type 2 diabetes according to their serum levels of ImP could be a useful strategy for diet interventions aimed to reduce the intestinal availability of histidine (the bacterial substrate for bacteria to produce ImP).

In paper III, I explored the role of microbiota on steatosis in humans. I was able to identify several bacteria associated with steatosis. However, in this project I did not perform any translational study. I think this missing part, which is potentially very interesting, needs to be expanded in future studies to identify causality and also mechanisms. By performing colonization of GF mice with feces from healthy subjects, with or without steatosis, I can potentially study the effect of microbiota on liver fat accumulation in mice by using a human microbiota before translating this into human studies. These future studies may potentially help to identify the microbial regulated metabolic pathways and molecules responsible of excessive fat accumulation the liver.

In paper III, I used two rather small but well-phenotyped cohorts of subjects with steatosis. Thus, my findings in the future need to be confirmed in a bigger independent cohort. One of the major issues in NAFLD research is the effect of potential bias, such as body weight on the features of NAFLD. Obesity/overweight is usually associated with NAFLD but a small portion of subjects with normal body weight develops NAFLD as well. Thus, a really interesting point will be to evaluate in subjects with NAFLD and with different body weights (normal weight vs. obese/overweight) microbiota composition and function to identify a common microbial NAFLD signature that is independent on other confounding factors such as obesity.

# 9 ACKNOWLEDGEMENT

**If you are among the people that jumped from the first page to this one shame on you! Go back and start again from page 1. It is an interesting thesis! If you instead read the entire thesis, you deserve to read this.**

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