Metabolic disorders: role of pregnane X receptor, fibroblast growth factor 19 and persistent organic pollutants

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Abstract

The incidence of obesity and correlated metabolic abnormalities has increased dramatically in the last decades and constitutes a huge burden on healthcare systems and economies. It is therefore important to increase our understanding of the mechanisms that underlie obesity and to develop safe and effective treatments for the management of these disorders.

Paper I focus on the role of the pregnane X receptor (PXR), which is encoded by the *Nr1i2* gene, in the regulation of whole-body metabolism. We carried out an extensive metabolic phenotyping of these mice to improve our understanding of the anti-obesity and anti-diabetic effects of PXR ablation. Our findings suggest that deletion of PXR improves metabolism through enhancement of skeletal muscle mass, which is associated with increased plasma levels of fibroblast growth factor (FGF) 15. We suggest that this hormone may be responsible for the stimulation of skeletal muscle growth in *Nr1i2*$^{-/-}$ mice. Larger skeletal muscles of PXR knockout mice suggest that pharmacological interventions to increase muscle size may be exploited for the management of metabolic disorders.

Paper II investigates the ability of FGF19, the human orthologue of FGF15, to promote skeletal muscle growth, given the importance of this organ in general metabolism and health. We demonstrated that human recombinant FGF19 promotes skeletal muscle growth by increasing the size of skeletal muscle fibers, but without affecting their number and type. In addition, we characterized the signaling pathway required for FGF19 hypertrophic activity. Importantly, we showed that treatment with FGF19 protects from skeletal muscle loss in animal models of muscle atrophy. Our results suggest potential therapeutic application of FGF19 to stimulate skeletal muscle growth and to protect from muscle-atrophy-related conditions.

Paper III examines the impact of a dietary mixture of persistent organic pollutants (POPs) at environmentally relevant levels on intestinal inflammation and immune system in the context of obesity. We revealed that, during obesity, exposure to a mixture of POPs stimulated further adipose tissue accumulation and affected the

immune system. In addition, POPs promoted intestinal inflammation and influenced faecal production. In obese mice with established intestinal inflammation (induced by treatment with dextran sodium sulphate), consumption of dietary POPs further aggravated it. These data suggest that dietary exposure to a mixture of POPs can impact health and metabolic outcome in obesity.

List of Publications

Paper I:

Bérengère Benoit, Martina Castelli, Emmanuelle Meugnier, Stéphanie Chanon, Etienne Lefai, Hubert Vidal, Jérôme Ruzzin (2019): "*Nr1i2*$^{-/-}$ mice are associated with increased circulating levels of fibroblast growth factor 15 and skeletal muscle hypertrophy". Manuscript.

Paper II:

Bérengère Benoit, Emmanuelle Meugnier, Martina Castelli, Stéphanie Chanon, Aurélie Vieille-Marchiset, Christine Durand, Nadia Bendridi, Sandra Pesenti, Pierre-Axel Monternier, Anne-Cécile Durieux, Damien Freyssenet, Jennifer Rieusset, Etienne Lefai, Hubert Vidal, Jérôme Ruzzin (2017): "Fibroblast growth factor 19 regulates skeletal muscle mass and ameliorates muscle wasting in mice". Nature Medicine, 2017. 23: p. 990. The first three authors share first authorship.

Paper III:

Martina Castelli, June Gudmestad, Florian Dingreville, Bérengère Benoit, Hubert Vidal, Jérôme Ruzzin (2019): "Mixture of persistent organic pollutants promotes accumulation of adipose tissue, affects immune system and induces inflammation-related changes in the intestine of C57BL/6 mice". Manuscript.

Abbreviations

AKT	Protein kinase B
BA	Bile acid
BMI	Body mass index
CVD	Cardiovascular disease
CYP	Cytochrome P450
DC	Dendritic cell
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DSS	Dextran sodium sulphate
EE	Energy expenditure
ERK	Extracellular-signal-regulated protein kinase 1/2
FA	Fatty acid
FGF	Fibroblast growth factor
FGFR	FGF receptor
FXR	Farnesoid X receptor
HCC	Hepatocellular carcinoma
HF	High-fat
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
LP	Lamina propria
LPS	Lipopolysaccharide
MAO	Metabolically abnormal obese
MCA	Muricholic acid
MHO	Metabolically healthy but obese
MLN	Mesenteric lymph node
mTOR	Mammalian target of rapamycin
NR	Nuclear receptor
OCP	Organochlorine pesticide
PCB	Polychlorinated biphenyl
POP	Persistent organic pollutant
PXR	Pregnane X receptor (a.k.a. *Nr1i2*)
REE	Resting energy expenditure
S6K1	S6 kinase1
SXR	Steroid and xenobiotic receptor (a.k.a. *NR1I2*)
T2D	Type 2 diabetes
Th	T helper
TLR	Toll-like receptor
Tregs	Regulatory T cells
VAT	Visceral adipose tissue
WAT	White adipose tissue

Contents

1 Introduction

1.1 Obesity

Overweight and obesity are defined as excessive fat accumulation that may impair health (World Health Organization 2018) and are classified according to the body mass index (BMI), which is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2). In adults, overweight is defined by a BMI equal or greater than 25 kg/m^2 and obesity is defined by a BMI equal or greater than 30 kg/m^2 (World Health Organization 2018). BMI provides a direct measure of excessive body weight. However, it may not reflect the actual degree of fat accumulation, because it does not allow to assess the contribution of bone density and muscle mass to the total body weight (Rothman 2008).

1.1.1 Prevalence of obesity

Although obesity was initially regarded as a problem of developed countries, it is now becoming more and more prevalent also in developing counties. According to the World Health Organization assessments, more than 1.9 billion adults older than 18 years (39% of the world's adult population) were overweight in 2016 and of these, over 650 million were obese (13% of the world's adult population). The worldwide prevalence of obesity nearly tripled between 1975 and 2016 (concerning men, women, adolescents and children) and the phenomenon has reached such alarming proportions that overweight and obesity were linked to more deaths than underweight (World Health Organization 2018). The global high incidence of these conditions has led to the concept of obesity epidemic (VanItallie 1994). Obesity and associated dysfunctions are estimated to have a considerable economic impact on health care systems globally (Chu et al. 2018). For example, in the USA the combined cost of overweight and obesity was estimated to be $113.9 billion in 2008 and was predicted to increase by an additional $48-66 billion by 2030 (Chu et al. 2018).

1.1.2 A multifactorial disease

While some forms of obesity are due to rare mutations in specific genes, multiple causes for weight gain have been established in the most common forms of obesity. Complex gene-gene and gene-environment interactions can influence the individual's susceptibility to common obesity (Bellisari 2008), and several obesity-associated genes (Rankinen et al. 2006; Bellisari 2008) have been identified. However, the rapid increase in the global occurrence of this disease has been too rapid to be explained by genetic changes (Wells 2006). Lifestyle factors, such as sedentariness and excessive energy intake, have been traditionally considered as primary factors (Hill 2006). Nonetheless, energy intake was described to have decreased during the last ten years (Ford and Dietz 2013), while our daily energy expenditure (EE) remained substantially unchanged compared to our hunter-gatherer ancestors (Pontzer et al. 2012). More recently, the role of environmental contaminants in the development of obesity has been investigated, and is now supported by a large body of scientific evidence (Casals-Casas and Desvergne 2011; Gore et al. 2015; Heindel et al. 2017).

1.1.3 Obesity-associated dysfunctions

Obesity is considered a risk factor for the development of metabolic complications such as insulin resistance, hypertension and dyslipidaemia, which may in turn result in an increased risk of cardiovascular disease (CVD) and type 2 diabetes (T2D) (Ferrannini 1998; Yip et al. 1998; Thomas et al. 2005). Insulin resistance occurs when insulin is not able to stimulate glucose disposal, and degenerates into T2D when an insulin-resistant individual is unable to secrete a sufficient amount of insulin to overcome this defect (Brown and Walker 2016). The obese state is often characterized by additional alterations, such as non-alcoholic fatty liver disease (Milić et al. 2014) and chronic low-grade inflammation (Hotamisligil 2017b).

The prevalence of these metabolic disturbances varies considerably among obese individuals, and this has led to the distinction of two different phenotypes: the metabolically healthy but obese (MHO) and the metabolically abnormal obese (MAO) (Messier et al. 2010). Despite having excessive accumulation of fat, MHO seem to be

protected from the development of metabolic complications, and show normal levels of insulin sensitivity and lipids, no hypertension and normal inflammation and immune profiles compared to MAO individuals (Brochu et al. 2001; Karelis et al. 2005; Stefan et al. 2008; Lynch et al. 2009). The risk of T2D, CVD and mortality among MHO individuals was found to be similar to that of healthy, normal-weight subjects, although not all studies agree (Karelis 2011). It has been estimated that MHO individuals may represent up to 30–40% of the obese population (Messier et al. 2010).

1.2 Inflammation in obesity

It is now widely accepted that inflammation is a key feature of obesity and T2D (Hotamisligil 2006). The field of immunometabolism, which studies interactions between immune system and metabolism, has grown rapidly in recent decades and has uncovered molecular mediators, signaling pathways and cellular players involved in the chronic state of low-grade inflammation associated with obesity and insulin resistance (Hotamisligil 2017a). This condition is due to a progressive shift from anti- to pro-inflammatory immune cells and cytokines (signaling molecules) associated with adipose tissue, liver, muscle, pancreas, central nervous system and intestine (Lee et al. 2018b). This highly pro-inflammatory environment has been directly linked to insulin resistance through cytokine-mediated activation of inflammatory signaling pathways (i.e. c-Jun N-Terminal kinase and IκB kinase-nuclear factor kappa B), which results in inhibition of insulin signaling and further stimulation of the pro-inflammatory response (Barnes and Karin 1997; Aguirre et al. 2002; Gao et al. 2002).

Inflammation in white adipose tissue (WAT), specifically in visceral adipose tissue (VAT), has been extensively studied since it is thought to play a major role in insulin resistance (Winer et al. 2016). Inflammation in WAT is an early event in obesity and increases gradually, in a process that involves recruitment and accumulation of several pro-inflammatory immune cells, such as M1 macrophages, cytotoxic CD8+ T cells, T helper (Th)1 cells, B cells, natural killer cells and neutrophils (Schipper et al. 2012). In parallel, the abundance of anti-inflammatory cells such as M2 macrophages, regulatory T cells (Tregs) and eosinophils is diminished (Schipper et al. 2012).

1.2.1 Intestinal inflammation in obesity

Recently, immunologic changes in the intestine have emerged as important contributors to obesity and insulin resistance. The intestine represents the largest compartment of the immune system in the body and it is exposed to a multitude of antigens and immune stimuli through the diet (Vighi et al. 2008). Intestinal immune cells constantly distinguish beneficial nutrients and commensal bacteria from harmful pathogens, and the maintenance of immunologic defence and intestinal homeostasis engages around 70% of the body's immune cells (Vighi et al. 2008).

Most immunological processes in the gut occur in the mucosa, which consists of the epithelium and the underlying lamina propria (LP) and muscularis mucosa (Fig. 1.1) (Mowat and Agace 2014). The composition of immune cells and their function varies considerably along the length of the intestine. This regional difference is due to segment-specific variations of environmental factors, such as availability of retinoic acid, flavonoids, short chain fatty acids, and microbiota composition (Mowat and Agace 2014). Importantly, segment specific expression of chemokines (signaling molecules that recruit responsive cells) promotes establishment of different immune cells throughout the intestine (Mowat and Agace 2014). In general, in the lean state, macrophages and dendritic cells (DCs) with anti-inflammatory properties and Tregs, which suppress inflammation, predominate (Winer et al. 2016). These cells maintain homeostasis and oral tolerance, which is the ability of the intestinal immune system to avoid unnecessary inflammatory responses against food antigens and commensal bacteria (Winer et al. 2016).

In addition to immune cells, specific epithelial cell types play a role in the maintenance of intestinal homeostasis. Among these, goblet cells are mucus-producing cells that are found in the entire intestine but are particularly abundant in the colon (Johansson et al. 2008; Johansson et al. 2011). They are responsible for the production of a mucus layer that protects the epithelium from intestinal bacteria, which normally are not able to penetrate it. In the colon, where the mucus layer is thickest, this physical barrier is composed of an inner dense layer attached to the epithelium and an outer, loose layer that is similar to the one found in the small intestine (Fig. 1.1). The integrity of the

mucus layer is important in maintaining the intestinal barrier function (Johansson et al. 2011).

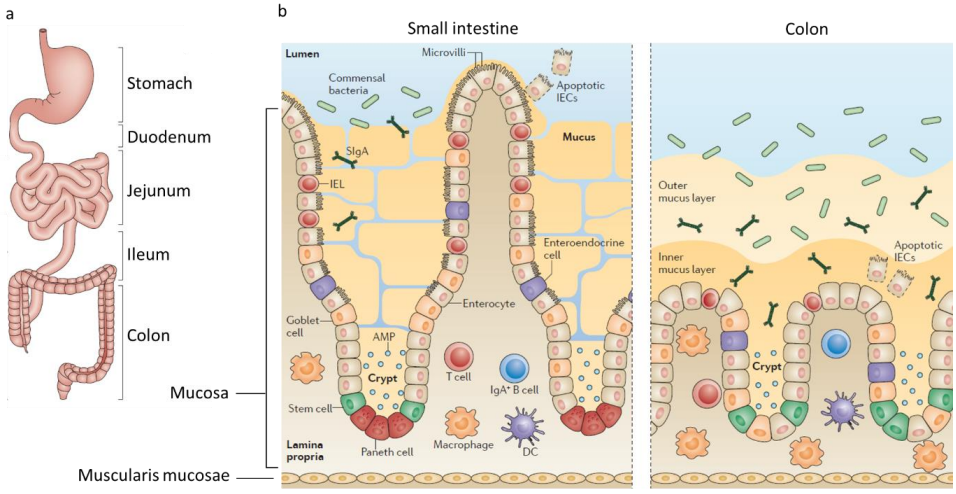

Figure 1.1: Segment-specific features of the intestine. *(a) The intestine is composed of different segments. (b) Duodenum, jejunum and ileum constitute the small intestine, that is primarily involved in nutrient absorption and shows long villi to maximise the absorptive area. The main function of the colon or large intestine is to reabsorb water and to act as a barrier for the microbiota. For this reason, the villi are short, and the epithelium is protected by two layers of mucus. Immune cells are present in the epithelium (intraepithelial lymphocytes) and in the underlying LP. Adapted from (Mowat and Agace 2014).*

Obesity is associated with changes in the abundance and type of intestinal immune cells, alterations of gut microbiota, impaired barrier function and consequent increased intestinal permeability, all of which contribute to intestinal and systemic inflammation (Winer et al. 2016).

1.2.1.1 Alterations of intestinal immune cells

It is well established that obesity affects the composition of adaptive immune cells in the LP of small and large intestine. The key changes associated with obesity in both human and mice are a decrease in anti-inflammatory Tregs, and an increase in pro-inflammatory Th1 cells and CD8+ T cells, which produce interferon-γ, a potent pro-inflammatory cytokine (Luck et al. 2015). Although with some inconsistencies between human and mice, and between small and large intestine, alteration of

additional immune cells was described. For example, pro-inflammatory γδ T cells were increased, whereas anti-inflammatory Th17 cells were reduced (Garidou et al. 2015; Luck et al. 2015). The role of mucosal innate cells such as macrophages and DCs in obesity is not well clarified. Although an increase in total macrophages and DCs was described in the small intestine of obese humans, no differences were reported between lean and obese mice (Winer et al. 2016). Overall, these inflammatory changes in immune cells contribute to decreased barrier function and worsening of obesity-related insulin resistance (Winer et al. 2016).

1.2.1.2 Microbiota

The gut microbiota contains tens of trillions of microorganisms that play an important role for general health. Increasing evidence described a role of intestinal bacteria in the development of obesity and glucose intolerance, as studies have demonstrated that germ-free bacteria are protected from high-fat (HF) diet-induced obesity (Bäckhed et al. 2004; Bäckhed et al. 2007). Moreover, obesity is associated with altered gut microbiota, specifically with an increase in the ratio of *Firmicutes* versus *Bacteroidetes* and in a general loss of bacterial richness compared to the lean state (Fig. 1.2) (Ley et al. 2005; Turnbaugh et al. 2006). One of the proposed mechanisms involves bacterial promotion of lipoprotein lipase activity in intestinal cells, which leads to increased triglyceride storage in WAT and liver (Bäckhed et al. 2004; Bäckhed et al. 2007). In addition, bacterial control of the metabolism of bile acids (BAs) could affect whole body metabolism resulting in enhanced weight gain, inflammation and hepatic glucose production (Thomas et al. 2009; Swann et al. 2011). Finally, it was suggested that bacteria may be more efficient in harvesting energy from the diet (Turnbaugh et al. 2006). Bacterial products such as the endotoxin lipopolysaccharide (LPS) have a potent local and systemic inflammatory effect, and different aspects of metabolic disorders, including intestinal inflammation, depend on the presence of microbiota (Winer et al. 2016).

1.2.1.3 Impaired barrier function

When the intestinal barrier function is compromised in the obese state, bacteria and bacterial metabolites such as LPS can easily cross the intestinal barrier (Fig. 1.2) (Cani et al. 2007; Cani et al. 2008; Amar et al. 2011), stimulating the innate immune system to induce inflammatory responses and ultimately leading to systemic inflammation (Cani et al. 2007). Pro-inflammatory changes in intestinal homeostasis have been proposed to be early, initiator events in obesity and insulin resistance (Ding and Lund 2011; Winer et al. 2016), as LPS infusion in mice recapitulates the same metabolic dysfunctions observed with HF feeding, including fat accumulation (Cani et al. 2007). In obesity, the increase in permeability is due to reduced expression of epithelial tight junction proteins, which is promoted by a pro-inflammatory environment (Fig. 1.2). For example, bacterial LPS binds to toll-like receptor (TLR)-4 present on intestinal epithelial cells (IECs) and innate immune cells and stimulates the production of pro-inflammatory cytokines, including tumor necrosis factor-α and interferon-γ (Winer et al. 2016). These cytokines can directly reduce the expression of tight junction proteins such as zona occludens-1 (Luck et al. 2015). The increase in intestinal permeability in obesity seems to require the presence of microbiota, since treatment of HF-fed mice with antibiotics prevents it (Cani et al. 2008). This hypothesis is supported by the observation that the intestinal segments characterized by the highest level of inflammation (i.e. jejunum, ileum and proximal colon) are also the portions with the highest levels of commensal bacteria (Winer et al. 2016).

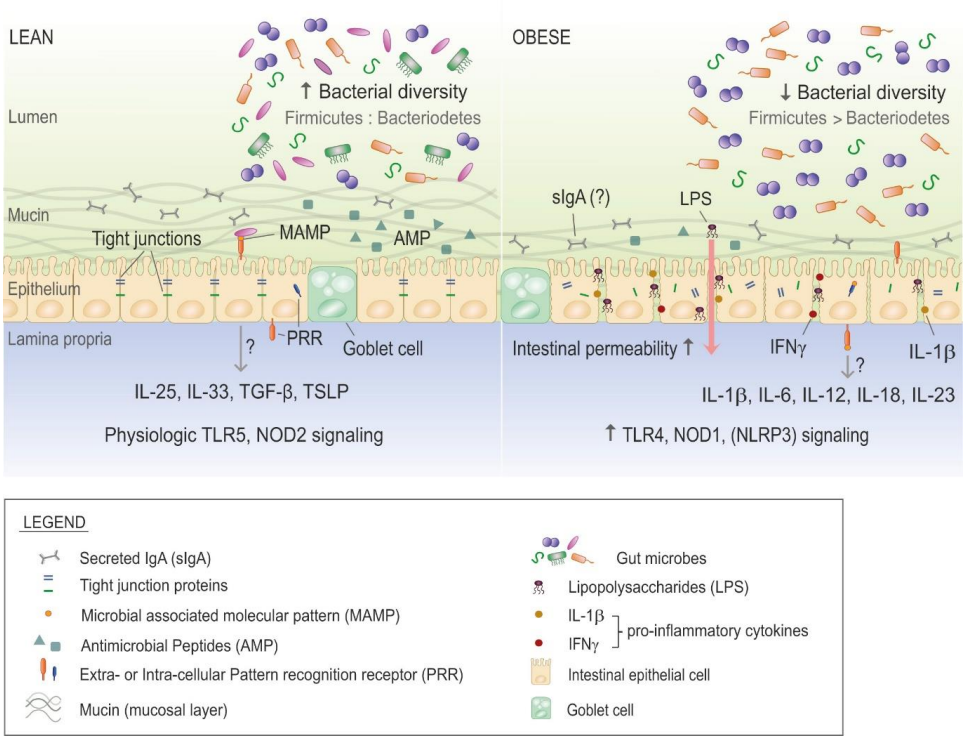

Figure 1.2: Changes in intestinal microbiota and intestinal permeability during obesity. *In lean conditions, the gut microbiota is highly diversified. IECs produce anti-inflammatory mediators and contribute to oral tolerance to commensal bacteria. Production of mucin and tight junction proteins ensures functionality of the intestinal barrier, avoiding bacterial translocation. During obesity, consumption of a HF diet decreases the diversity of the gut flora and alters the balance between bacterial species. The resulting bacteria and bacterial products stimulate TLR-4 signaling and release of inflammatory cytokines. HF-diet also results in reduced mucin production and expression of tight junction proteins, promoting penetration of bacteria past the gut barrier. In these conditions, leakage of bacterial products such as LPS into the circulation occurs, causing systemic inflammation (Winer et al. 2016).*

1.2.1.4 Oral tolerance in obesity

Mesenteric lymph nodes (MLNs) are secondary lymphoid organs embedded in VAT (Pond and Mattacks 1998) and drain lymph from the intestine (Cifarelli and Eichmann 2018). The migration of DCs from intestinal LP to MLNs is essential for the maintenance of oral tolerance (Maloy and Powrie 2011). Oral antigens are loaded onto intestinal DCs that migrate to the MLNs (Pabst and Mowat 2012), where they induce Tregs (Sun et al. 2007). These cells return to the intestinal LP, where they promote tolerance and reduce inflammation (Hadis et al. 2011). This process induces also

systemic immune suppression, possibly through the ability of Tregs to leave the bowel through the lymphatic system and then seed into other nodes (Pabst and Mowat 2012).

Decrease in the size of inguinal lymph nodes, impaired lymphatic transport and diminished intestinal DCs migration to lymph nodes was described in obese mice (Kim et al. 2008; Weitman et al. 2013), together with defective oral tolerance (Mito et al. 2006). Moreover, administration of dietary antigens to mice with lowered immunological tolerance affected CD4+ T cells in VAT and promoted glucose intolerance (Wang et al. 2010). Therefore, it is possible that diminished oral tolerance in obesity, together with alterations in gut immunity, contributes to low-grade systemic inflammation and metabolic dysfunction.

An integrated view of the intestine-related inflammatory changes that are thought to occur in the onset of obesity is presented in Fig. 1.3.

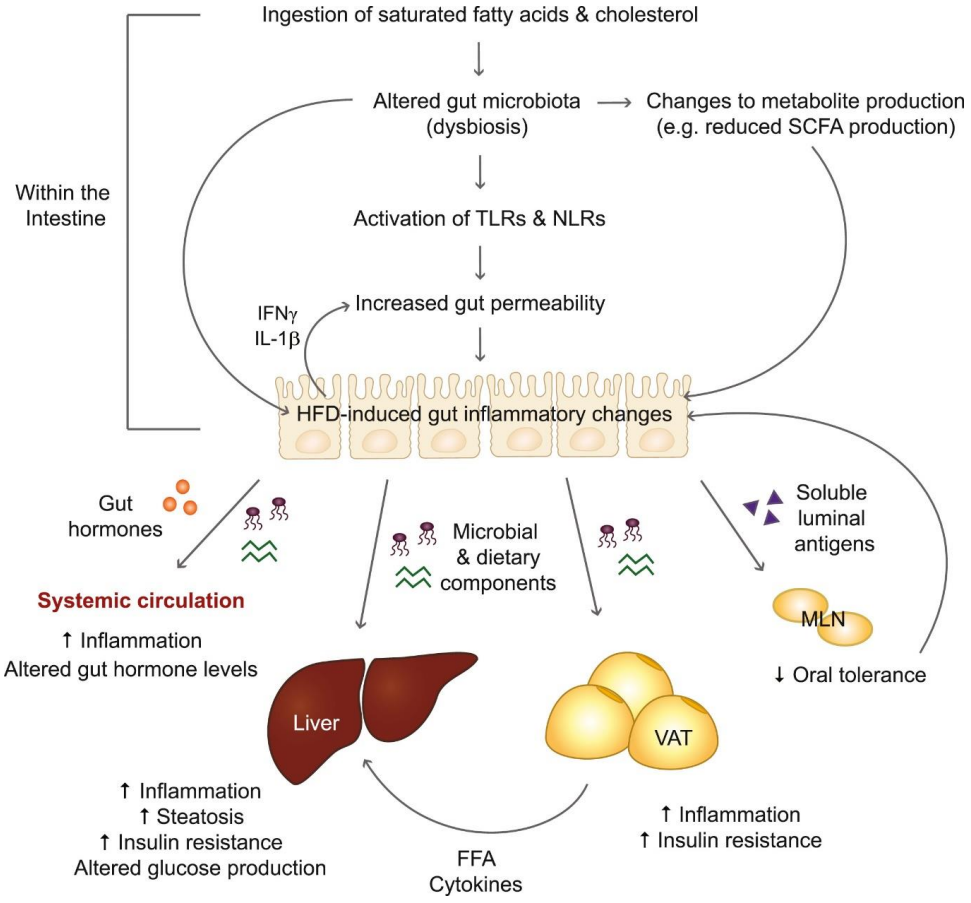

Figure 1.3: Diet-induced inflammatory changes leading to obesity. *The ingestion of a fatty acid (FA)- and cholesterol-rich diet induces gut dysbiosis, in which bacteria with anti-inflammatory properties are reduced. The altered microbial community stimulates the innate immune system through TLRs signaling in IECs and immune cells, which secrete pro-inflammatory molecules. These cytokines directly reduce the intestinal barrier function, causing leakage of intestinal antigens. This results in systemic inflammation, metabolic alterations in liver and adipose tissue, and diminished oral tolerogenic responses in the MLNs, which further fuels inflammation. Adapted from (Winer et al. 2016).*

1.2.2 Dextran sodium sulphate-induced colitis as a model of intestinal inflammation

Several mouse models have been developed for the study of intestinal inflammation in the context of inflammatory bowel disease (IBD) (Wirtz and Neurath 2000; Kiesler et al. 2015), which is a chronic inflammatory disorder of the intestine (Xavier and Podolsky 2007). IBD is caused by a combination of abnormal immune response in the

intestinal mucosa and diminished barrier function of the epithelium in individuals with a genetic predisposition (Xavier and Podolsky 2007). The clinical features of IBD in humans are highly diverse, and there is currently no model that encompasses all the aspects of the disease. Instead, each model more closely represents specific IBD-like intestinal features.

One of the most commonly used models of intestinal inflammation is the dextran sodium sulphate (DSS) model, in which water soluble DSS is administered to mice with drinking water to induce colitis (Wirtz et al. 2007; Chassaing et al. 2014). DSS is a sulphated polysaccharide with variable molecular weight, with the 40 to 50 kDa DSS being the most effective in causing colitis (Wirtz et al. 2007; Chassaing et al. 2014). DSS is directly toxic to the IECs and interferes with the intestinal barrier function resulting in increased permeability (Chassaing et al. 2014). The action of DSS is restricted to the colon, in particular to its distal portion, which contains high numbers of bacteria (Chassaing et al. 2014). Common aspects of the colitis are: weight loss, diarrhoea, rectal bleeding, intestinal ulcerations and infiltrations of granulocytes, shortening of the colon and splenomegaly (abnormal enlargement of the spleen) (Wirtz et al. 2007; Chassaing et al. 2014).

This model is very common in IBD research because it is rapid, simple and reproducible. In addition, it gives the advantage of recreating acute, chronic and relapsing models of inflammation simply by modifying the DSS concentration and time of administration. This model is particularly useful when studying the contribution of the innate immune system to IBD, since T and B cells are not a prerequisite for the development of the DSS-induced colitis, unlike in human IBD (Chassaing et al. 2014). Conversely, proper interaction between microbiota and associated ligands with innate immune system is a key element that determines the development and severity of colitis (Round and Mazmanian 2009).

1.2.3 Immune function in obesity

Recent findings suggest that obesity may adversely impact immunity and pathogen defence (Andersen et al. 2016). These changes are linked to worsening in the

progression of chronic diseases, reduced immunity from infections and diminished vaccine efficacy (Karlsson and Beck 2010).

Secondary lymphoid organs, such as spleen, lymph nodes and mucosa-associated lymphoid tissue, are the sites where lymphocytes, after maturation in the thymus, are taken up and can be activated in response to pathogens (Ley and Kansas 2004). Normal immune cells maturation and activation by antigens require structural integrity of the immune tissue (Takahama 2006). This is often compromised in the obese and insulin resistant state, because the fatty acids (FAs) mobilized from WAT in these conditions can accumulate in lymphoid organs, as well as other tissues, disrupting tissue integrity (Yang et al. 2009; Kanneganti and Dixit 2012).

In addition, obesity has been related to diminished function of immune cells. It was suggested that T cells of obese mice can respond to a reduced range of pathogens compared to normal-weight mice (Yang et al. 2009). In obese humans, reduced capacity of lymphocytes to respond to antigen stimulation has been reported (Lamas et al. 2002; Lynch et al. 2009; Yang et al. 2009). In addition, obesity seems to be associated with the inability to maintain influenza-specific CD8+ memory T cells (Karlsson et al. 2010). Further supporting a negative impact of obesity on immunity, several studies described an improvement in immune function after dietary restriction and weight loss (Tanaka et al. 2001; Lamas et al. 2004).

1.3 Persistent organic pollutants and metabolic disorders

The hypothesis that relates environmental chemicals to obesity was firstly formulated in 2002 by Baillie-Hamilton, who suggested that the increased production of chemicals was associated to the obesity epidemic (Baillie-Hamilton 2002). Shortly after, the term "obesogen" was introduced to describe chemicals that alter the regulation of energy balance in favour of lipid accumulation and weight gain (Grün and Blumberg 2006). Since then, an increasing number of chemicals with metabolic-disrupting properties has been identified, including pollutants that cause diabetes (Thayer et al. 2012).

1.3.1 Persistent organic pollutants

Persistent organic pollutants (POPs) include several lipophilic, halogenated compounds that are resistant to biodegradation, persist in the environment and bioaccumulate in food webs and living organisms (Fisher 1999). As they move up the food chain, POPs increase in concentration, a process known as biomagnification (Fisher 1999). Examples of POPs include organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs) and dioxins (Lee et al. 2014). Although most POPs have either been banned or strictly regulated, humans are still exposed to mixtures of these pollutants, mainly through contaminated food, especially fat-rich products such as fish, meat and milk (Fisher 1999; Verner et al. 2013).

1.3.2 Experimental and epidemiological evidence for the role of mixtures of POPs in metabolic disorders

The involvement of mixtures of POPs in obesity and other metabolic disorders is now supported by different studies. In adult rodents, chronic exposure to mixtures of POPs containing OCPs, PCBs, polychlorinated dibenzodioxins and polychlorinated dibenzofurans at environmental levels leads to increased visceral fat accumulation and insulin resistance (Ruzzin et al. 2010; Ibrahim et al. 2011), possibly through alteration in the regulation of genes involved in inflammation, mitochondrial function, lipid oxidation, and lipogenesis. These studies are particularly important because they mimic human exposure in terms of mixture and doses, and therefore provide evidence that human exposure to low-dose mixture of POPs can have detrimental effects on metabolism. In addition, extensive literature on the effect and mechanisms of single POPs on metabolism has been reviewed (Jackson et al. 2017), but will not be presented in details here.

In a cross-sectional study in the US general population, the six most commonly detected POPs (two polychlorinated dibenzodioxins, one PCB, and three metabolites of OCPs) had a strong association with the prevalence of T2D (Lee et al. 2006). Furthermore, two prospective studies determined that POPs mixtures were associated with a three to five times increased risk of T2D (Lee et al. 2010; Lee et al. 2011a).

While no clear correlation was described for exposure to mixture of POPs and prevalence or risk of obesity, a correlation was described between *p,p'*-dichlorodiphenyldichloroethylene (DDE) and highly chlorinated PCBs and higher BMI after 18 years (Lee et al. 2011b). Moreover, *p,p'*-DDE, dioxin and less chlorinated PCBs predicted future risk of abdominal obesity (Lee et al. 2012), and maternal exposure to DDE was associated with increased BMI in female offspring (Karmaus et al. 2009). Although they suggest a link between POPs and obesity, results of these epidemiological studies should be interpreted carefully since the effect of diet and exercise on obesity cannot be completely eliminated.

1.3.3 Role of POPs in obesity-associated inflammation

Inflammation is a key feature of obesity and T2D (Hotamisligil 2006). So far only one study has demonstrated that consumption of a mixture of POPs through the diet induces chronic inflammation in WAT (Ibrahim et al. 2011). It has been proposed that the release of POPs from necrotic adipocytes (which are more numerous in obesity) could stimulate macrophages, leading to chronic inflammation in WAT (Lee et al. 2014). Since POPs can accumulate within lipids in virtually all tissues, this process could be relevant also in other tissues. Additional studies reviewed in (Lee et al. 2018a) showed a link between individual POPs and inflammation in adipose tissue. Finally, MAO, which are characterized by higher levels of chronic systemic inflammation and have a higher risk of developing T2D compared to MHO, show a higher burden of POPs (in particular dioxin and non-dioxin-like PCBs) than MHO, further supporting a pro-inflammatory role of POPs in obesity (Gauthier et al. 2014).

1.4 Pregnane X Receptor as regulator energy metabolism

Pregnane X receptor (PXR) is a nuclear receptor (NR) encoded by the *Nr1i2* gene. It was firstly described as a NR activated by natural steroids and synthetic glucocorticoids and anti-glucocorticoids (Kliewer et al. 1998). The human orthologue is often termed the steroid and xenobiotic receptor (SXR) and is encoded by the *NR1I2* gene (Blumberg et al. 1998). PXR/SXR is highly expressed in the liver and intestine, where it regulates

the expression of several genes involved in drug and energy metabolism (Hariparsad et al. 2009).

1.4.1 PXR as xenosensor

PXR is a major regulator of enzymes involved in the biotransformation, metabolism, and elimination of xenobiotics (synthetic chemical substances that are normally not found within an organism) and natural occurring substances (Kliewer et al. 2002). This NR can be activated by a multitude of ligands due to its unusually large binding pocket, that can accommodate structurally diverse molecules (Watkins et al. 2001). Examples of naturally occurring ligands for PXR are the steroid pregnenolone (Kliewer et al. 1998) and the BA lithocolic acid (Xie et al. 2001). PXR can also be stimulated by prescription drugs such as rifampicin, and by environmental contaminants such as chlordane and PCBs (Kliewer 2003). There are important differences in PXR activation profiles across different species (Jones et al. 2000; Lille-Langøy et al. 2015). For example, mouse PXR is efficiently activated by pregnenolone but not rifampicin. Conversely, human SXR is activated by rifampicin but not pregnenolone. Once activated by a ligand, PXR dimerizes with retinoid X receptor (Squires et al. 2004) and stimulates the transcription of genes that are involved in xenobiotic detoxification in both liver and intestine (Maglich et al. 2002). The main target gene of PXR is cytochrome P450, family 3, subfamily A (*Cyp3a*), which is co-expressed with PXR and which is responsible for the metabolism of around 50% of all prescription drugs (Kliewer 2003). PXR is activated by nearly all chemicals that are known to stimulate *Cyp3a* expression, and these chemicals are also substrates for CYP3A enzymes (Kliewer 2003).

1.4.2 PXR as regulator of metabolism

In addition to its established role as xenosensor, it has recently emerged that PXR also regulates lipid and glucose metabolism. The discovery of this new function came from the initial observation that patients treated with potent SXR agonists such as rifampicin, tamoxifen, carbamazepine, and rifaximin showed alterations of serum and hepatic

lipids (Eirís et al. 1995; Grieco et al. 2005; Cheng et al. 2012; Gao and Xie 2012) and of glucose tolerance (Lahtela et al. 1984; Lahtela et al. 1985; Chang and Waxman 2006; Hukkanen 2012).

1.4.2.1 PXR regulates hepatic lipid metabolism

PXR activation has been related to the development of hepatic steatosis (Bitter et al. 2015), which is an abnormal accumulation of fat in the liver of obese individuals that exacerbates insulin resistance and glucose intolerance (Hakkola et al. 2016). PXR affects several genes involved in energy metabolism in the liver and this ultimately results in simultaneous repression of mitochondrial β-oxidation and stimulation of lipogenesis (Hakkola et al. 2016). Although with some differences regarding the involved transcriptional changes, activation of SXR also leads to repression of mitochondrial β-oxidation and stimulation of lipogenesis (Moreau et al. 2009). Surprisingly, also the ablation of PXR/SXR promotes hepatic steatosis, although the mechanisms involved and the morphologic features of the steatosis are different (Bitter et al. 2015).

1.4.2.2 PXR regulates hepatic glucose metabolism

Several studies demonstrated that PXR regulates glucose homeostasis, although no clear mechanism has been proposed yet. Some studies in mice described beneficial effects of PXR activation on glucose homeostasis, such as decrease in fasting glucose levels (Nakamura et al. 2007) and prevention of HF-diet-induced glucose intolerance (Ma and Liu 2012). In addition, PXR ablation increased the severity of glucose intolerance induced by HF-diet (Spruiell et al. 2014b). The beneficial effect of PXR activation on glucose metabolism could be explained by a suppression of hepatic gluconeogenesis, which has been described to be under PXR control (Bhalla et al. 2004; Kodama et al. 2004; Kodama et al. 2007). However, detrimental effects of PXR activation on different glucose-related parameters were reported in rats (Rysä et al. 2013; Ling et al. 2016). In addition, another study showed that PXR knockout improves glucose homeostasis in HF-diet-induced and genetic models of obesity, while

transgenic activation of PXR exacerbates diabetes in *ob/ob* mice (He et al. 2013). Form this study it was evident that, although PXR is expressed mainly in liver and intestine, its ablation has profound consequences on whole-body metabolism, as insulin sensitivity was improved in liver, WAT and skeletal muscle. Therefore, the negative consequences of PXR activation on glucose metabolism may involve systemic secondary effects. Since obesity is a factor that can affect glucose tolerance and insulin sensitivity, some studies have investigated whether PXR affects weight gain under HF-diet.

PXR ablation reduced fat accumulation in different obesity models (He et al. 2013; Spruiell et al. 2014b). The reduced fat mass has been explained in terms of increased mitochondrial FA β-oxidation and consequent elevation of metabolic rate in the absence of PXR (He et al. 2013). In addition, it was recently suggested that PXR prevents HF-diet-induced obesity through stimulation of intestinal fibroblast growth factor (FGF) 15 production, which leads to reduction of BA output and impaired intestinal absorption of lipids (Zhao et al. 2017).

Given the newly discovered role of PXR in metabolism, understanding the molecular mechanism that underpin the metabolic role of PXR may reveal novel therapeutic targets for the management of metabolic disorders.

1.5 The FGF family and FGF15/19

The FGF family consists of 22 proteins that are involved in several biological functions such as development, differentiation and metabolism (Fig. 1.4) (Ornitz and Itoh 2015). The first FGFs to have evolved belong to the intracellular FGF subfamily, which is composed of intracellular non-signaling proteins that act as co-factors for voltage-gated Na-channels (Ornitz and Itoh 2015). With the acquisition of a signal peptide for secretion, secreted FGFs were generated (Itoh and Ornitz 2008). The secreted FGFs are further divided in canonical FGFs and endocrine FGFs. Canonical FGFs comprise five subfamilies of proteins that act in an autocrine/paracrine way to regulate cell proliferation, differentiation and survival (Ornitz and Itoh 2015). These FGFs bind to heparin/heparan sulphate proteoglycans, which limit their diffusion through the

extracellular matrix, and act as co-factors regulating specificity and affinity of FGFs (Ornitz 2000). Endocrine FGFs have reduced affinity for heparin/heparan sulphate proteoglycans and as a result they are free to circulate and target distant organs, where they act as endocrine hormones to regulate phosphate, BAs, carbohydrates and lipid metabolism, in addition to cellular proliferation, differentiation and survival (Ornitz and Itoh 2015). These FGFs require the presence of cofactors such as α- and β-Klotho to bind their receptors (Potthoff et al. 2012; Smith et al. 2014). The majority of FGFs signals through FGF tyrosine kinase receptors (Ornitz and Itoh 2015). There are four known FGF receptors (FGFR1-4), of which three (FGFR1, FGFR2 and FGFR3), are present in two variants (IIIb or IIIc) (Ornitz and Itoh 2015).

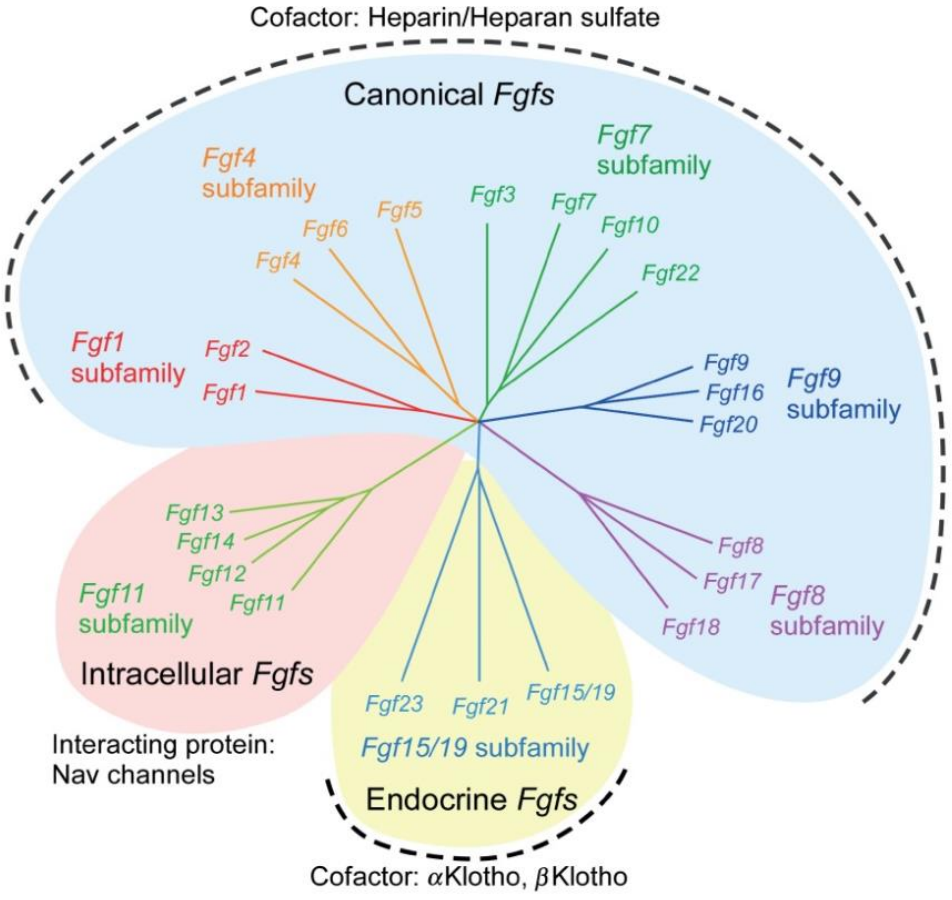

Figure 1.4: The FGF sub-families. *(Ornitz and Itoh 2015).*

Mouse FGF15 and the human orthologue FGF19 belong to the endocrine subfamily of FGFs, together with FGF21 and FGF23 (Ornitz and Itoh 2015). Although FGF15/19 is able to bind to FGFR1c, FGFR2c, FGFR3c and FGFR4 in vitro (Wu et al. 2010b), this FGF exerts it biological action mostly through FGFR4 (Yu et al. 2000; Ito et al. 2005; Katafuchi et al. 2015) and FGFR1 (Lan et al. 2017) and in the presence of the cofactor β-Klotho, which is essential for the biological effects of FGF19 (Wu et al. 2010b).

1.5.1 FGF15/19 as regulator of BA homeostasis

Despite their relatively low (53%) amino acid sequence identity (McWhirter et al. 1997; Nishimura et al. 1999), mouse FGF15 and human FGF19 are orthologues. FGF15/19 is physiologically involved in in the regulation of BA homeostasis (Fig. 1.5). In the post-prandial state, FGF15/19 is actively secreted from the small intestine of mice and humans in response to the increased influx of BAs. FGF15/19 targets the liver (Inagaki et al. 2005), where it inhibits transcription of cytochrome P450 family 7 subfamily A member 1 (*Cyp7a1*/*CYP7A1*), which is the rate-limiting enzyme of the classical BA synthetic pathway, leading to suppression of BA production (Holt et al. 2003; Inagaki et al. 2005). In humans, the postprandial release of BAs is followed by a peak in the levels of serum FGF19. This peak occurs before the repression of BA synthesis (LUNDÅSEN et al. 2006). FGF15/19 also stimulates gallbladder filling (Choi et al. 2006). The importance of FGF15/19 in the maintenance of BA homeostasis is well illustrated by the phenotype of FGF15-deficient mice, characterized by increased *Cyp7a1* expression and activity and a corresponding increase in BA synthesis and in faecal BA excretion (Inagaki et al. 2005). In addition, even though *Cyp7a1* is known to be repressed by farnesoid X receptor (FXR)-dependent signaling in the liver (Goodwin et al. 2000), activation of FXR in the intestine and not in the liver is required to suppress *Cyp7a1* (Kim et al. 2007). This suggests that hepatic FGF15/19 signaling may play a major role in the feedback regulation of BA synthesis. Furthermore, FGF19 administration to FXR-deficient mice also results in the suppression of BA synthesis (Miyata et al. 2011). These observations suggest that FGF15/19 could negatively regulate BA metabolism through FXR-independent mechanisms. FGF15/19 acts through a receptor complex consisting of FGFR4 and β-Klotho (Fig. 1.5), and loss of

either FGFR4 or β-Klotho in mice results in a phenotype that resembles that of FGF15-deficient mice with elevated *Cyp7a1* expression (Yu et al. 2000; Ito et al. 2005).

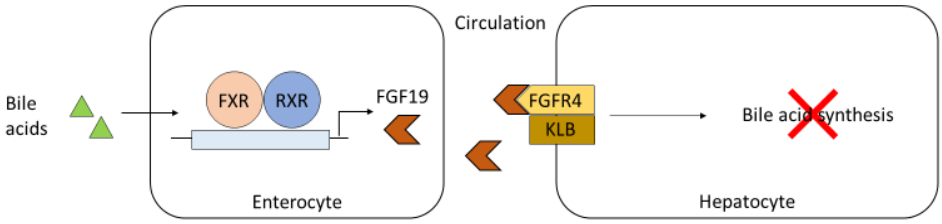

Figure 1.5: FGF15/19 regulates post-prandial BA homeostasis. *(Martina Galatea Castelli).*

FGF15/19 is a known transcriptional target of intestinal FXR, which is activated by BAs (Holt et al. 2003; Johansson et al. 2008). Accordingly, BA-induced increase in FGF15/19 production in the small intestine was described to be mediated by FXR (Holt et al. 2003; Inagaki et al. 2005). Nonetheless, mRNA expression profiles of FXR and FGF15/19 in the intestine do not coincide entirely, and intestinal-specific ablation of FXR strongly reduces, but does not eliminate FGF15/19 expression (Stroeve et al. 2010). Moreover, even if lipids are more powerful than carbohydrates in raising BA levels, ingestion of lipids was reported to be less effective in raising plasma FGF19 levels compared to ingestion of carbohydrates (Morton et al. 2014). Altogether these observations suggest that postprandial FGF19 production involves other mechanisms in addition to BA-induced FXR activation.

1.5.2 FGF15/19 as regulator of metabolism

1.5.2.1 FGF19 ameliorates the obese phenotype

Transgenic overexpression of FGF19 in mice results in elevated metabolic rate and lower adiposity, despite a higher food intake (Tomlinson et al. 2002). In addition, FGF19-transgenic mice are resistant to HF diet-induced obesity, dyslipidaemia, insulin resistance and glucose intolerance. A similar metabolic phenotype is obtained also by administration of recombinant human FGF19 to HF diet-fed mice (Fu et al. 2004). In these early studies it was suggested that the liver constitutes the major target for FGF19,

given its central role in lipid and carbohydrate metabolism and that it is responsible for a large quota of the metabolic rate (Rolfe and Brown 1997). Indeed, hepatic FA β-oxidation was found to be increased, and the reduction in adiposity was explained in terms of elevated metabolic rate (Tomlinson et al. 2002; Fu et al. 2004). The improvement of glucose homeostasis was initially suggested to be secondary to the reduced fat deposition in adipose tissue and other tissues (Tomlinson et al. 2002), given the known detrimental role of fat accumulation on insulin sensitivity (Kahn and Flier 2000). More recent research has explored the role of FGF19 further and revealed that this hormone is able to directly influence glucose homeostasis through a suppression of hepatic gluconeogenesis (Shin and Osborne 2009; Potthoff et al. 2011). FGF19 was also reported to act on WAT through the FGFR1/β-Klotho receptor complex, leading to induction of extracellular-signal-regulated protein kinase (ERK)1/2 phosphorylation and increased adipocyte glucose uptake in mice (Kurosu et al. 2007). However, the activation of FGF19 signaling in WAT was less effective compared to that of the liver. The contribution of WAT to FGF19-mediated metabolic effects seems to be minimal (Kurosu et al. 2007). Studies where FGF15/19 was administered centrally and systemically have shown that this hormone can regulate metabolism also by acting on the brain (Fu et al. 2004; Morton et al. 2013; Ryan et al. 2013; Marcelin et al. 2014), and a recent study (Lan et al. 2017) demonstrated that FGF19 action on the brain, and not on liver and adipose tissue, is essential for long-term weight loss and beneficial glycaemic effects.

So far only one study has contextually compared the effects of transgenic overexpression of FGF19 and FGF15 on diet-induced obesity (Zhou et al. 2017b). This study showed that both FGF19 and FGF15 were able to increase EE, reduce adiposity and improve glucose tolerance and insulin sensitivity in mice, although FGF19 showed a more powerful effect compared to FGF15.

1.5.2.2 FGF15/19 participates in the regulation of the postprandial response

Similarly to insulin, FGF19 stimulates hepatic protein and glycogen synthesis in mice (Kir et al. 2011). The same study also showed that mice lacking FGF15 had a reduction

in hepatic glycogen content, supporting a physiological role of this enterokine in glycogen metabolism. However, whereas insulin levels peak early after feeding, FGF15/19 peaks around three hours after a meal (LUNDÅSEN et al. 2006) and exerts its action on protein and glycogen synthesis through a different signaling pathway. Activation of the FGFR4/β-Klotho receptor complex by FGF15/19 leads to activation of an ERK1/2 pathway, while insulin signals through the insulin receptor-phosphoinositide 3-kinase/protein kinase B (AKT) pathway (Kir et al. 2011). Another important difference is that while insulin stimulates FA synthesis (Porstmann et al. 2008; Li et al. 2010), FGF19 inhibits it (Bhatnagar et al. 2009).

The metabolic effects of FGF15/19 are summarized in Fig. 1.6.

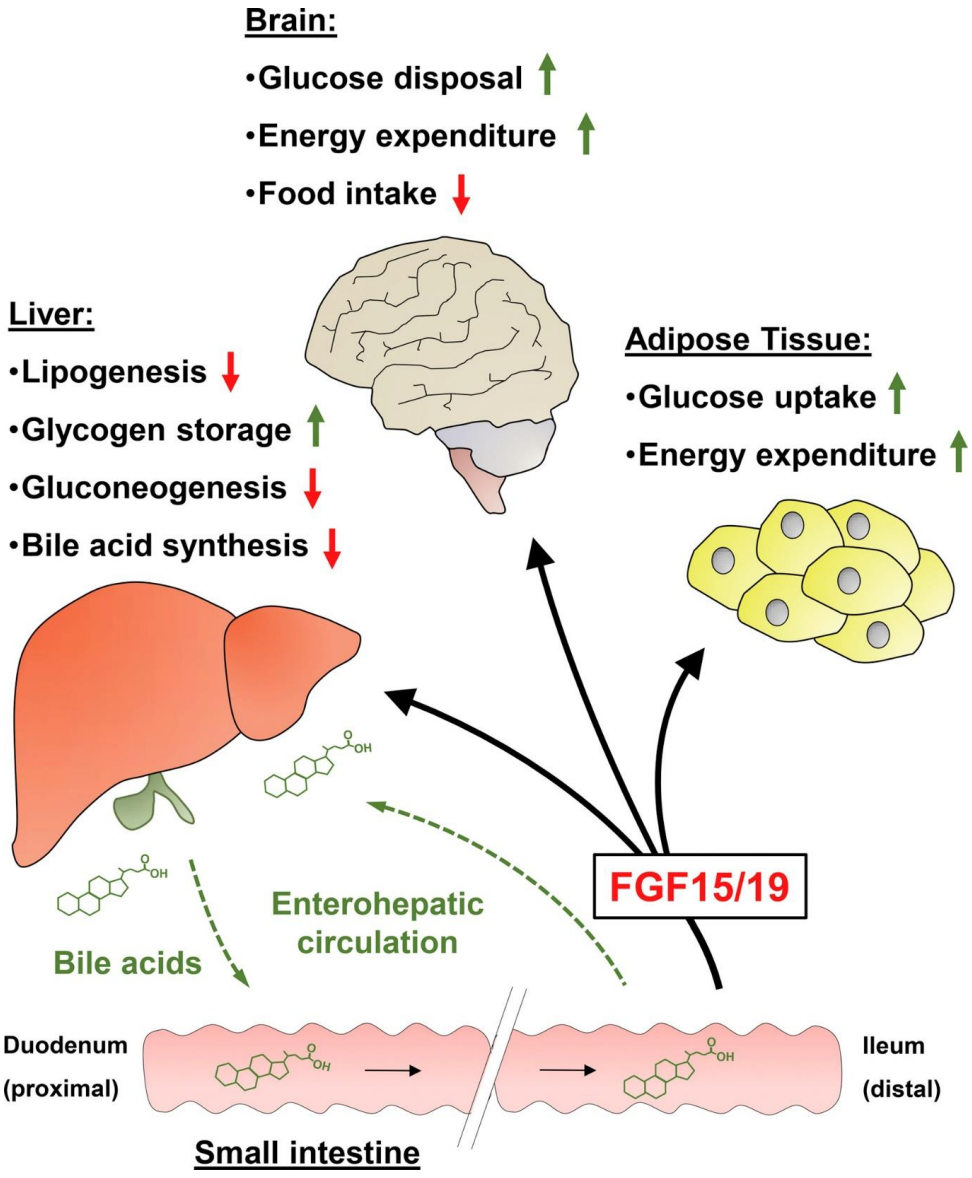

Figure 1.6: Metabolic action of FGF15/19. *FGF15/19 affects metabolic homeostasis by targeting the liver, WAT and the brain (Jahn et al. 2015).*

Considering the beneficial effects of FGF15/19 on metabolism, this molecule appears as a potential candidate for the treatment of metabolic disorders.

1.6 The role of skeletal muscle in metabolism

Skeletal muscle is the largest and most dynamic tissue of the human body, where it constitutes around 40% of total weight (Rolfe and Brown 1997). This tissue contains 50–75% of all body proteins (Frontera and Ochala 2015) and together with the heart is responsible for almost 30% of resting energy expenditure (REE) (Gallagher et al. 1998).

Skeletal muscle consists of several muscle fibers (i.e. myofibers) where each myofiber is a single multinucleated muscle cell that results from the proliferation and differentiation of adult skeletal muscle stem cells (Fig. 1.7) (Frontera and Ochala 2015). The size of a muscle is determined by the number and size of individual muscle fibers (Frontera and Ochala 2015). While the number of fibers is set at birth and does not show appreciable variations during adult life, the size and the type of the fibers can vary considerably in response to different stimuli (Rowe and Goldspink 1969; Pearson 1990).

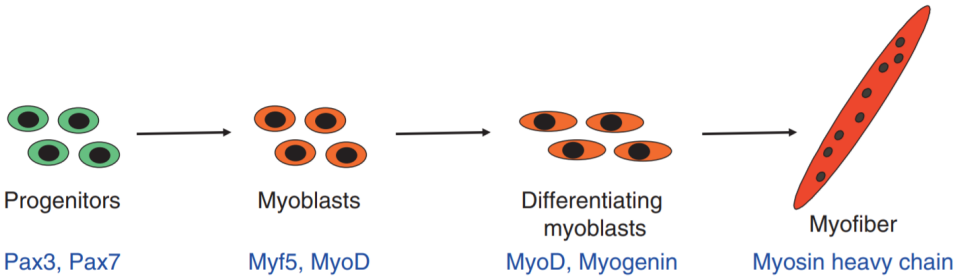

Figure 1.7: Myofiber differentiation. *Progenitor cells express Pax3/Pax7. Progenitor cells become committed to the muscle lineage by expression of Myf5 and/or MyoD and are called myoblasts. Myoblast proliferate and differentiate into multinucleated myofibers in a process that involves expression of myosin heavy chain proteins and fusion into multinucleated myofibers (Krauss 2017).*

1.6.1 Muscle mass and fiber composition affect whole-body metabolism

Muscle fibers in mammals are highly heterogeneous regarding their biochemical, mechanical, and metabolic characteristics, and different muscles show different fiber composition (Frontera and Ochala 2015). The main distinction has been made between slow-twitch (type I) and fast-twitch (type II) fibers (Baskin et al. 2015). While slow-

twitch fibers show low fatigability, high oxidative capacity and prefer FAs as substrate for ATP production, fast-twitch fibers display a higher fatigability, higher strength of contraction, lower oxidative capacity and primarily generate ATP through anaerobic glycolysis (Bassel-Duby and Olson 2006; Schiaffino and Reggiani 2011). The presence of fibers with different properties in the same muscle is very important because it allows the muscle to adapt to various metabolic and mechanical demands. Moreover, the fiber composition of skeletal muscles affects systemic energy consumption. For example, endurance exercise is associated with a fast- to low-twitch shift of fibers, which results in enhanced oxidative capacity (Fig. 1.8) (Baskin et al. 2015). Also resistance training enhances the oxidative and glycolytic capacity of fast-twitch fibers (LeBrasseur et al. 2011). Resistance training impacts systemic metabolism also by stimulating hypertrophic growth of muscle, which results in elevated REE (Speakman and Selman 2003; LeBrasseur et al. 2011).

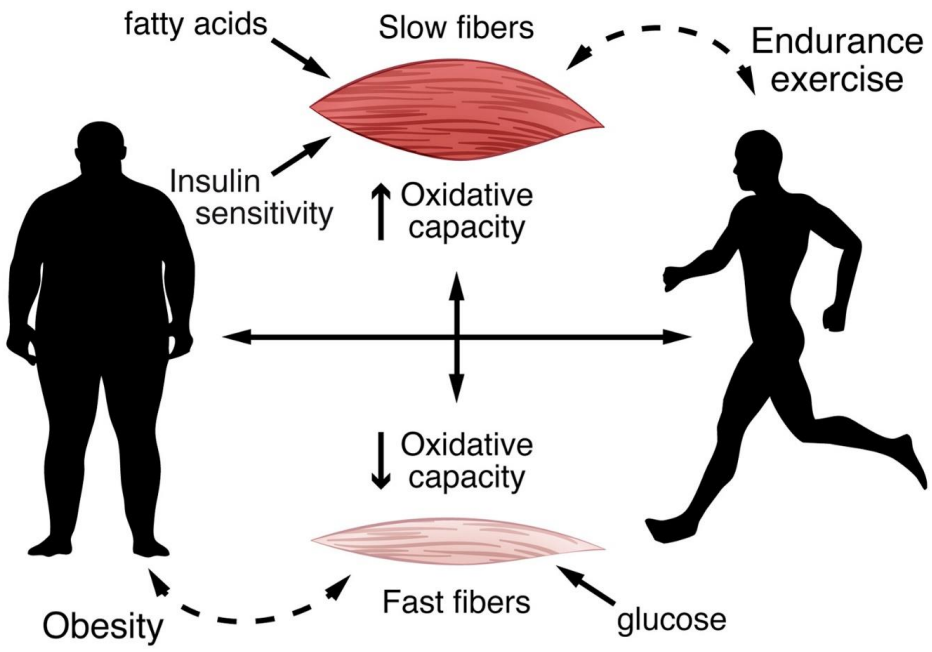

Figure 1.8: Impact of skeletal muscle on whole-body metabolism. *Modified from (Baskin et al. 2015).*

1.6.2 Sarcopenia

Loss of muscle mass (also known as muscle atrophy or sarcopenia) and a switch from slow- to fast-twitch fibers (Fig. 1.8) are observed in conditions such as obesity and diabetes (Simoneau et al. 1995; Mootha et al. 2003; Kim et al. 2010). The importance of skeletal muscle on general metabolism and health is illustrated by studies where diabetic or obese patients on resistance training had improved insulin sensitivity (Zanuso et al. 2010) and developed larger muscles and a higher REE (Willis et al. 2012). In addition to metabolic disorders, muscle atrophy characterizes a number of conditions including cancer, neurological disorders, inflammation-related diseases and aging, where it is associated with a poor quality of life and increased risk of mortality (Anker et al. 1997; Powers et al. 2016).

Skeletal muscle biology is extremely important for general metabolism and health. The discovery of treatments able to protect from muscle atrophy represents an attractive strategy for the management of the above-mentioned pathologies.

2 Aim

The aims of this PhD work were to:

- Improve our understanding of the anti-obesity and anti-diabetic effects of PXR ablation (Paper I)

- Investigate the ability of FGF19 to promote skeletal muscle growth (Paper II)

- Investigate the impact of a dietary mixture of POPs on intestinal inflammation and immune system in the context of obesity (Paper III)

3 Results

3.1 *Nr1i2*$^{-/-}$ mice have larger skeletal muscles and elevated circulating FGF15 (Paper I)

In this work we have carried out an extensive metabolic phenotyping of *Nr1i2*$^{-/-}$ mice, in order to understand how PXR regulates metabolism.

After 22 weeks of standard low-fat diet feeding, *Nr1i2*$^{-/-}$ mice became leaner than their *Nr1i2*$^{+/+}$ counterparts despite a higher food intake. The lower average body weight of *Nr1i2*$^{-/-}$ mice was substantially due to a significant reduction of WAT depots, while the weight of liver and brown adipose tissue remained unaffected. In agreement with reduced fat accumulation, *Nr1i2*$^{-/-}$ mice showed reduced circulating levels of triglycerides and cholesterol and improved glucose tolerance and insulin sensitivity. In addition, PXR ablation was associated with an increase in REE. As the expression of genes involved in EE in brown adipose tissue, WAT and skeletal muscles, as well as body temperature were not significantly different between *Nr1i2*$^{-/-}$ and wild type mice, the enhanced metabolic rate was not related to a stimulation of thermogenic processes. Importantly, *Nr1i2*$^{-/-}$ mice were characterized by increased skeletal muscle mass and physical activity. The enlarged muscles did not seem to directly depend on the absence of PXR in this tissue, since *NR1I2* silencing in primary human muscle cells did not result in larger myotubes. Interestingly, *Nr1i2*$^{-/-}$ mice displayed elevated levels of circulating FGF15, which was associated with a suppression of the classic BA synthetic pathway and concurrent stimulation of the alternative synthetic pathway. Plasma total BAs were increased in the absence of PXR, with a predominance of the more hydrophilic BAs. In *Nr1i2*$^{-/-}$ mice, the phenotype characterized by reduced adiposity, augmented muscle mass and FGF15 levels was maintained also under HF feeding and in 17-month-old mice.

3.2 FGF19 is a regulator of skeletal muscle mass (Paper II)

In this work we investigated whether FGF19, which is biologically active in mice and more stable than its mouse orthologue FGF15, can affect skeletal muscle and protect from sarcopenia.

Adult mice that received daily injections of recombinant human FGF19 (0.1 mg/kg) for a period of seven days, were leaner compared to vehicle-treated mice and showed enhanced skeletal muscle mass and strength. The augmented muscle mass was due to hypertrophy of both slow- and fast-twitch fibers, without modification of the fiber number and type composition. The hypertrophic effect of FGF19 was also observed in three-week-old mice. *In vitro* exposure of primary human muscle cells revealed that FGF19 did not affect cell proliferation and expression of myogenic factors. When the exposure occurred during the differentiation process or on fully differentiated myotubes, the area covered by the myotubes was substantially increased. This effect was observed with physiological and pharmacological doses of FGF19. Both *in vivo* and *in vitro*, fiber hypertrophy was associated with the stimulation of an ERK1/2-mammalian target of rapamycin (mTOR)-S6 kinase (S6K)1 pathway. Inhibition of this signaling pathway either at the ERK1/2 or mTOR level abolished the hypertrophic effect of FGF19 *in vitro*. The presence of β-Klotho in skeletal muscle was essential for FGF19 action, since FGF19 treatment of mice with a whole-body or muscle-specific deletion of *Klb* (the gene that encodes for β-Klotho in mice) did not result in gain of muscle mass. This was confirmed also by silencing of *KLB* (the gene that encodes for β-Klotho in humans) in primary human muscle cells, which blunted FGF19-induced hypertrophy. Treatment of mice and primary human muscle cells with FGF21 (another member of the endocrine FGFs subfamily that is closely related to FGF19) did not produce hypertrophic growth of muscle fibers.

Finally, FGF19 treatment significantly improved skeletal muscle mass and strength in three different animal models of sarcopenia (glucocorticoid-induced, aging- and obesity-related sarcopenia).

3.3 Mixture of POPs affects immune system and promotes gut inflammation in the context of obesity (Paper III)

In this work, we exposed mice to a control diet, a HF diet and a HF diet supplemented with a mixture of POPs at environmental levels for 12 weeks. We assessed the impact of POPs on weight gain, on immune cells in the spleen and MLNs and on intestine. In addition, the role of POPs in intestinal inflammation in obesity was evaluated with the use of a DSS-induced colitis model combined with control, HF diet and HF diet supplemented with POPs.

As expected, mice in the HF group accumulated more adipose tissue compared to Control mice. Despite a similar energy intake, exposure to a mixture of POPs exacerbated the adipose tissue accumulation induced by HF diet. HF diet feeding did not alter immune cell composition with the exception of splenic B cells, that were increased compared to mice on Control diet. The addition of POPs to the HF diet resulted in a further increase in splenic B cells and in a decrease in splenic CD4+ T cells. In the MLNs, immune cells remain unaffected under HF diet-feeding. However, the presence of POPs affected the composition of T cells, reducing and increasing the abundance of CD4+ and CD8+ T cells, respectively, compared to the HF group.

Compared to Control group, animals fed with HF diet had a lower faecal output, while the length of the colon and the weight of the spleen were not significantly affected. Interestingly, mice that consumed POPs through the diet showed elevated faecal output and a strong reduction in colon length and increase in the weight of the spleen compared to mice on the HF diet.

In the DSS-induced colitis model, the HF diet did not substantially worsen intestinal inflammation, although the presence of blood in the faeces was anticipated compared to the Control group. However, despite a similar DSS intake, the presence of POPs in the HF diet worsened colon shortening and splenomegaly and further anticipated the appearance of blood in the faeces.

4 Discussion

4.1 *Nr1i2*$^{-/-}$ mice are associated with increased circulating levels of FGF15 and skeletal muscle hypertrophy

The role of PXR in metabolism has been studied extensively in recent years. The most relevant studies (He et al. 2013; Spruiell et al. 2014b) have focused on the ability of PXR deletion to protect from diet-induced obesity and insulin resistance. Conversely, the effect of PXR ablation on standard low-fat diet was either not investigated or did not result in a substantial change in metabolic phenotype of the animals (He et al. 2013; Spruiell et al. 2014b)

4.1.1 Enhanced muscle mass contributes to improved metabolic homeostasis in PXR deficient mice

In this study we show for the first time that absence of PXR has a profound effect on metabolism also when mice are fed with a standard low-fat diet, and that this effect persists throughout the life span of the mice. The body weight reduction in *Nr1i2*$^{-/-}$ mice was essentially due to smaller adipose tissue depots, which is consistent with an increase in EE in these mice. Previously, enhanced EE in HF diet-fed *Nr1i2*$^{-/-}$ mice versus *Nr1i2*$^{+/+}$ mice was explained in terms of augmented hepatic FA β-oxidation (He et al. 2013; Spruiell et al. 2014b), while thermogenic processes did not seem to be important. Increased FA oxidation in PXR knockout mice is indeed in line with the proposed role of PXR as stimulator of lipogenesis and inhibitor of FA β-oxidation (Hakkola et al. 2016). In our work we show that thermogenic processes are not involved in the improved metabolic phenotype of *Nr1i2*$^{-/-}$ mice fed a standard low-fat diet. Although we did not examine the rate of FA oxidation in the liver, *Nr1i2*$^{-/-}$ mice on standard low-fat diet exhibit larger skeletal muscles, which is a highly metabolically active tissue. Lean mass is an important determinant of REE (Rolfe and Brown 1997; Gallagher et al. 1998), which is the energy cost of maintaining whole-body homeostasis at rest and accounts for 60%-70% of daily EE (Hall et al. 2012). Therefore, the increase in skeletal muscle mass contributes to the higher EE in the absence of PXR, which in turn determines lower adiposity. Augmented fat-free mass and REE have been

associated with higher energy intake (Hopkins et al. 2016). Hence, the increase in food intake in *Nr1i2*$^{-/-}$ mice could reflect the increased energy demand generated by larger muscle mass. *Nr1i2*$^{-/-}$ mice maintained on standard feeding show improved insulin sensitivity as shown by the insulin tolerance test and by the lower levels of insulin required to maintain normal levels of fasting glucose. In addition, they display improved glucose clearance during a glucose tolerance test. In our model, these changes could be at least in part explained by the enhanced lean mass. Skeletal muscle is the primary site for insulin-stimulated glucose disposal (Thiebaud et al. 1982), and therefore improved glucose clearance from the blood could reflect the larger muscle size. The increase in insulin sensitivity in *Nr1i2*$^{-/-}$ mice can also be a consequence of the increase in muscle mass, which results in higher REE, lower adiposity and circulating lipids, which in turn have a positive effect on whole-body insulin sensitivity (Kahn and Flier 2000; Belfort et al. 2005).

In agreement with previous studies (He et al. 2013), *Nr1i2*$^{-/-}$ mice were protected from HF diet-induced obesity and showed improved glucose tolerance and insulin sensitivity. Interestingly, enlarged skeletal muscles in *Nr1i2*$^{-/-}$ mice were maintained also under HF feeding. Therefore, larger muscles may contribute to prevention of diet-induced obesity and to the improvement of glucose homeostasis by elevating EE in the absence of PXR.

4.1.2 Are larger skeletal muscles of Nr1i2$^{-/-}$ mice due to higher circulating FGF15?

We have demonstrated that FGF19 promotes muscle hypertrophy in mice (Benoit et al. 2017) and hypothesized that the elevated levels of circulating FGF15 in *Nr1i2*$^{-/-}$ mice may be responsible for the increased size of skeletal muscles. This is suggested by the similar functions that FGF15 and FGF19 carry out in mice and humans despite their rather low degree of sequence identity (50%) (McWhirter et al. 1997; Nishimura et al. 1999). Different studies suggested similar effects on the liver, where they regulate BA synthesis (Inagaki et al. 2005), promote protein and glycogen synthesis (Kir et al. 2011) and suppress gluconeogenesis (Potthoff et al. 2011). In addition, FGF19 induces body

weight loss and improves insulin sensitivity in mice (Tomlinson et al. 2002), and confers resistance to diet-induced and genetic obesity (Fu et al. 2004). FGF15 exerts a similar protective action against diet-induced obesity (Zhou et al. 2017b). Furthermore, both FGF15 and FGF19 can bind to all FGFR in the presence of β-Klotho (Ornitz and Itoh 2015). However, compared to FGF19, the systemic effects and mechanisms of FGF15 actions on metabolism were not thoroughly investigated because of the instability of this protein (Kir et al. 2011; Angelin et al. 2012; Katafuchi et al. 2015), and only recently some structural and functional differences have been discovered. In a recent study, both FGF19 and FGF15 prevented diet-induced obesity, but FGF19 seemed more effective and only FGF19 reversed diabetes in *db/db* mice (Zhou et al. 2017b). These functional differences were explained in terms of distinct structural features of the human and mouse orthologues. The rodent FGF15 contains an unpaired cysteine in a region that is critical for the interaction with FGFR. The presence of this residue results in a formation of a disulphide-homodimer, which could hinder efficient binding of FGF15 to its FGFR (Zhou et al. 2017b).

Another relevant question is whether FGF15 would be able to promote muscle growth at the concentration found in *Nr1i2*$^{-/-}$ mice. Injection of mice with 0.1 mg/kg FGF19 for one week induces a degree of hypertrophy (Benoit et al. 2017) that is similar to that observed in the absence of PXR. However, injection of FGF19 results in a FGF19 plasma concentration of 17.8 ng/mL (unpublished data), which is >100 times above the level of FGF15 in *Nr1i2*$^{-/-}$ mice (153.2 pg/mL). Furthermore, a recent study showed that mice with a liver-specific deletion of β-Klotho, which display abnormally high levels of circulating FGF15 (up to 20 ng/mL), did not show an increase in lean mass (Lan et al. 2017). These observations challenge our hypothesis that FGF15 could be a regulator of skeletal muscle mass in *Nr1i2*$^{-/-}$ mice.

Furthermore, the elevated activity of *Nr1i2*$^{-/-}$ mice compared to wild type mice is an important factor that likely contributes to the hypertrophy of skeletal muscle mass (Speakman and Selman 2003) and to the increased EE in these mice, since EE is also determined by activity energy expenditure (Hall et al. 2012).

4.1.3 Is the phenotype of *Nr1i2*$^{-/-}$ mice determined by the increased circulating FGF15?

The metabolic phenotype of PXR knockout mice in this study and of mice with elevated levels of FGF19 share many similarities, including reduced adiposity, improved lipid and glucose homeostasis, enhanced EE, stimulation of the alternative synthetic pathway of BAs and enlarged skeletal muscles (Tomlinson et al. 2002; Fu et al. 2004; Wu et al. 2011; He et al. 2013; Benoit et al. 2017). Furthermore, exposure of mice to both FGF19 and FGF15 results in higher metabolic rate and improvement of metabolic homeostasis (Zhou et al. 2017b). Therefore, one hypothesis is that the metabolic features of *Nr1i2*$^{-/-}$ mice are essentially mediated by the increase in FGF15 levels. So far only our study described elevated FGF15 plasma levels in *Nr1i2*$^{-/-}$ mice. A previous study (Zhao et al. 2017) described increased levels of FGF15 mRNA and protein in the ileum of *Nr1i2*$^{-/-}$ mice. In this study the increase in FGF15 in *Nr1i2*$^{-/-}$ mice would protect from diet-induced obesity by suppressing hepatic BA production and consequently reducing intestinal lipid absorption, providing a mechanism by which FGF15 could prevent obesity in the absence of PXR. Interestingly, in humans FGF19 seems inversely associated with the degree of visceral adiposity (Hu et al. 2018).

Besides possible divergences in the action of FGF15 and FGF19, which were highlighted in section *4.1.2*, there are also some subtle differences between *Nr1i2*$^{-/-}$ mice and mice treated with FGF15/19. For example, while all the studies with increased circulating levels of FGF15/19 describe positive glycaemic effects (Tomlinson et al. 2002; Fu et al. 2004; Wu et al. 2009; Wu et al. 2010a; Wu et al. 2011; Adams et al. 2012; Ge et al. 2012; Wu et al. 2013; Lan et al. 2017; Zhou et al. 2017b), this is not the case for all the studies with PXR knockout mice (Nakamura et al. 2007; Spruiell et al. 2014b). Absence of PXR and enhancement of FGF15/19 levels have different outcomes on hepatic lipid metabolism, as FGF15/19 treatment protects from hepatic steatosis (Tomlinson et al. 2002; Alvarez-Sola et al. 2017; Zhou et al. 2017a) while PXR ablation was reported to both prevent and worsen it (Nakamura et al. 2007; He et al. 2013; Spruiell et al. 2014a; Zhao et al. 2017).

Clearly, while increased FGF15 could contribute to the phenotype observed in our *Nr1i2*$^{-/-}$ mice, the regulation of metabolic homeostasis in these animals is far more complex. Determining the contribution of FGF15 to the phenotype of *Nr1i2*$^{-/-}$ mice would help to understand the mechanisms behind their metabolism.

4.1.4 Does PXR regulate intestinal FGF15?

FGF15/19 production in the small intestine is under the control of different molecular regulators, such as FXR and Diet1 (Somm and Jornayvaz 2018). Interestingly, the transcription level of both these regulators was not affected in the ileum of *Nr1i2*$^{-/-}$ mice (unpublished data). Therefore, the observation that FGF15 is elevated in *Nr1i2*$^{-/-}$ mice raises the question whether PXR is a regulator of FGF15 in the intestine. Overexpression of PXR and stimulation with rifampicin promoted FGF19 expression in a human adenocarcinoma cell line (Wistuba et al. 2007), although it seems that the ability of PXR to activate FGF19 may be specific of cancer cells (Wang et al. 2011). Conversely, our own study and the work of (Zhao et al. 2017) suggest that PXR negatively regulates FGF15. Since *Nr1i2*$^{-/-}$ mice do not express PXR in both liver and intestine, higher FGF15 could either be a direct consequence of the lack of PXR in the intestine or be a secondary effect of PXR deletion in the liver.

4.1.5 PXR ablation affects BA homeostasis

BAs play an important role in intestinal absorption of lipids and fat-soluble vitamins and are synthesized in the liver starting from cholesterol (de Aguiar Vallim et al. 2013). Our results suggest that in the absence of PXR, hepatic synthesis of BAs is affected, as the classic and alternative pathways seem suppressed and stimulated, respectively. While repression of *Cyp7a1*, which is key in the regulation of the classic pathway (Schwarz et al. 1996; de Aguiar Vallim et al. 2013), is determined by elevated FGF15 in *Nr1i2*$^{-/-}$ mice, stimulation of the alterative pathway is more complex to explain. The classical BA pathway is highly regulated and can be manipulated by BAs administration, while the alternative pathway seems to be a constitutive pathway which is normally not easily induced (Chiang 2002). However, this pathway has not received

much attention since in humans, unlike mice, it accounts for a very small percentage of the total BA synthesis (de Aguiar Vallim et al. 2013). A study reported stimulation of the alternative pathway in mice injected with FGF19 (Wu et al. 2011). Therefore, FGF15 in *Nr1i2*$^{-/-}$ mice may have a role in the induction of this pathway. Alternatively, another explanation would be that PXR is directly involved in the regulation of the alternative pathway. PXR ablation affected also the amount and the composition of circulating BAs. *Nr1i2*$^{-/-}$ mice display an increase in total plasma BAs, which is essentially due to an increase in β-muricholic acid (MCA) and ω-MCA, in line with a stimulation of the alternative pathway. Hydrophilic BAs such as MCAs, are less efficient than hydrophobic BAs in promoting the absorption of dietary lipids (Wang et al. 2003), which is coherent with reduced fat accumulation and circulating lipids observed in *Nr1i2*$^{-/-}$ mice. However, these results should be interpreted carefully, since intestinal BAs were not analysed in our study. Furthermore, composition of circulating BAs is influenced also by BA transport in the liver, kidney and intestine (Zollner et al. 2006; Thomas et al. 2008), which were not assessed here.

Besides their primary role in intestinal lipid absorption, BAs are present at low levels in the peripheral circulation and act as signaling molecules, influencing lipid and glucose homeostasis, EE and inflammation by acting on several organs (Shapiro et al. 2018). As the hydrophobicity of BAs affects their ability to activate or repress BA-responsive receptors (de Aguiar Vallim et al. 2013), changes in the composition of BAs, like those observed in the absence of PXR in our study, could also influence whole-body metabolism.

4.2 FGF19 regulates skeletal muscle mass and ameliorates muscle wasting in mice

Skeletal muscle is important for whole body homeostasis, as it is a tissue with a high metabolic rate, that is responsible for around 30% of REE (Gallagher et al. 1998). Loss of muscle mass has known negative consequences on general health, and it is associated with different conditions such as obesity (Kim et al. 2010), cancer and aging, where it is thought to have an important role in the overall physical decline (Anker et

al. 1997; Powers et al. 2016). It is therefore of primary importance to find strategies to fight loss of muscle mass.

4.2.1 FGF19 promotes hypertrophy of muscle fibers

Effects of FGFs on skeletal muscle were described previously in the literature. In particular, previous studies have addressed FGFs effect on adult muscle stem cells, also named satellite cells (Pawlikowski et al. 2017). The majority of these cells are dormant in adult skeletal muscles, but they are activated following an injury and actively proliferate and differentiate to produce new myofibers (Murphy et al. 2011). FGF2 and FGF6 were shown to stimulate the proliferation of injury-activated muscle stem cells (Pawlikowski et al. 2017). Previous to our work, only one study investigated the role of FGF19 in skeletal muscle biology, showing that FGF19 increased the myogenic capacity of freshly isolated, injury-activated old satellite cells (Yousef et al. 2014). The same study also reported a positive effect of FGF19 on proliferation of cultured human myoblasts. Conversely, we found no effect of FGF19 on the proliferation rate of cultured myoblasts (Benoit et al. 2017), suggesting that this hormone does not regulate the proliferation of satellite cells. In agreement with the study from (Yousef et al. 2014), we determined that FGF19 does not affect the differentiation rate of cultured myoblasts. In our work we reported for the first time an effect of FGF19 on the size of differentiated human muscle cells. This finding was confirmed in vivo, where FGF19 induced muscle growth by promoting hypertrophy of the existing muscle fibers, without affecting their numbers (Benoit et al. 2017).

4.2.2 FGF19 stimulates the ERK-mTOR signaling pathway

Muscle size is determined by a delicate balance between protein synthesis and degradation (Mukund and Subramaniam 2020). In hypertrophic conditions, in which the size of existing muscle fibers is increased, there is a net shift towards proteins synthesis (Saini et al. 2009). mTOR is a central regulator of hypertrophy in skeletal muscle and the major regulator of protein synthesis (Bodine et al. 2001). Activated mTOR stimulates protein synthesis through activation and inhibition of its downstream

targets S6K1 and eIF4E-4EBP1, respectively. mTOR is positively regulated by phosphoinositide 3-kinase/AKT in skeletal muscle and activation of the AKT/mTOR pathway was found to be essential for hypertrophy (Bodine et al. 2001). In addition, AKT promotes protein synthesis independently of mTOR by directly inactivating glycogen synthase kinase-3β (Cross et al. 1995; Alessi et al. 1996). AKT activation seems sufficient to stimulate increase in muscle mass (Egerman and Glass 2014), and AKT was shown to be activated by FGFR signaling (Ong et al. 2001). In our study however, AKT and glycogen synthase kinase-3β were not affected by FGF19 treatment, suggesting that this is not the pathway that regulates FGF19-induced hypertrophy. In contrast, FGF19 stimulated phosphorylation of ERK1/2, a well-established target of FGF19 (Kurosu et al. 2007; Kir et al. 2011), and produced hypertrophy via stimulation of mTOR ad its downstream target S6K1. Phosphorylation of ERK1/2 was necessary for FGF19-induced atrophy, since ERK1/2 inhibition prevented it in myotubes (Benoit et al. 2017).

Different studies revealed a direct role of ERK1/2 in the regulation of hypertrophy, with somewhat controversial observations. Activation of the ERK1/2 signaling pathway had a pro-atrophic effect *in vitro* (Rommel et al. 1999). Another study reported that ERK1/2 induced atrophy in cultivated myotubes and in cancer cachexia through suppression of myogenesis (Penna et al. 2010). More in line with our study, (Shi et al. 2009) showed that inhibition of ERK1/2 signaling in vitro decreases myotube size and protein content in muscle fibers, while activation of the ERK1/2 pathway is associated with enhanced protein synthesis. In this study, ERK1/2 inhibition seems to induce atrophy by directly decreasing protein synthesis and by downregulating AKT hypertrophic signaling (Shi et al. 2009). Indeed, ERK1/2 stimulates protein synthesis at the translational level (Pyronnet et al. 1999). FGF19 was previously reported to induce protein synthesis through increased ERK1/2 signaling (Kir et al. 2011). However, in this study the effect on protein synthesis was independent of mTOR (Kir et al. 2011), whereas inhibition of mTOR abolished the hypertrophic effect of FGF19 in our study (Benoit et al. 2017). In this study we provide evidence that FGF19-

ERK1/2-mTOR- S6K1 signaling promotes hypertrophy, but the mechanisms remain to be clarified.

4.2.3 Side effects of FGF19 intervention

The finding that FGF19 increases muscle mass ad protects from muscle loss in different atrophy models has important clinical implications. However, similarly to other members of the FGF family, FGF19 has been implicated in the development and progression of liver tumors (Nicholes et al. 2002). In this study, transgenic overexpression of FGF19 in skeletal muscles promoted hepatocellular proliferation, which degenerated in hepatocellular carcinoma (HCC) after 10-12 months. Importantly, increased hepatocellular proliferation was achieved also after injection of wild type mice with recombinant FGF19 for 6 days (Nicholes et al. 2002). Later studies determined that the proliferative action of FGF19 in the liver is mediated by FGFR4 (Wu et al. 2010a; Wu et al. 2010b; French et al. 2012). Some studies investigated the role of FGF19 in HCC development in mice by using adeno-associated virus system (Ge et al. 2012; Zhou et al. 2014; Zhou et al. 2017b). This method results in circulating FGF19 levels up to 1500 ng/mL (Zhou et al. 2014), which is more than 80 times higher than plasma FGF19 levels found in our study (17.8 ng/mL). In other studies, cellular proliferation in the liver was stimulated in mice injected with FGF19 for a short period of time (Nicholes et al. 2002; Wu et al. 2010a; Wu et al. 2010b; Wu et al. 2011; Ge et al. 2012). Although we have used a FGF19 dose that is at least 10 times lower (Benoit et al. 2017) than the dose reported in the above-mentioned studies, circulating levels of FGF19 are in the same range of that found in mice that developed HCC (Nicholes et al. 2002). Furthermore, although livers of mice treated with FGF19 did not show any visible sign of cancer, cell proliferation was not evaluated in our study. The possibility that FGF19 increases the risk of HCC at the dose that promotes muscle hypertrophy cannot therefore be ruled out at present. Importantly, it is possible to engineer FGF19 molecules that lack or minimize this side effect, while maintaining the ability to regulate BAs and metabolism (Wu et al. 2010a; Zhou et al. 2014).

4.3 Mixture of POPs affects immune system and promotes gut inflammation in the context of obesity

The contribution of POPs to the onset of obesity and insulin resistance is suggested by several epidemiological studies (Pelletier et al. 2002; Lee et al. 2007a; Lee et al. 2011b; Lee et al. 2012; Dirinck et al. 2014; Lee et al. 2014). In addition, experimental studies described mechanism by which single POPs can affect metabolism (reviewed in (Jackson et al. 2017)). However, epidemiological studies only establish correlation between exposure levels and metabolic outcome, and organisms are rarely exposed to one single compound. Instead, humans and other organisms are exposed to a multitude of POPs, generally at relatively low concentrations. Only a few studies have established a causative link between exposure to a mixture of POPs and obesity and insulin resistance (Ruzzin et al. 2010; Ibrahim et al. 2011).

4.3.1 Selection of the mixture of POPs

OCPs, including dichlorodiphenyltrichloroethane (DDT) and its metabolites, PCBs and dioxin-like PCBs are represented in the POPs mixture used in our studies. The composition of the POPs mixture was selected based on the POPs that were found to be elevated in MAO compared to MHO individuals, suggesting a negative relationship between POP burden and obesity-related health outcomes, such as inflammatory diseases, increased cardiometabolic risk and decreased insulin sensitivity (Gauthier et al. 2014; Dusanov et al. 2018). In addition, some of these pollutants (DDTs and OCPs in particular) prevented normal insulin-stimulated glucose uptake in cultured adipocytes (Ruzzin et al. 2010). The concentrations of individual POPs in the diet were in the range of POPs concentrations detected in food items from different locations (Dirtu and Covaci 2010; Polder et al. 2010; Törnkvist et al. 2011; Cimenci et al. 2013; Chen et al. 2015). In addition, we previously determined that the concentration of some representative POPs in adipose tissue of mice fed with HF/POPs diet (unpublished data) did not exceed those found in adipose tissue of adult human subjects (Kiviranta et al. 2005; Yu et al. 2011; Malarvannan et al. 2013; Achour et al. 2017). Therefore, POP concentrations used in this study were not excessive and should reproduce real-life exposure levels.

4.3.2 POPs mixture: obesogenic effect

One interesting finding in our work was that inclusion of POPs in the HF diet aggravated adipose tissue expansion in mice. Since the energy intake was not different between mice that consumed HF and HF/POPs diets, this suggests an obesogenic effect of the POPs mixture used in our study. Indeed, some of the pollutants included in our mixture have known obesogenic effects when administered singularly. For example, PCB 153 (Chapados et al. 2012; Taxvig et al. 2012), p,p'-DDT (Moreno-Aliaga and Matsumura 2002) and p,p′-DDE (Mangum et al. 2015) stimulate adipogenesis, while DDE and oxychlordane stimulate lipogenesis (Howell and Mangum 2011). Injection of mice with PCB 153 exacerbates HF diet-induced obesity (Wahlang et al. 2013). In addition, different POPs were positively associated with increased BMI or waist circumference in epidemiological studies. Examples are dioxin-like PCBs such as PCB 105 (Glynn et al. 2003) and PCB 118 (Pelletier et al. 2002; Glynn et al. 2003; Dhooge et al. 2010), non-dioxin-like PCBs, such as PCB 138 and PCB 153 (Pelletier et al. 2002; Lee et al. 2007a), DDE (Pelletier et al. 2002; Glynn et al. 2003; Lee et al. 2006; Lee et al. 2007a; Dhooge et al. 2010; Lee et al. 2011b; Lee et al. 2012; Dirinck et al. 2014), DDT (Elobeid et al. 2010) and OCPs such as oxychlordane and trans-nonachlor (Hue et al. 2007; De Roos et al. 2012; Lee et al. 2012).

Previous studies have examined the effect of exposure of a mixture of POPs on weight gain, showing different results. (Ruzzin et al. 2010) showed that exposure of rats to environmentally relevant POPs for a relatively short period of time increased body weight. Similarly, a study by (Ibrahim et al. 2011) demonstrated that consumption of salmon fillets containing several POPs promotes fat accumulation. In other studies, exposure to mixtures of POPs did not induce adipose tissue expansion (Ibrahim et al. 2012; Midtbø et al. 2013). Differences in the composition and in the concentration of single POPs of the mixtures used in the different studies may explain different outcomes in the development of obesity. In addition, in some of the above-mentioned studies, experimental groups did not only differ in the levels of POPs, but also in nutrient composition (Ibrahim et al. 2011; Ibrahim et al. 2012; Midtbø et al. 2013). Since nutrition can influence the accumulation of POPs in adipose tissue (Myrmel et

al. 2016), and more importantly can affect the toxicity of POPs (Hennig et al. 2012), these studies do not allow to distinguish between effect due to POPs and altered nutritional composition. In our study, the nutritional composition of HF and HF/POPs diets was identical, which allows us to identify effects that are unique for POPs exposure.

4.3.3 POPs promote intestinal inflammation

Pro-inflammatory events in the intestine seem to have an important role in promoting obesity and insulin resistance (Winer et al. 2016). Since the intestine is the first exposure site for dietary POPs (Fisher 1999; Linares et al. 2010), it could be vulnerable to the effect of these chemicals. This study provides initial evidence that POPs can induce intestinal inflammation in the context of obesity. Intestinal inflammation was essentially evaluated in terms of degree of colon shortening, which is a major feature to evaluate the severity of intestinal inflammation in experimental animal models (Wirtz et al. 2007; Chassaing et al. 2014). Shortening of the colon was reported also in ulcerative colitis patients (Gore 1992), and was explained in terms of enlargement and contraction of the muscolaris mucosae, but the mechanisms remain unclear (Gore 1992). Splenomegaly is associated with intestinal inflammation in animal models of IBD (Wirtz et al. 2007; Chassaing et al. 2014) and it has been reported also among obese patients (Heneghan et al. 2013) and HF diet-fed rodents (Altunkaynak et al. 2007; Aboura et al. 2017; de Jesus Felismino et al. 2018).

In our pilot study we did not explore possible mechanisms by which POPs may promote inflammation in the gut. Inflammation is associated with alterations in the abundance and type of intestinal immune cells, changes in gut microbiota, impaired barrier function and consequent increased intestinal permeability (Winer et al. 2016). Studies reported that POPs such as PCB 153 (Phillips et al. 2018) and POPs found in fish oil (Hong et al. 2017) stimulate inflammatory pathways in intestinal cells. In addition, interactions between POPs and intestinal microbiota could have an impact on obesity. It was hypothesized that the microbiota could modulate absorption, distribution, metabolism, and excretion of POPs by different mechanisms such as

alteration of the compound bioavailability from food, direct activations of pollutants, alterations of the host biotransformation capacity and also changes in the enterohepatic circulation (Snedeker and Hay 2012). Conversely, POPs may regulate the species composition of the microbiota. The ability of microbes to biodegrade several organic chemical substances including halogenated compounds and pesticides has been documented (Megharaj et al. 2011). Therefore, the constant exposure to POPs in the intestine could favour the presence of species that effectively metabolize these chemicals, as was suggested by cross-sectional (Lee et al. 2011c) and experimental (Choi et al. 2013; Zhang et al. 2015) studies. Interestingly, intestinal mucus barrier was also suggested to be a target for environmental pollutants (Gillois et al. 2018).

Furthermore, different NRs are expressed in the intestine, where they regulate nutrient uptake, xenobiotic metabolism and mucosal function (Schmidt and Mangelsdorf 2008). NRs are involved in the regulation of energy metabolism (Sonoda et al. 2008), as well as different aspects of intestinal inflammation in IBD (Klepsch et al. 2019). Since NRs can also be activated by different classes of environmental contaminants (Lille-Langøy et al. 2015; Toporova and Balaguer 2019), they could mediate POPs-induced intestinal inflammation in obesity.

4.3.4 POPs affect the immune system

The ability of POPs to impact immune system is documented in the literature. In Inuit individuals characterized by high levels of contamination, levels of POPs correlated positively with markers of inflammation (Schæbel et al. 2017). This suggested that POPs contribute to the development of chronic diseases occurring in this population, such as rheumatoid arthritis, atherosclerosis and CVD (Schæbel et al. 2017). A correlation was observed also between background exposure to PCBs and prevalence of rheumatoid arthritis in women (Lee et al. 2007b) and between developmental exposure to POPs and immune system development (Hertz-Picciotto et al. 2008), alterations in white blood cell counts in children (Oulhote et al. 2017) and increased risk of asthma in offspring (Hansen et al. 2014). Experimental studies have also examined the effects of single POPs on different aspects of immune function. For

example, exposure of mice to a high dose of DDE caused diabetes and affected T cell function (Cetkovic-Cvrlje et al. 2016), while dioxin exposure during development decreased allergic sensitization (Tarkowski et al. 2010).

In all these studies POPs caused or were related to malfunctioning of the immune system/increased inflammation, which are common also among obese individuals (Hotamisligil 2006; Karlsson and Beck 2010; Andersen et al. 2016). In our study we showed that, compared to the HF diet alone, dietary POPs affect the abundance of some sets of adaptive immune cells in spleen and MLNs. As immune cells from these organs can be recruited in and communicate with metabolic tissues such as VAT and gut (Wu et al. 2014; Magnuson et al. 2018), exposure to POPs may contribute to the exacerbation of obesity and metabolic disorders through an effect on immune cells. B cells, CD4+ and CD8+ T cells consist of several subsets of immune cells that differ in the type of adipokines they produce and in their role in immunity (Asghar and Sheikh 2017). As the specific immune cell subtypes were not analysed in our pilot study, the full extent of POPs' effect on the immune system cannot be interpreted from our data at present and deserves further investigation.

5 Conclusions

To specifically answer the aims of my PhD work:

- Improve our understanding of the anti-obesity and anti-diabetic effects of PXR ablation (Paper I)

Using a *Nr1i2*$^{-/-}$ model, we showed that ablation of PXR improves metabolism through enhancement of skeletal muscle mass. This feature of PXR knockout mice is new and suggests that pharmacological interventions to increase muscle size may be exploited for the management of metabolic disorders. In our model, the increase in skeletal muscle is associated with elevated plasma levels of FGF15, a hormone with known effects on metabolism that may also be responsible for the stimulation of skeletal muscle growth. Ablation of PXR was also associated with differences in level and type of circulating BAs, which could also influence metabolism through their ability to interact with NRs involved in metabolic regulation.

- Investigate the ability of FGF19 to promote skeletal muscle growth (Paper II)

We demonstrated that treatment with human recombinant FGF19, unlike FGF21, induces hypertrophic growth of skeletal muscle fibers without affecting their number and composition. FGF19-mediated muscle hypertrophy is dependent on the presence of β-Klotho in the muscle and on the activation of the ERK1/2-mTOR-S6K1 signaling pathway. Importantly, FGF19 administration significantly ameliorated skeletal muscle mass and strength in three different animal models of muscle atrophy (glucocorticoid-induced, aging- and obesity-related sarcopenia).

- Investigate the impact of a dietary mixture of POPs on intestinal inflammation and immune system in the context of obesity (Paper III)

We revealed that challenging the diet-induced obesity model with a mixture of POPs promoted further adipose tissue deposition, affected B cells and CD4+ T cells in the spleen and changed the composition of T cells in MLNs. In addition, POPs induced classical signs of intestinal inflammation, such as colon shortening and splenomegaly,

and affected faecal production. Exposure to POPs exacerbated intestinal inflammation also when obese mice were challenged with DSS. This study provides initial evidence that dietary exposure to a mixture of POPs at environmentally relevant levels can worsen health and metabolic outcome in obesity.

6 Future perspectives

6.1 FGF15 regulation and role in *Nr1i2*$^{-/-}$ mice

FGF15 is produced in the small intestine (Inagaki et al. 2005), which is also the major site of PXR expression, together with the liver (Hariparsad et al. 2009). In the *Nr1i2*$^{-/-}$ model, the ileal mRNA expression of *Fgf15* is increased compared to wild type mice, which raises the hypothesis that PXR negatively regulates FGF15. This hypothesis can be tested with the generation of an intestinal specific PXR knockout, which would allow to assess the effect of PXR ablation in the intestine, while its expression is still maintained in the liver.

We described that *Nr1i2*$^{-/-}$ mice, which are characterized by elevated circulating FGF15 levels, have enlarged skeletal muscles. The murine FGF15 and human FGF19 share many common functions, including potential binding to all FGFRs (Ornitz and Itoh 2015), and we recently demonstrated that FGF19 promotes hypertrophy in skeletal muscles (Benoit et al. 2017). Expression of FGFRs and β-Klotho in skeletal muscle of *Nr1i2*$^{-/-}$ mice should be assessed to determine if this tissue is a potential target of FGF15. Generation of a double *Nr1i2*$^{-/-}$ and *Fgf15*$^{-/-}$ knockout will allow to determine if FGF15 exerts hypertrophic activity in *Nr1i2*$^{-/-}$ mice. In addition, the use of this double knockout would also allow to determine if and which metabolic features are determined by elevated FGF15 in *Nr1i2*$^{-/-}$ mice.

6.2 Further characterization of FGF19 action

In our study we did not identify the FGFR that mediates FGF19 effect on skeletal muscles. FGF19 treatment in muscle-specific knockouts for each of the FGFRs would allow to determine which receptor is activated by FGF19. In addition, human muscle cells in which individual FGFRs have been silenced could be treated with FGF19. Moreover, since the activation of FGF19 signaling converges on important regulators of protein synthesis, it would be interesting to assess the rate of protein synthesis in muscles of mice treated with FGF19 compared to vehicle.

It would be relevant to evaluate whether the effect of FGF19 persists after the treatment and for how long. Importantly, proliferative effects in the liver should be evaluated at the end of the treatment and during a period of time after the end of the intervention.

In our study we showed that FGF19 administration restored muscle mass in three different models of muscle atrophy. However, we did not study the pathways involved in the different atrophy models and the FGF19 signaling involved in the rescue of atrophy. It would therefore be important to determine which pathways are involved in the atrophy models and if the decrease in the size of the muscles is attributed to decreased protein synthesis, increased protein degradation, or both. It would be interesting to determine if FGF19 counteracts atrophy only by increasing protein synthesis or also by affecting the rate of protein degradation in the atrophy models, and what signaling is involved in these possible mechanisms.

6.3 Further characterization of the effects of POPs on immunometabolism in obesity

We described that exposure to POPs in a diet-induced obesity model induces intestinal inflammation, which was evaluated in terms of colon shortening. It would be interesting to further describe POPs-induced inflammation in the intestine by assessing intestinal permeability and modifications in the microbiota and in the immune compartment. Exposure to POPs also determined an increase in faecal production compared to consumption of a HF diet. Assessing faecal composition would allow to determine if POPs can affect metabolism through modification of intestinal absorption of nutrients. The inflammatory role of POPs in the intestine was also suggested in the DSS-induced colitis model, where pollutants aggravated intestinal colon shortening and splenomegaly. However, the design of the DSS experiment in our study was not optimal, since at day 7 all the DSS groups showed similar body weight loss and all mice in the groups had blood in the faeces. Reducing the time of DSS treatment or using a lower DSS concentration could possibly reveal more differences between the different diet groups during DSS treatment.

Finally, the abundance of some adaptive immune cells was affected by POPs, showing that environmental contaminants can impact the immune system. However, the specific immune cell subtypes were not analysed in our pilot study. Analysing cytokine profiles of immune cells in MLNs and spleen would provide more information regarding the role of POPs on immunity and inflammation in the context of obesity.

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Paper I

I

Bérengère Benoit, **Martina Castelli**, Emmanuelle Meugnier, Stéphanie Chanon, Etienne Lefai, Hubert Vidal, Jérôme Ruzzin (2019).
Nr1i2$^{-/-}$ mice are associated with increased circulating levels of fibroblast growth factor 15 and skeletal muscle hypertrophy.
Manuscript.

Paper II

Bérengère Benoit, Emmanuelle Meugnier, **Martina Castelli**, Stéphanie Chanon, Aurélie Vieille-Marchiset, Christine Durand, Nadia Bendridi, Sandra Pesenti, Pierre-Axel Monternier, Anne-Cécile Durieux, Damien Freyssenet, Jennifer Rieusset, Etienne Lefai, Hubert Vidal, Jérôme Ruzzin (2017).
Fibroblast growth factor 19 regulates skeletal muscle mass and ameliorates muscle wasting in mice.
Nature Medicine, 2017. 23: p. 990.
The first three authors share first authorship.

II

Paper III

Martina Castelli, June Gudmestad, Florian Dingreville, Bérengère Benoit, Hubert Vidal, Jérôme Ruzzin (2019).
Mixture of persistent organic pollutants promotes accumulation of adipose tissue, affects immune system and induces inflammation-related changes in the intestine of C57BL/6 mice.
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