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Role of environmental factors on intestinal barrier dysfunctions reported in obesity

Bárbara Graziela Postal

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Role of environmental factors on intestinal barrier dysfunctions reported in obesity

par

Bárbara Graziela Postal

Dirigée par le Dr Armelle Leturque et Dr. Véronique Carrière

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Devant un jury composé de :

Pr Philippe CARDOT

Président

Dr Stéphanie BLANQUET-DIOT

Rapporteur

Dr Thomas GAUTIER

Rapporteur

Pr Christine POITOU-BERNERT

Examinatrice

Pr Robert BAROUKI

Examinateur

Dr Véronique CARRIERE

Co-Directrice de thèse

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Abbreviations

Å	Angstrom
βNF	β-naphthoflavone
AIP	AhR-interacting protein
AhR	Aryl hydrocarbon receptor
AhRR	AhR repressor
AIEC	Adherent-invasive <i>Escherichia coli</i>
AJs	Adherens junctions
AKK	<i>Akkermansia muciniphila</i>
AKT	Protein kinase B
ARNT	AhR nuclear translocator
ASBT	Sodium-dependent bile acid transporter
ATG	Autophagy-related genes
ATG16L1	Autophagy related gene 16 like 1
BMI	Body Mass Index
bHLH	Basic helix–loop–helix
Bves	Blood vessel epicardial substance
CD	Crohn's disease
CD3 / 36	Cluster of differentiation 3 or 36
CYP1A1	Cytochrome P450 family-1 subfamily-A polypeptide-1
CYP1A2	Cytochrome P450 family-1 subfamily-A polypeptide-2
CYP1B1	Cytochrome P450 family-1 subfamily-B polypeptide-1
DIM	Diindolylmethane
DSS	Dextran sodium sulfate
EGTA	Ethylene glycol-bis(β-aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
Elk-1	ETS Like-1 protein
Epi	Epithelium
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinases
FAO	Food and Agriculture Organization
FD4	Fluorescein isothiocyanate-dextran 4 kDa
FGF15/19	Fibroblast growth factor 15/19
FICZ	6-formylindolo-[3,2-b]-carbazole
FXR	Farnesoid X receptor
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
GuK	Guanylate kinase
GWAS	Genome-wide association scanning
HFD	High fat diet
HOMA-IR	Homeostasis Model assessment of insulin Resistance
HSP90	90-kDa heat shock protein
IBD	Intestinal bowel disease
I3C	Indole-3-carbinol
IAid	Indole-3-aldehyde
IBABP	Ileal bile acid-binding protein

ABBREVIATIONS

ICZ	Indolo-[3,2-b]-carbazole
IDF	International Diabetes Federation
IECs	Intestinal Epithelial Cells
IELs	Intestinal Intraepithelial Lymphocytes
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IFN- γ	Interferon- γ
IL-1 β	Interleukine-1 beta
IL-6	Interleukine-6
IL-8	Interleukine-8
IL-10	Interleukine-10
IL17a	Interleukine-17a
IL-22	Interleukine-22
ILC	Innate lymphoid cells
iNOS	Inducible nitric oxide synthase
JAM	Junctional adhesion molecule
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
KO	Knock-out
LBP	lipopolysaccharides-binding protein
LBD	Ligand-binding domain
LC3	Light Chain 3A
LDL	low density lipoprotein
LP	Lamina propria
LPS	Lipopolysaccharides
MAGUK	Membrane-associated guanylate kinase
MALT	Mucosa-associated lymphoid tissue
MAP	Mitogen-activated protein
MARVEL	MAL and related proteins for vesicle trafficking and membrane link
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MDCK	Madin-Darby Canine Kidney
MEK	Mitogen-activated protein kinase kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MPO	Myeloperoxidase
MRP3	Multidrug resistance protein 3
NF- κ B	Nuclear factor-kappa B
NK	Natural killer
NLR	NOD-like receptor
NLS	Nuclear localization signal
NOD 1/2	Nucleotide-binding oligomerization domain 1/2
OST α/β	Organic solute transporter alpha/beta
p38MAPK	p38 mitogen-activated protein kinase
PA	Palmitic acid
PAHs	Polycyclic aromatic hydrocarbons
PAHO	Pan American Health Organization
PAS	PER-ARNT-SIM

ABBREVIATIONS

PDZ	Post synaptic density-95/Drosophila disc large/Zonula occludens-1
PER	Periodic circadian protein
PGE2	Prostaglandin E2
PKA	Protein kinase A
PKC	Protein kinase C
PP2A	Protein phosphatases 2A
PPM	Post-prandial lipid micelles
PrPc	Cellular prion protein
Reg3- β	Regenerating islet-derived protein 3-beta
Reg3- γ	Regenerating islet-derived protein 3-gamma
ROCK	Rho-associated protein kinase
RYGB	Roux-en-Y gastric bypass
SAA	Serum amyloid A
SCFA	Short-chain fatty acids
SHP	Small heterodimer partner
SFB	Segmented Filamentous Bacteria
Ser	Serine
SH3	Src homology-3
SIM	Single-minded protein
SMAD	Small Mothers Against Decapentaplegic
STAT3	Signal transducer and activator of transcription 3
TAMP	Tight junction-associated MARVEL proteins
TNBS	Trinitrobenzenesulfonic acid
TCD4	T helper cells
TCD8	Cytotoxic T cells
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEER	Transepithelial electrical resistance
TGF- β	Transforming growth factor-beta
Thr	Threonine
TJs	Tight Junctions
TLRs	Toll like receptors
TNF- α	Tumor necrosis factor-alpha
Tyr	Tyrosine
UC	Ulcerative colitis
UV	Ultraviolet
XREs	Xenobiotic response elements
ZO	Zonula occludens
WHO	World Health Organizations

Preface

The involvement of the intestine in metabolic diseases such as diabetes and obesity has been emphasized in several studies. Recently our team described for the first time, inflammation in the small intestine of obese subjects (Monteiro-Sepulveda *et al.*, 2015), which is characterized by infiltration of T lymphocytes into the epithelium leading to an increased susceptibility to epithelial cells dysfunctions. Moreover, our team has shown that obese subjects present subtle intestinal barrier alterations in the basal state and an increased jejunal permeability revealed by a lipid load, associated with inflammatory and metabolic status (Genser *et al.*, 2018). These results are in accord with murine studies, which have showed that obese mice present a higher permeability of the intestinal barrier, a higher endotoxemia and a higher level of circulating proinflammatory cytokines compared to the control mice (Brun *et al.*, 2007; Cani *et al.*, 2007; Cani *et al.*, 2008).

The mechanisms driving this intestinal barrier dysfunction present in obesity remain unclear. The intestine is the main barrier separating the external to the internal environment in the human body and it is a gateway for pathogens and antigens. Thus, nutrients and/or environmental factors are important to intestinal homeostasis and could be involved in intestinal disturbance.

In this context, we investigated some factors that are sensitive to environmental changes and could be implicated on intestinal dysfunction reported in obesity. We studied the role of a transcription factor known to trigger an immune response to environmental changes, the Aryl hydrocarbon receptor (AhR) (Stange and Veldhoen 2013) and the role of lipid supply on small intestine disturbance. During my thesis, I focused on the effects of the activation of AhR and palmitic acid supply on intestinal homeostasis by studying intestinal epithelial disruption and the initiation of intestinal inflammation. My goal was to study the early stages involved in the occurrence of these events before the onset of obesity and to look beyond the cause-effect relationships for the molecular mechanisms involved.

INTRODUCTION

Introduction

1) Obesity

Overweight and obesity are described as abnormal or excessive fat accumulation that may impair health (World Health Organization (WHO), 2018). Obesity is a state of excess adipose tissue mass. This condition usually translates into excessive body weight (Purnell 2000). Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify overweight and obesity in adults. It is established by a person's weight in kilograms divided by the square of their height in meters (kg/m^2). Overweight is defined as a BMI greater than or equal to $25 \text{ kg}/\text{m}^2$ while obesity is defined as a BMI greater than or equal to $30 \text{ kg}/\text{m}^2$. According to the World Health Organization obesity distinguishes in 4 types in terms of severity, according to BMI: class I for a BMI between 30 and $34.9 \text{ kg}/\text{m}^2$, class II for a BMI between 35 and $39.9 \text{ kg}/\text{m}^2$, and class III for a BMI $\geq 40 \text{ kg}/\text{m}^2$ (WHO, 2018). In turn, class I obesity is associated with a “moderate risk”, class II with a “high risk”, and class III with a “very high risk” of mortality.

Obesity and overweight have reached epidemic proportions (WHO, 2018). In 2016, a report from the World Health Organization indicated that more than 1.9 billion adults, 18 years and older, were overweight. Of these, over 650 million were obese. The pandemic of obesity is also developing in emerging countries, affecting now children, who 41 million under the age of 5 were overweight or obese in 2016 (WHO, 2018). According to a report by the Food and Agriculture Organization of the United Nations (FAO) and the Pan American Health Organization (PAHO), more than half of the Brazilian population is overweight. Based on data from the WHO, overweight in adults increased from 51.1% in 2010 to 54.2% in 2016 in Brazil. The upward trend was also recorded in the national obesity assessment. In 2010, 17.8% of the Brazilian population was obese while in 2016 the index reached 20% (VIGITEL, 2017). In addition, it is also reported an increase in overweight children in this country. It is estimated that 7.3% of children under five are overweight (FAO, 2016). In France, the ObEpi report indicates that 32% of French adults are overweight and 15% are obese (ObEpi, 2012).

Obesity and overweight are associated with an increase of prevalence of comorbidities such as hypertension, diabetes and dyslipidemia (characterized by high cholesterol and high

triglyceride plasma levels). Many obese subjects will develop type-2 diabetes. According to a report from International Diabetes Federation (IDF, 2015) Brazil has around 14.3 million of people with diabetes and more than 3.3 million cases of diabetes in France in 2015, a number that increases year after year concomitantly with the expansion of obesity. Furthermore, high rates of dyslipidemia are seen among the Brazilian population. Gigante et al., conducted research experiments on 49,395 adults living in the state capitals and federal districts and reported 16.5% cases of dyslipidemia (Gigante *et al.*, 2009) (Silva *et al.*, 2014). Thus, obesity and its associated metabolic diseases are a global concern and countries need to join their effort in order to limit and cure these pathologies.

The word “obesity” (from the Latin *obesitas*) indicates the most common behavioral condition leading to obesity, the overeating. In fact, *obesitas* is the condition of the *obesus*, a word that is composed of *ob* (ie, over) and *esus*, ie the past participle of *edere* (ie, to eat) (Purnell 2000). However the etiology of this pathology is complex and cannot be reduced to voluntary overconsumption of nutrients (Rotge *et al.*, 2017). Indeed obesity is a multifactorial pathology resulting from multiple genetic and epigenetic factors associated with environmental factors such as changes in diet and lack of physical activity resulting in a positive energy balance. The control of body weight and composition have to consider energy intake, energy expenditure and fat deposition, which are interconnected and under an integral regulation by the neural and endocrine systems, where different neuropeptides and hormones participate (Gonzalez-Muniesa *et al.*, 2017). A majority of cases of obesity is associated with an imbalance between food intake and energy expenditure due to deregulation of the mechanisms involved in their control. Several agents modify these regulatory processes: environmental factors, overall diet quality, level of physical activity, the gut microbiota, endocrine disruptors, reproductive factors, drugs, as well as intra-uterine and epigenetic intergenerational effects (Gonzalez-Muniesa *et al.*, 2017). Reducing food intake or increasing physical activity can lead to a negative energy balance and a cascade of central and peripheral compensatory adaptive mechanisms that preserve vital functions (Heymsfield and Wadden 2017).

Several studies, including those of our team, have also demonstrated that obesity is characterized by low-grade systemic (Rodriguez-Hernandez *et al.*, 2013; Magalhaes *et al.*, 2015; Monteiro-Sepulveda *et al.*, 2015) and tissue (Dalmas *et al.*, 2011; Dalmas *et al.*, 2014; Monteiro-Sepulveda *et al.*, 2015) (Vernon *et al.*, 2011; Stolarczyk 2017) inflammation. This

inflammatory response is characterized by a systemic increase of molecular markers of inflammation of innate and adaptive immunity (activation of blood immune cells, recruitment of activated immune cells to "inflamed" tissues and finally repair of the tissues) (Ellulu *et al.*, 2017). Regarding intestinal inflammation itself, recent work conducted by our team shows for the first time, that human obese subjects presenting with an increased height of intestinal villi and therefore the absorption surface, display an inflammation of the jejunum characterized by infiltration of T lymphocytes into the epithelium leading to an increased susceptibility to epithelial cells dysfunctions such as reduced enterocyte insulin sensitivity (Monteiro-Sepulveda *et al.*, 2015). Moreover, this work showed a correlation between systemic insulin resistance (HOMA-IR) and mucosal T cell infiltration in non-diabetic obese subjects (Monteiro-Sepulveda *et al.*, 2015). Thus, chronic inflammation, although present at a subclinical level, would contribute to the impairment of cellular functions and metabolic complications associated with obesity.

Initial work in rodents has suggested links between immune cell recruitment, intestinal permeability, microbiota changes, and metabolic complications (Cani *et al.*, 2008). It is well known that obesity is associated with increased risk factors for gastrointestinal diseases (Teixeira *et al.*, 2012). As stated by Bischoff (2011), the gastrointestinal health can present some major criteria and specific signs (effective digestion and absorption of food, absence of gastrointestinal illness, normal and stable intestinal microbiota, effective immune status, and status of well-being) (Bischoff 2011). Perturbations of these signs may negatively influence the gut barrier function. The presence of such disorders being reported in obese subjects suggests impaired intestinal homeostasis. In obesity, an alteration of the intestinal barrier, a change in the composition of the intestinal microbiota and a chronic inflammatory state coexists, making it difficult to identify the triggering factors. Several observations indicate that an alteration of the intestinal barrier would be an early event that would occur before the onset of metabolic disorders and inflammation. A passage of lipopolysaccharide (LPS) through the transcellular route, but also paracellular in the case of an alteration of the intestinal barrier, is proposed (Guerville and Boudry 2016). Besides, our team has described also that obese subjects present subtle barrier alterations in the basal state, however, increased jejunal permeability is revealed by a lipid load and associated with inflammatory and metabolic status (Genser *et al.*, 2018). In addition, studies have shown that obese mice present

a higher permeability of the intestinal barrier, higher endotoxemia of the portal system and a higher level of proinflammatory cytokines compared to the control mice (Brun *et al.*, 2007; Cani *et al.*, 2007; Cani *et al.*, 2008). These findings reinforce the hypothesis of possible links between lipid and low-grade intestinal inflammation, which can be also correlated to intestinal barrier disruption.

Thus whether the molecular mechanisms involved in the onset of intestinal and low-grade systemic inflammation remains largely unknown several shreds of evidence revealed the importance of intestine in this process.

2) Intestine

The gastrointestinal (GI) tract is a multifunctional organ with a complex structure and diverse physiological roles. Rather than a single organ, it is an ecosystem composed of three main components: host cells, microbiota and environmental cues, including nutrients. A main function of the gastrointestinal tract is to provide energy as well as micro- and macronutrients to the rest of the body. In recent years, however, it became apparent that the GI tract is much more than just a digestive and absorptive organ. Indeed, it is an important line of defense against natural toxins and chemicals. It also contains trillions of bacteria and maintains a balance between tolerogenic and inflammatory signals. The GI tract is also the largest endocrine organ of our body by the amount and diversity of signaling molecules that it secretes and which communicate with the rest of the body, including the brain. All these functions are strongly related to the particular structure and morphology of the GI tract (Le Gall *et al.*, 2018).

In humans and other vertebrates, the digestive system consists of a tubular gastrointestinal tract and related digestive organs. The general anatomy of the gastrointestinal tract is shown in Figure 1. The digestive system begins with the oral cavity and is followed by the pharynx, esophagus, stomach, small intestine consisting of three segments (duodenum, jejunum and ileum), colon and ends with the rectum and anus. The digestive tract is associated with other organs such as the liver, gallbladder or pancreas that are also involved in physiological processes, especially during digestion by the secretion of different hormones and digestive enzymes.

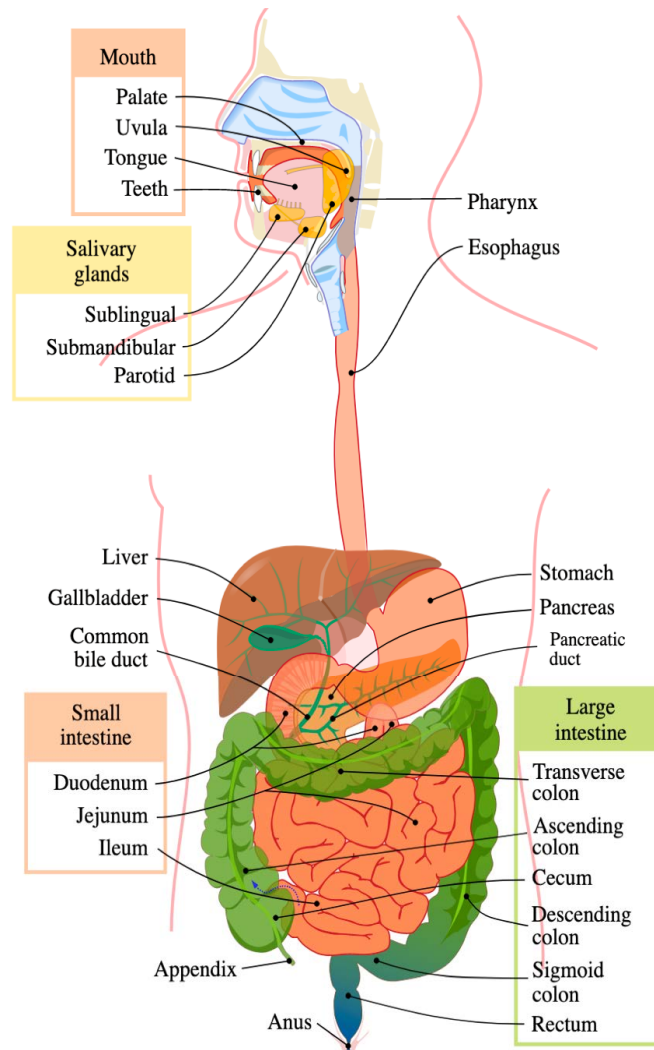


Figure 1: Organs and structures in the gastrointestinal tract.

The digestive tract includes the oral cavity, followed directly by the pharynx and esophagus, stomach, small intestine, large intestine and anus. These organs are interconnected and interact with each other via afferent nerves with the liver, gallbladder and pancreas to ensure in particular the digestive function.

(<https://biologydictionary.net/digestive-system/>)

The food is crushed by the teeth, compacted by the tongue and moistened by the saliva produced by the salivary glands. The result of the combined action of these organs forms what is called the bolus. Following swallowing comes the esophagus, a muscular tube that transfers the food bolus to the stomach, where digestion continues. The bolus is then transformed into chyme, a whitish porridge, which, under the effect of contractions of the stomach, will pass into the small intestine where a battery of digestive enzymes continues the digestive process. In the proximal part of the small intestine, chemicals from the liver, pancreas (pancreatic juices) and gallbladder (bile) mingle with the chyme and break down the chyme into absorbable nutrients. The jejunum is the major site of macronutrient absorption, while colon ensures the absorption of electrolytes and water. The remaining product forms the feces, which can remain in the rectum for several hours before finally being evacuated through the anus.

In humans, longitudinally, the intestinal tract can be divided into the small intestine (duodenum, jejunum and ileum) and the large intestine (cecum and colon), the small intestine is 6 m long and about 2 cm in diameter and the large intestine is 1.5 m long and about 4 cm in diameter. In the mouse, the small intestine measures between 35 and 45 cm and the large intestine measures between 10 and 15 cm. Several folding structures make it possible to considerably increase the exchange surface between the epithelium and the intestinal lumen. The mucosa is itself folded into villi 1 mm high. At the microscopic level, the apical membrane of intestinal epithelial cells is composed of regular microvilli formed by evaginations of the plasma membrane. The colonic epithelium differs from that of the small intestine because it is devoid of villi. It consists of epithelium and crypts.

The gastrointestinal tract also contains a large network of neurons that are interconnected and allow the regulation of digestive functions. This network of nerve cells is called the submucosal plexus (or Meissner's plexus) in the submucosa and the myenteric plexus (or Auerbach's plexus) between the inner and outer muscular layers.

The intestinal micro-anatomical morphology varies along the gastrointestinal tract but there are common features in the overall organization of the tissue (Figure 2). Transversely, from the external serosa to the lumen, the intestine is constituted by 2 muscle layers and covered by mucosa. The mucosa from lumen to internal layer consists of a monostriated epithelium, lamina propria and *muscularis mucosae*. It is largely innervated and vascularized with microvessels and lymph vessels.

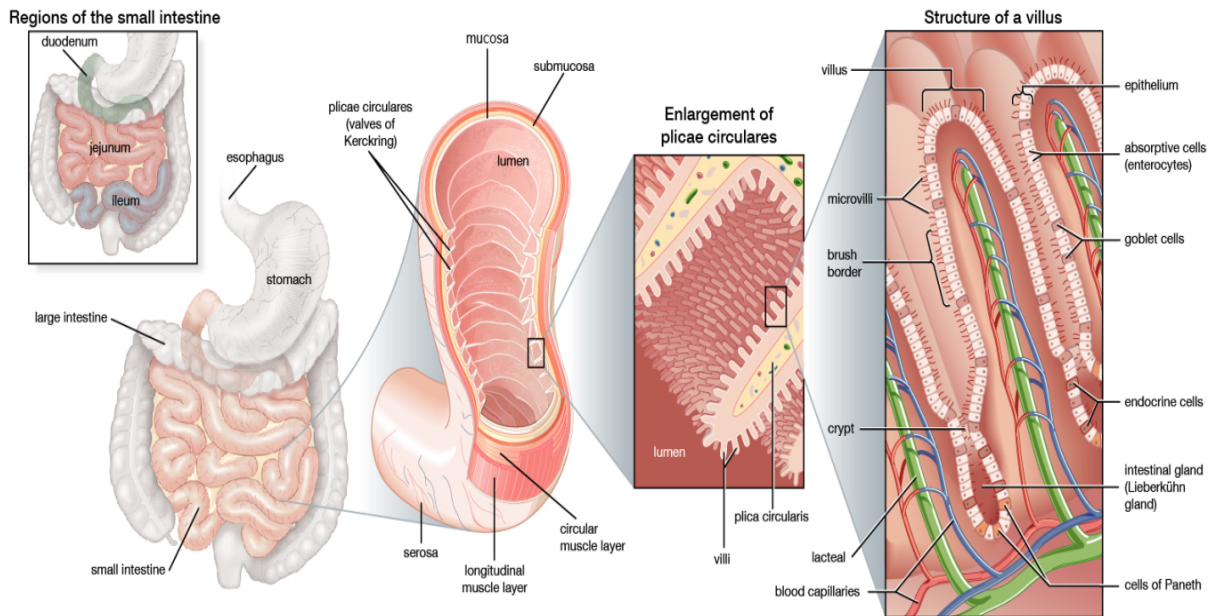


Figure 2: Anatomy and surface of the small intestine in humans.

The small intestine measures about 6 meters in length, is wrapped in the abdominal cavity with folds forming intestinal loops. The intestinal mucosa presents convolving valves which allow to triple the exchange surface between the epithelium and the intestinal lumen. These structures form villi which increase this surface in factor 10. Concerning a cytological view, each enterocyte contains microvilli on its apical surface, these folds of the plasma membrane will multiply the surface by 500. The surface of the small intestine epithelium is estimated at 32 m², the equivalent of a badminton court. (Encyclopaedia Britannica, 2014)

The muscularis ensures peristalsis that allows the advancement of the bolus along the gastrointestinal tract. It consists of two layers of smooth muscle cells: the circular inner layer and the longitudinal outer layer.

The surface of the small intestine is greatly enlarged through mucosal protrusions called villi. These cells migrate from the crypt proliferative compartment to the villus tip where they undergo the replacement of epithelial cells eliminated by anoikis, which is a particular form of cell death triggered by the loss of adhesion between the cell and the extracellular matrix at the apex of the villi (Vachon 2018), cell are then shed into the lumen (Figure 3). The renewal of the epithelium takes approximately 3-5 days in humans (Umar 2010; Darwich *et al.*, 2014). It is the intestinal stem cells at the base of the Lieberkühn crypts that allow this constant renewal. The progeny of these stem cells then migrate along the crypto-villous axis and differentiate into several cell types.

Several types of differentiated epithelial cells cover the villi: absorptive enterocytes, M cells which act as antigen-presenting cells and a gateway to the microorganisms (Lelouard *et al.*, 2001), goblet cells producing mucous, tuft cells, which play an essential role in the initiation of the type 2 immune response during parasitic infections (Gerbe *et al.*, 2016; Banerjee *et al.*, 2018) and hormone-secreting enteroendocrine cells (Crosnier *et al.*, 2006; van der Flier and Clevers 2009). Paneth cells settle at the crypt bottoms after differentiation and exert innate immunity function by producing anti-microbial peptides. In addition, the epithelium also contains lymphocytes. Underlying the epithelium, the lamina propria (LP) is a connective tissue, composed of collagen and elastin fibers that contain numerous blood and chyliferous capillaries through which absorbed nutrients transit. It contains smooth muscle cells, fibroblasts and immune cells.

Besides, the intestine is the largest surface of the lining of the human body and is a critical interface between the host and the external environment. The gut epithelium consists of a single layer of intestinal epithelial cells (IECs) that are crucially important for nutrient uptake and provide a barrier against harmful substances. A fundamental function of the intestinal epithelium is to act as a protective barrier that regulates interactions between the luminal contents and the remainder of the body while controlling the absorption and secretion functions necessary for its digestive activity.

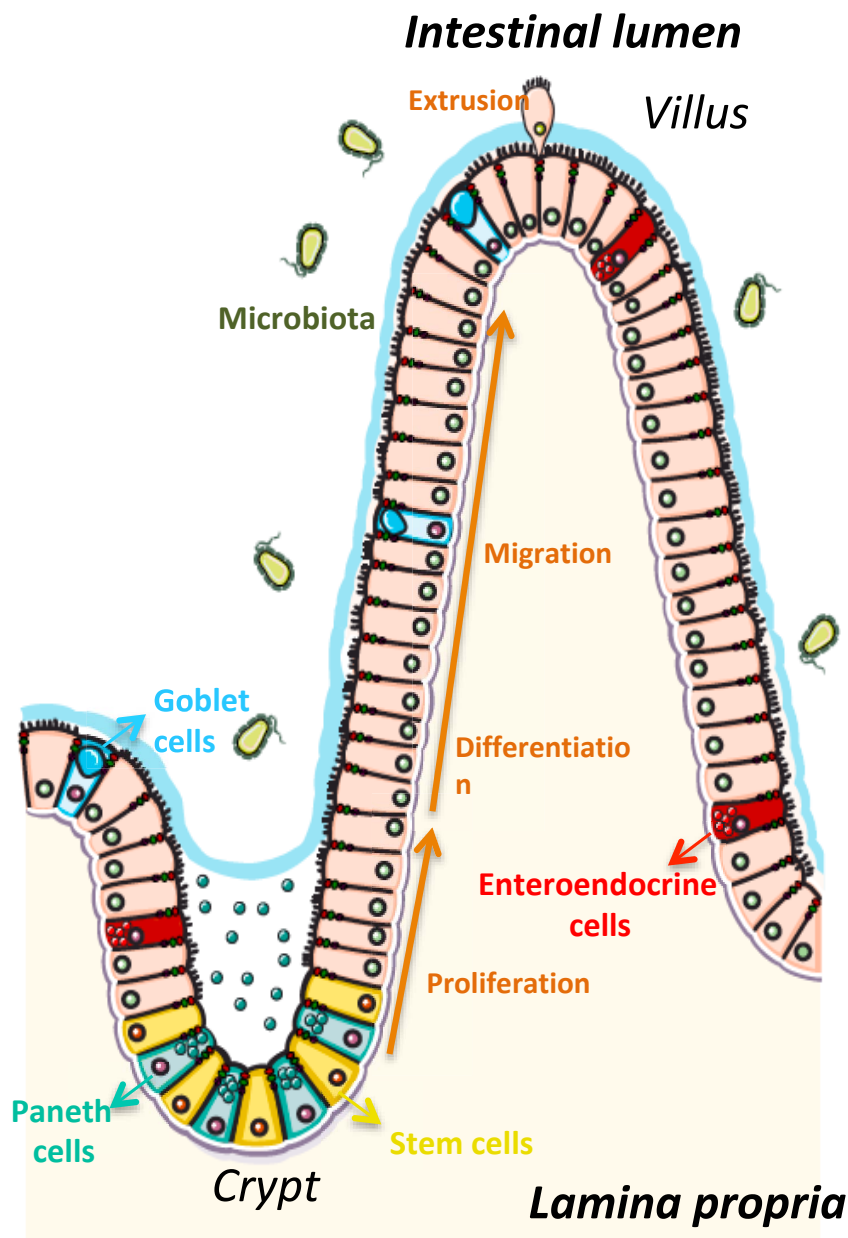


Figure 3: Architecture of the small intestinal epithelium and schematic representation of its different cell types.

The small intestine presents a crypt-villus structure. The crypt, which is the proliferative compartment, contains stem cells and Paneth cells. The intestinal epithelial stem cells in the crypts produce the proliferating cells that differentiate while migrating up the villus, except for Paneth cells. Villi include enterocytes, M cells (at the ileum), enteroendocrine cells, mucus-secreting goblet cells, and tuft cells. Secretory goblet cells and Paneth cells secrete mucus and antimicrobial proteins, respectively, to promote the exclusion of microbiota from the epithelial surface. Differentiated cells are extruded into the intestinal lumen through a process called anoikis. The intestinal epithelium is based on the lamina propria which is populated by immune cells. (Adapted from Le Gall *et al.*, 2018)

Epithelial barrier function requires a contiguous layer of cells as well as junctions sealing the cellular space between epithelial cells. The intestinal tissue needs to be highly regenerated in order to maintain its integrity against constant physical, chemical and biological pressures (Le Gall *et al.*, 2018; Olivares-Villagomez and Van Kaer 2018).

The intestinal barrier is the largest interface between the body and the external environment. It is a complex structure presenting around 300-400 m² of surface area and composed of four main sections: the microbiological, chemical, immunological and physical barriers (Figure 4). The main function of this barrier is to limit the access of intestinal lumen contents, including bacterial components of the microbiota, to the blood circulation and the internal environment (Odenwald and Turner 2013).

In the following chapters, I described the importance of microbiological, chemical, immunological and physical barrier for intestine functions.

2.1) Microbiological barrier

The digestive tract is populated by microorganisms, mainly bacteria but also viruses, yeasts, fungi and archaea, whose abundance increases from duodenum to the colon. The intestine is home, both permanently and transiently, to a complex microflora. Many studies have focused on the dynamic and complex interactions between pathogens and the intestinal epithelium, which often leads to disturbances in the intestinal barrier, altered fluid and electrolyte transport and the induction of an inflammatory response. Since the technological advances allowing the more and more precise characterization of the metagenome, the demonstration of a dysbiosis in several inflammatory or metabolic diseases made it possible to hypothesize about the implication of the host-microbiota relationship in the occurrence of these pathologies and in particular via its role in maintaining the integrity of the intestinal barrier (Groschwitz and Hogan 2009).

The gut microflora, which is composed of approximately 10¹¹ number of bacteria per ml of content, approximately the number of body cells (Sender *et al.*, 2016), is now considered as a functional human pseudo-organ. Comprised of 500-1000 species, this complex ecosystem is established in humans from birth, stabilizes over time around the age of 2 years and remains stable over time. Although disruptive episodes such as antibiotics may exist, a return to equilibrium is possible, it is the phenomenon of resilience (Villanueva-Millan *et al.*, 2015). Moreover, although the composition of the gut microbiota in terms of

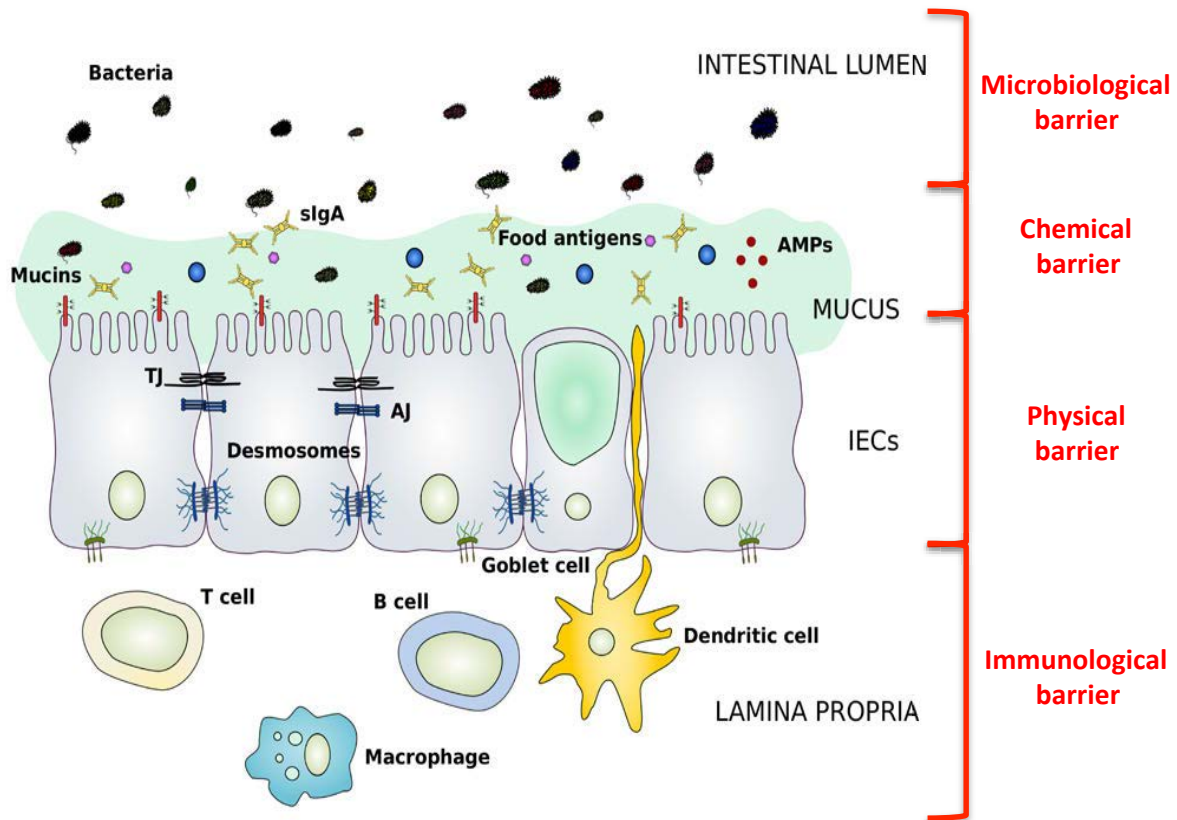


Figure 4: Schematic representation of the main components of the intestinal barrier

The intestinal barrier is a semipermeable structure that allows the immune sensing and the uptake of essential nutrients, while being restrictive against pathogenic molecules and bacteria. The microbiological barrier is formed by microorganisms, mainly bacteria but also viruses, yeasts, fungi and archaea, with a maximum concentration gradient reached in the colon. It is an essential component of intestinal barrier function that influences epithelial metabolism, proliferation and survival. The mucus layer forms a chemical barrier covering and protecting the intestinal epithelium. Antimicrobial peptides (AMPs) and secretory IgA molecules (sIgA) are secreted in the mucus layer as immune-sensing and regulatory molecules. The intestinal epithelial cells (IECs) form a continuous monolayer and physical barrier. Cells are tightly attached to each other by junctional complexes, such as the tight junctions (TJs), the adherens junctions (AJs) and desmosomes which ensure the maintenance of the integrity of the intestinal barrier. The lamina propria contains immune cells (e.g. T cells, B cells, macrophages and dendritic cells) from the adaptive and innate immune system forming the immunological barrier. It takes part in the immunological defense mechanisms of the intestinal barrier. AMP, antimicrobial peptide; sIgA, secretory immunoglobulin A; IECs, intestinal epithelial cells; TJ, tight junction; AJ, adherens junction

(Adapted from Vancamelbeke and Vermeire, 2017)

species is specific to each individual, it remains nevertheless similar between individuals in terms of abundance of large phylogenetic groups (Shreiner *et al.*, 2015).

This microbiological barrier is an essential component of intestinal barrier function that influences epithelial metabolism, proliferation and survival. The normal gut microflora provides protection against infection, educates the immune system, ensures tolerance to foods, and contributes to nutrient digestion and energy harvest and is important in the induction of the host innate response (Huang *et al.*, 2013; Moya and Ferrer 2016).

The abundance of intestinal microbiota is distributed mainly in five phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia (Figure 5) (Rajilic-Stojanovic and de Vos 2014). The phylum Firmicutes is mainly composed of bacteria belonging to the Clostridia group of clusters XIVa and IV including respectively the groups *Eubacterium rectal-Clostridium coccoides* and *Clostridium leptum*. The group *Eubacterium rectal-C. coccoides* includes species belonging to the genera *Clostridium*, *Eubacterium*, *Rhuminococcus* and *Butyrivibrio* while the group *C. leptum* includes the species *Faecalibacterium prausnitzii*, *Ruminococcus albus* and *R. flavefaciens*. Another diverse, group of the Firmicutes is the class *Bacilli* that includes the genera of *Lactobacillus*, *Enterococcus*, and *Streptococcus*, which are dominant in the upper part of the gastrointestinal tract. The second most represented phylum is that of Bacteroidetes comprising several species of Bacteroides: *B. thetaiotaomicron*, *B. fragilis*, *B. ovatus* and *B. caccae* (Rajilic-Stojanovic and de Vos 2014). Identification of microbiota composition has received a considerable boost since the developing of high-throughput sequencing tool of the bacterial gene coding for 16S ribosomal RNA. The microbiota of an individual contains about 750,000 genes, compared to 25,000 genes for the human genome, giving to the microbiota its importance in the regulation of physiological functions (Shreiner *et al.*, 2015).

More than 90% of the intestinal bacterial species are not cultivable since the majority lives in a particular physicochemical and anaerobic environment. Such conditions are difficult to set up in the laboratory. However, several approaches exist to study the microbiome and its function in a physiological and physiopathological context (Jubelin *et al.*, 2018). Systems such as the TIM (TNO gastrointestinal Model), SHIME (Simulator of the Human Intestinal Microbial Ecosystem) or the *in vitro* model of piglet colon (PigutIVM), allow dynamic studies of changes in microbiota composition as well as microbiota-dependent digestion of nutrients in anaerobic conditions including controlled pH, temperature,

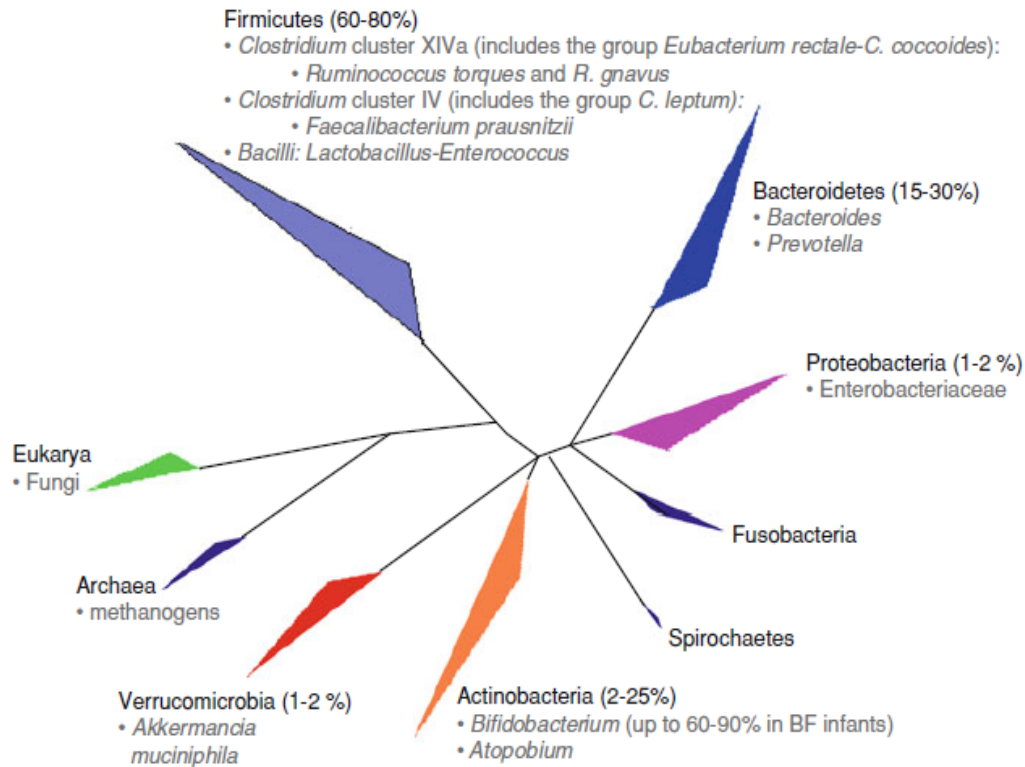


Figure 5: The schematic presentation of a phylogenetic tree of microorganisms, with the main bacterial phyla, composing the microbiota present in the human colon.

The relative abundance of major phyla of human intestinal microbiota is shown. In parenthesis, the proportions of the total microbiota are expressed as a percentage. The major groups, genera and bacterial species that have been implicated in some human pathologies are included in the phylogenetic tree. BF: breast-fed. (Cheng *et al.* 2011)

flow rates etc (Fleury *et al.*, 2017; Dupont *et al.*, 2018; Jubelin *et al.*, 2018). Several of these models have for objectives to adapt epithelial cells containing a module for studying host-bacteria cross-talk.

The protective role of the intestinal microbiota against colonization by pathogens potentially deleterious to the body goes through different actions. Indeed, symbiotic bacteria limit pathogen colonization by competing for adherence to epithelial surfaces, producing antimicrobial compounds, and stimulating mucin production. Also, commensal bacteria limit the accessibility of pathogenic bacteria to nutrients by competitive effects and modulate responses of host cells to inhibit the growth of pathogens. Bacteria communicate and interact with body cells but also with other bacteria via the quorum sensing which is a mode of communication using chemicals secreted and captured by bacteria to coordinate their metabolism and other processes complexes such as the formation of a biofilm (Srivastava *et al.*, 2017).

The intestinal microbiota provides crucial functions for the host such as nutrient acquisition and energy regulation and influences processes such as predisposition to obesity, immune homeostasis, inflammation, repair and angiogenesis. In eubiosis status, a mutualistic and symbiotic relationship exists between the intestinal microbiota and the host organism. Commensal bacteria contribute to the development and maturation of the immune system, the synthesis of certain vitamins, the production by epithelial cells of antimicrobial peptides, secretory IgA, cytokines, mucus and promote the integrity of the epithelial barrier (Duerkop *et al.*, 2009; Ihara *et al.*, 2017). The microbiota contributes to the metabolic functions of the host by biotransformation of different food substrates to obtain energy for survival and bacterial growth and to generate metabolites that are absorbable and usable by intestinal epithelial cells. Among these substrates, the undigested food fibers by the host will be supported by the bacteria thus providing the main source of energy for the microbiota in the form of carbohydrates (Makki *et al.*, 2018). The glycolytic bacteria transform the carbohydrates into pyruvate via glycolysis and then into short-chain fatty acids (SCFA), the final products of fermentation such as butyrate, propionate and acetate (Morrison and Preston 2016). Acetate and propionate pass into the blood compartment, reach the liver and then the peripheral organs where they will be used as substrates for gluconeogenesis and lipogenesis. An imbalance in the richness and diversity of bacterial communities is observed in several

metabolic and inflammatory pathologies (Young 2012; Wang *et al.*, 2017; Valdes *et al.*, 2018) and its role in intestinal barrier function seems to be crucial.

I present below some examples showing how bacteria species or metabolites produced by bacteria modulate of intestinal functions in particular barrier function and immune response.

Butyrate produced by gut microbiota has been described as having protective properties for the colonic mucosa (Bedford and Gong 2018; Wu *et al.*, 2018). It affects cellular proliferation and differentiation, increases intestinal blood flow, and inhibits inflammation by reducing the expression of proinflammatory cytokines such as IL-1 β , TNF- α and IL-8 and inducing IL-10 or TGF- β anti-inflammatory cytokines (Bedford and Gong 2018). Also, butyrate decreases oxidative stress and enhances the colonic epithelial barrier by participating in the formation of tight junctions in the colonic epithelium (Hou *et al.*, 2014).

Studies with axenic mice or intestinal flora depleted mice by antibiotic administration have been performed in order to establish the role of gut microbiota in epithelial barrier dysfunction. Some of these studies have shown that these mice are more susceptible to chemically induced inflammation (Natividad and Verdu 2013). Enteric bacteria can disrupt the intestinal barrier either directly, by binding to cell surface molecules and inducing changes in TJ protein expression. Bacterial fragments such as LPS produced by gram-negative bacteria are also able to increase permeability via activation of TLR receptors present on intestinal epithelial cells (Guo *et al.*, 2013).

A direct role of an adherent and invasive pathogenic strain of *Escherichia coli* (AIEC) on the intestinal epithelial barrier has been observed (Shawki and McCole 2017). The role of this bacterial species on the intestinal barrier involves several cellular and molecular actors, including increased permeability to ions and macromolecules, changes in membrane localization of ZO-1 and E-cadherin (Wine *et al.*, 2009). Beyond these direct effects, the AIEC following its translocation through the epithelium is able to activate the secretion of cytokines by the immune cells of the lamina propria and to increase the secretion of TNF- α , which could also contribute to amplifying the disturbances of the barrier (Eaves-Pyles *et al.*, 2008).

A commensal bacterium, the *Akkermansia muciniphila* (AKK), has been studied concerning its involvement in various pathologies such as type 1 diabetes, IBD and obesity

(Derrien *et al.*, 2017). Some papers showed that this bacterium could play a beneficial effect on the homeostasis of the intestinal epithelium and the decrease in its abundance is correlated with the pathologies cited above (Reunanen *et al.*, 2015; Derrien *et al.*, 2017). On epithelial cell models in culture, AKK strengthened epithelial monolayer integrity (Reunanen *et al.*, 2015). AKK has also the ability to control gut microbiota, to secrete long-chain fatty acids, to increase mucus production and to stimulate immune defenses (Derrien *et al.*, 2017).

2.2) Chemical barrier

The first layer of defense in the epithelium of the gut is formed by a layer of mucus, which is critical for the limitation of the exposure of epithelial cells to the microbiome (Chelakkot *et al.*, 2018). The mucus layer forms a semipermeable, highly hydrated gel composed of 95% water, electrolytes, lipids, immunoglobulins, antimicrobial peptides and glycoproteins called mucins. The mucus is synthesized and secreted by the goblet cells, which form about 5% of the small intestine epithelial cells and up to 15% of the distal colon epithelial cells. Because of that, the goblet cells are responsible for the constant renewal of this mucus layer (Birchenough *et al.*, 2015; Johansson and Hansson 2016).

The secretion rate of mucus in the gastrointestinal tract is estimated at about 10 liters per day, so it is produced and then eliminated continuously to ensure permanent protection of the underlying structures (Cone 2009).

The thickness of the mucus layer is determined by the balance between the level of synthesis/secretion and degradation/elimination and differs along the digestive tract. The small intestine presents one layer while the colon consists of two distinct layers. The first one is an adherent inner layer, firmly attached to the epithelial cells, free of bacteria and having a thickness of about 50 μ m at 100 μ m in the rodent. The second layer presents in the colon is a looser outer layer potentially colonized by commensal bacteria up to 100 μ m and 700 μ m thick in mice and rats, respectively (Atuma *et al.*, 2001; Johansson *et al.*, 2008) (Figure 6). The colonic epithelium is therefore in continuous interaction with an abundant microbiota. Various factors such as hormones, neuropeptides and inflammatory mediators such as prostaglandin E2 (PGE2) or inducible nitric oxide synthase (iNOS) can modulate the thickness of the mucus layer by stimulating the secretion of mucins (Phillipson *et al.*, 2008).

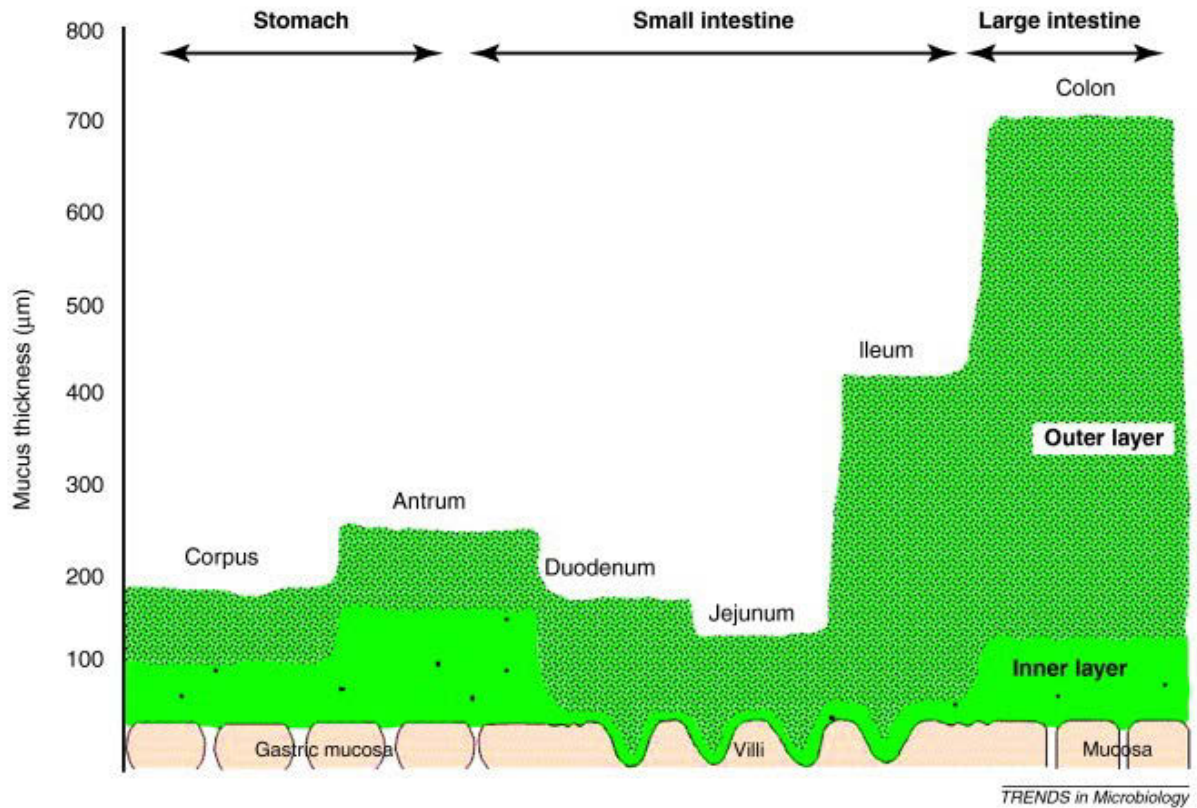


Figure 6: Schematic representation of the mucus layers thickness along the rat gastrointestinal tract.

The intestinal epithelium surface is covered by two layers of mucus: the inner most firmly attached to the mucosa and is practically devoid of bacteria; the outer layer, loosely adherent and potentially colonized by commensal bacteria (as schematized with black dots). (Adapted from Juge 2012)

The mucus consists essentially of water and mucins. Mucins form a glycoprotein network that prevents the deep penetration of pathogenic microorganisms and provides a habitat for commensal bacteria through the interaction between bacterial surface adhesins and mucin glycans. These glycanic residues act as receptors for pathogens that are then trapped in the mucus and then eliminated via the flow of mucus generated by peristalsis (Juge 2012).

Thus, the mucus layer contributes to the protection of the epithelium and protects the underlying epithelial cells against unwanted substances and invasion of pathogenic bacteria and acts as a lubricant to minimize shear stress on the physical barrier. Besides, it is a dynamic defense barrier containing antimicrobial peptides (immunological barrier) that helps prevent contact between bacteria and the epithelial layer.

2.3) Immunological barrier

The organized lymphoid tissues unencapsulated and composed by immune cells and are found associated with the mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts, they are called MALT (mucosa-associated lymphoid tissue). In addition to MALT, a diffuse lymphoid tissue composed of widely distributed immune cells can be found in the lamina propria of the mucosal surfaces. The mucosal immune system contains the largest reservoir of immune cells in the body. It is in close contact with the outside environment, including nutrients, microbiota, pathogens, and environmental antigens, and this requires fine-tuning of the immune response to enable efficient responses against pathogens while maintaining tolerance to innocuous stimuli (Yap and Marino 2018). Thus, the development and progression of the complex defense system and immunological responses at this lymphoid tissue should be regulated, avoiding an exacerbated and harmful reaction to the body against the antigens, while at the same time promoting an effective response against pathogens when necessary.

Recent studies have highlighted the central role of the intestine as a site of immunological events participating in the progress of several diseases, including metabolic diseases such as diabetes and obesity. The intestinal mucosal immune system forms the largest part of the body's immune tissues. The vast majority of immune cells within the intestinal epithelium are lymphocytes that are referred to intestinal intraepithelial lymphocytes

(IELs), which are the T cells resided interspersed among the epithelial cells (Olivares-Villagomez and Van Kaer 2018). IELs are at the interface between the external environment and the body and provide immediate immune protection against initial entry and spreading of pathogens, thereby contributing to the intestinal barrier. Besides, the IELs are resident in the intestinal epithelium and they express several characteristic surface receptors, which interacts with those produced by IECs and thus assists in recruiting IELs to the gut mucosa (Olivares-Villagomez and Van Kaer 2018). IELs also need to display regulatory functions to avoid excessive inflammation that could endanger the integrity of the intestinal barrier (Cheroutre *et al.*, 2011).

In the small intestine, the intestinal immune system is composed of an initiation compartment of the immune response, defined as the organized gut-associated lymphoid tissues (GALTs). GALT is constituted of dense lymphoid tissue, represented by lymphoid follicles isolated in the large intestine or forming aggregates of germinal centers with lymphoid follicles as in Peyer's plaques (Figure 7) (Mowat 2003; Pearson *et al.*, 2012). The Peyer's patches representing groups of non-encapsulated lymphoid follicles located between the mucosa and the submucosa of the small intestine, in particular in the ileum. Peyer's patches and lamina propria are both drained by lymphatic vessels to the mesenteric lymph nodes (Mowat 2003; Olivares-Villagomez and Van Kaer 2018). The intestinal immune system has a wide variety of cell types and can be schematically separated into an innate component consisting of epithelial cells, macrophages, dendritic cells, neutrophils and NK cells, and an adaptive component consisting of lymphocytes. The immune barrier is provided by the coordinated action of these two components that act synergistically to control the integrity of the lumen/internal environment interface (Cheroutre *et al.*, 2011). The dendritic cells, having captured the antigens, and the lymphocytes sensitized by these antigens then migrate into the mesenteric lymph nodes. Activated lymphocytes then infiltrate the effector sites of the intestinal epithelium and in particular intestinal villi.

In the colon, the lymphoid islands allow the development of an immune response against antigens penetrating by the M cells and contact the lymphocytes and dendritic cells with production of anti-immunoglobulin type A (IgA) antibodies.

Thereby, an imbalance in the homeostasis of the intestinal immune system can result in inflammation of the intestine. The intestinal inflammation is characterized by infiltration of immune cells (intraepithelial lymphocytes, macrophages, etc) into the mucosa and by the

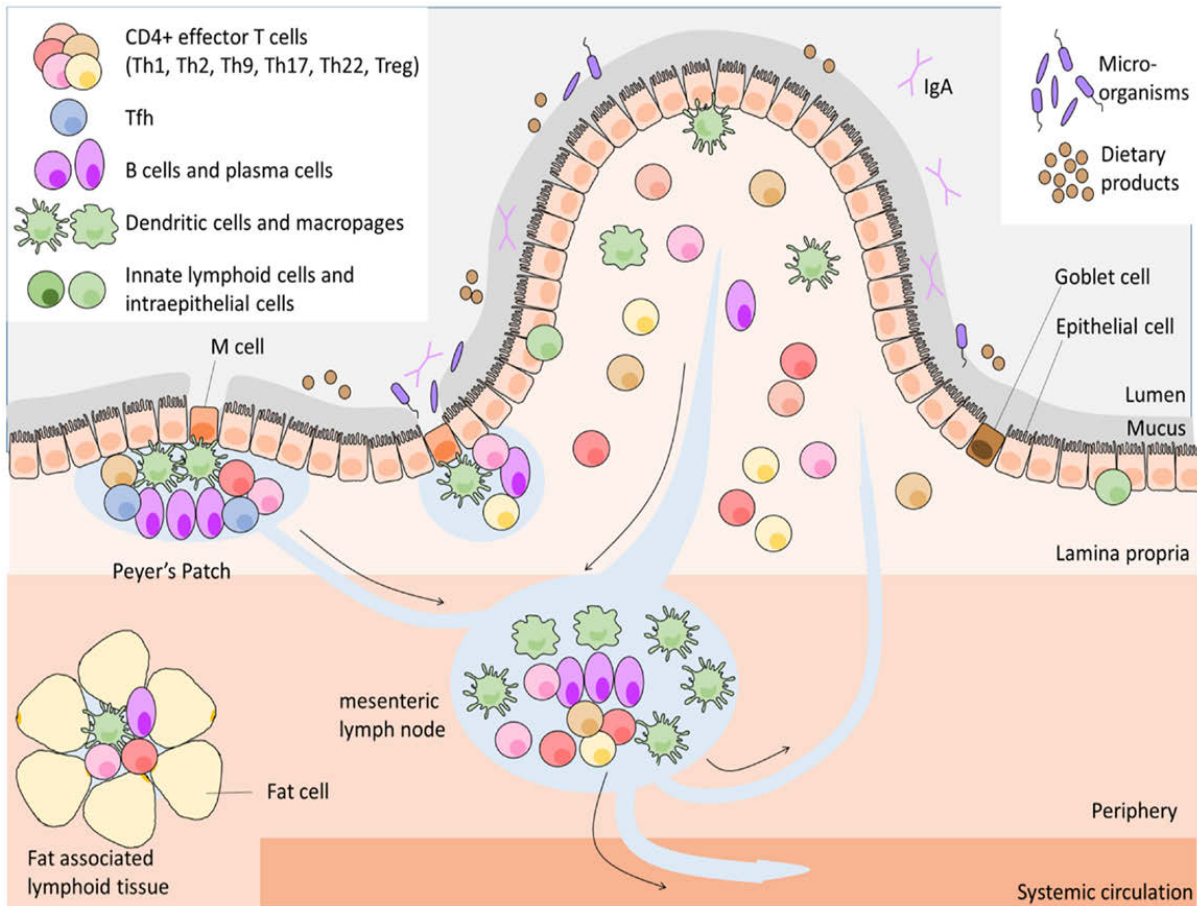


Figure 7: Architecture of gut-associated lymphoid tissue (GALT)

The GALT contains one of the largest lymphoid cell population found anywhere in the body. GALT is distributed along the intestinal tract and is separated from the luminal content. The intestine presents immune cells dispersed in the lamina propria but also interposed between the epithelial cells (intraepithelial lymphocytes). It also has Peyer's patches of lymphoid follicles rich in B and T cells. The passage of bacteria through the M cells at the top of the dome triggers an immune response. The GALT displays three mainly functions: provides antigenic samples from throughout the GI tract; optimizes the opportunities for naïve lymphocytes to encounter antigens, and finally supports the activated lymphocytes and initiates their differentiation.

(Brucklacher-Waldert *et al.*, 2014)

production of pro- or anti-inflammatory cytokines, by several cell types, including enterocytes (Cader and Kaser 2013; Silva *et al.*, 2016; Lee *et al.*, 2018). However, the mechanisms driving to this intestinal dysfunction which seems to be present in several diseases is not yet elucidated and deserves to be more studied.

2.4) Physical barrier

The physical barrier provides a physical constraint and allows the tightness between cells in the intestinal epithelium, resulting in the physical inhibition of the microbial invasion of the mucosa. The physical barrier function is provided by the monolayer of intestinal epithelial cells (IECs) closely interconnected by intercellular junctions as well as by the mucus layer, a common component of the physical and chemical barrier, which covers the apical surface of intestinal epithelial cells (Okumura and Takeda 2018).

The intestinal epithelium is a single layer of cells (IECs) lining the gut lumen and has two critical functions. The first one is to control the communication between the intestinal lumen and the body. This function is essential to prevent the passage of harmful intraluminal entities including foreign antigens, microorganisms and their toxins (Groschwitz and Hogan 2009; Lee *et al.*, 2018). Its second function is to act as a selective filter allowing the translocation of essential dietary nutrients, electrolytes and water from the intestinal lumen into the circulation. The intestinal epithelium mediates selective permeability via two major routes: transepithelial/transcellular and paracellular pathways (Groschwitz and Hogan 2009).

Transcellular permeability is generally involved in the absorption and transport of nutrients, including sugars, amino acids, peptides, electrolytes, short chain fatty acids, minerals, and vitamins through the epithelial cells. As the cell membrane is impermeable, this process is predominantly mediated by specific transporters or channels located at the apical and basolateral membranes (Suzuki 2013).

Paracellular permeability is associated with passive transport in the space between epithelial cells and is stringently regulated to permit the passage of only certain solutes and fluids, creating a selectively permeable barrier (Suzuki 2013). In the presence of an intact epithelial cell layer, the paracellular pathway between cells is sealed. It is ensured by intercellular junction complexes localized at the apical part of the lateral membrane and along

the lateral membrane (Groschwitz and Hogan 2009; Turner 2009; Le Gall *et al.*, 2018; Lee *et al.*, 2018).

2.4.1) Cell-cell junctions and paracellular permeability

Intestinal epithelial cells, and in particular enterocytes, are specialized, and by differentiation acquire a highly polarized phenotype. The cells present characteristic microvilli at their apical pole and their nucleus is located at the basal pole of the cell. In addition, the intestinal epithelial cells establish strong junctions between them. These cell-cell junctions are essential for the physical maintenance of the organs and constitute an important signaling platform. There are four types of intercellular junctions with distinct functions: communicating junctions (gap junctions), desmosomes, adherens junctions (AJs) and tight junctions (TJs), the last three being connected to the cytoskeleton and involved in cell-cell adhesion (Figure 8).

At the cellular apical pole, the establishment of tight junctions allows delimitation between the apical and the basolateral pole of the plasma membrane and confers to the intestinal epithelium its barrier function face to the external environment. Below tight junctions, the cells form between them adherent bonds, desmosomes and communicating junctions (Figure 8). Extracellular matrix-cell interactions take place at the basal pole.

Although forming complex multiprotein platforms, these junctions are not fixed structures. They are flexible, dynamic and highly regulated. This is essential in view of the permanent renewal of the intestinal epithelium, integrating dynamic events such as the extrusion of an epithelial cell or the migration of cells along the villi, or even peristalsis in certain regions of the intestine.

2.4.1.1) Tight Junctions

Among the different types of cellular junctions that occur between intestinal epithelial cells, tight junctions (TJs) are those that play a predominant role in maintaining the intestinal barrier. The TJs are responsible for sealing the intercellular space and regulating selective paracellular ionic solute transport.

Tight junctions are multi-protein complexes connected to the actin microfilaments. These protein complexes are composed of transmembrane proteins, peripheral membrane (scaffolding) proteins and regulatory molecules that include protein kinases. They are

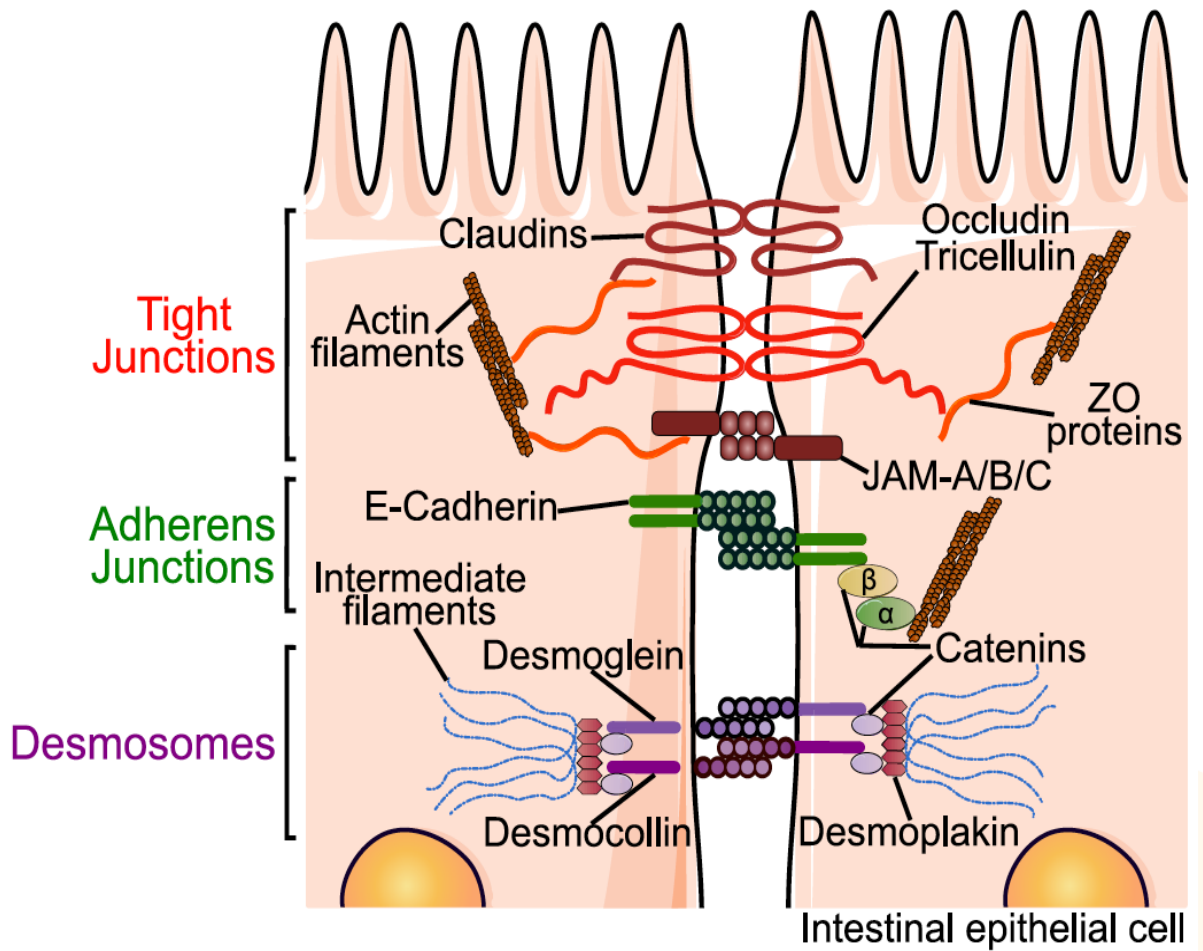


Figure 8: Intercellular junctions' structure.

Simplified scheme of the three major junctions which form the apical junctional complex, involved in cell–cell adhesion in intestinal epithelial cells: tight junctions (TJs), adherens junctions (AJs), and desmosomes. Tight junctions are responsible for controlling the paracellular permeability through transmembrane proteins: claudins normally ensure a selective permeability to cations and anions, whereas occludin and tricellulin control mainly the passage of macromolecules. The TJs are connected to the actin cytoskeleton *via* adapter proteins such as zonula occludens (ZO) proteins. In adherens junctions, E-cadherin is connected to actin by the catenin complex, whereas the molecular complex forming desmosomes is connected to intermediate filaments of keratins.

(Le Gall *et al.*, 2018)

intercellular contacts that "seal" the space between two adjacent epithelial cells to separate the tissue compartments. They are the most apical junctions and they form a kind of adherent belt all around the cell. Nevertheless, they do not only have a role of barrier, they are also important in signaling pathways, involved in the regulation of the cytoskeleton, the control of cell proliferation and the setting up of the cellular polarity which allows defining two functionally distinct domains of the plasma membrane, the apical pole and the baso-lateral pole (Gonzalez-Mariscal *et al.*, 2008).

The biochemical characterization of the tight junction showed that it is composed of a complex of multiple proteins that include transmembrane proteins, cytoplasmic plaque proteins, signaling proteins, and adapters that link it to the actin cytoskeleton (Figure 9). The interaction of TJ proteins with the actin cytoskeleton is essential for the maintenance of the TJ structure and permits the cytoskeletal regulation of TJ barrier integrity (Turner 2009; Lee 2015). The transmembrane proteins are of particular interest. These proteins are the only components of the junction that have intramembranous and extracellular portions, which probably give them the ability to mediate the main functions of the firm junction: barrier and pore.

The tight junctions limit solute flux along the paracellular pathway and are the principal determinant of mucosal permeability. The permeability of TJs varies in different segments of intestine and low solute permeability characterize the well-formed TJs. This permeability can be determined by measuring the paracellular fluxes of ions and small molecules. TJs permeability to ions determines the transepithelial electrical resistance (TEER). It is important to understand the specific barrier properties of the tight junctions, which can be defined in terms of size selectivity and charge selectivity (Lu *et al.*, 2013).

The members of TJ transmembrane proteins can be separated in three groups: 1) the single transmembrane domain proteins, including junctional adhesion molecule (JAM-A) as the main family agent, 2) the triple transmembrane domain protein, Bves (blood vessel epicardial substance) and 3) the four-transmembrane domain proteins of the claudin and TAMP (tight junction-associated MARVEL proteins) families, which include occludin and tricellulin, as the main representatives of TAMP family (Gunzel and Yu 2013; Garcia-Hernandez *et al.*, 2017). The tight junctions are also subject to constant remodeling. Indeed, occludin and ZO-1 have been shown to diffuse permanently within the plasma membrane to continually form effective junctions (Shen *et al.*, 2008).

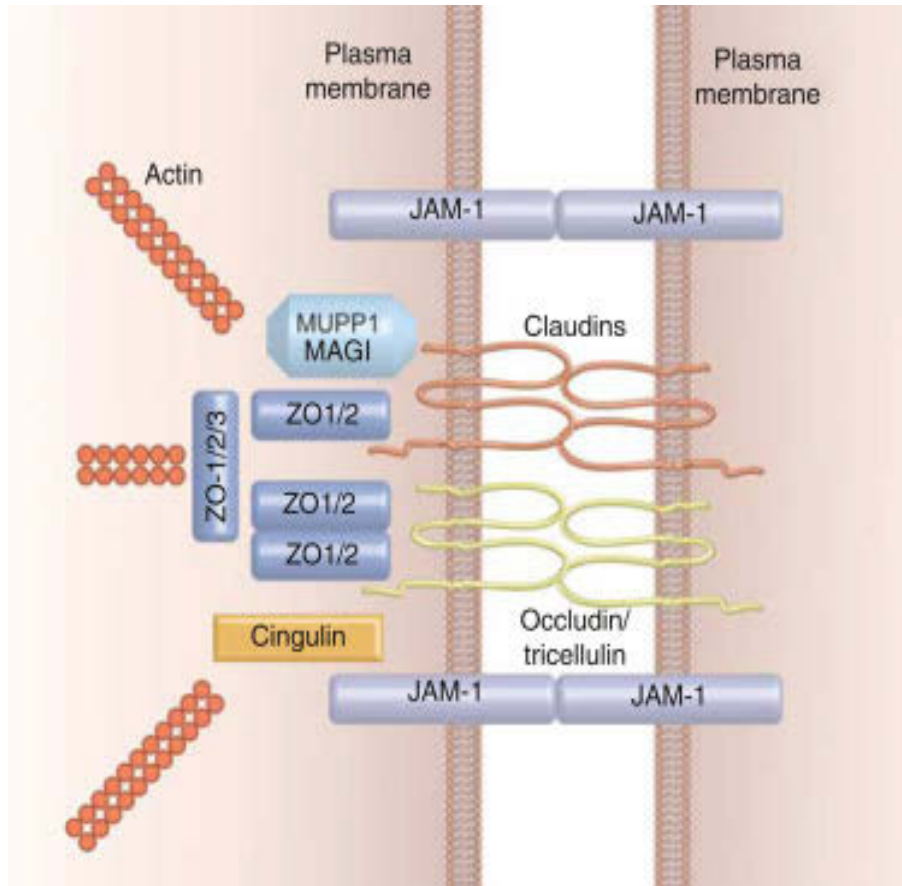


Figure 9: Schematic representation of the basic structural transmembrane components of tight junctions.

Tight junctions control the paracellular permeability. Claudins ensure a selective permeability to cations and anions, whereas occludin and tricellulin control the permeability to macromolecules. Moreover, these proteins are connected to the actin cytoskeleton through several adaptor proteins such as ZO proteins. ZO-1 or ZO-2 is important for clustering of claudins and occludin, resulting in the formation of tight junctional strands. The ZOs and cingulin proteins can provide a direct link to the actin cytoskeleton.

(Niessen *et al.*, 2007)

In the following subchapters, I will only present the junction proteins that I studied in my works. A subchapter is also devoted to the notion of pore and leak pathways.

a) Claudins

Within TJs, claudins appear to be the main structural components and the major determinants of paracellular ion conductance. Claudins are proteins with four transmembrane domains expressed in a tissue-specific manner, and mutation or deletion of individual family members can have profound effects on organ function. There are about 20 claudin isoforms, many of which are often expressed in the same cells; their variable expression profile makes it possible to regulate the barrier function (Odenwald and Turner 2017). The claudin isoforms have distinct charge-selectivity depending on the amino acid charge present in the proteins sequence of their extracellular domain (Krause *et al.*, 2008).

Differential expression patterns of claudin members in the GI tract are likely to contribute to local diversity of ion paracellular flow (Figure 10) (Van Itallie and Anderson 2004; Lu *et al.*, 2013). The claudins can be divided into two main categories, pore-sealing and pore-forming claudins. The increased expression of the pore-sealing claudins (1, 3, 4, 5, 7, and 19) leads to increased tightness of epithelial monolayer reflected by an increased TEER and decreased solute permeability across the monolayer. On the other hand, pore-forming claudins (2 and 15) are able to form paracellular anion/cation pores as well as water channels leading to a decreased epithelial tightness and resulting in an increased solute permeability and decreased TEER (Khan and Asif 2015).

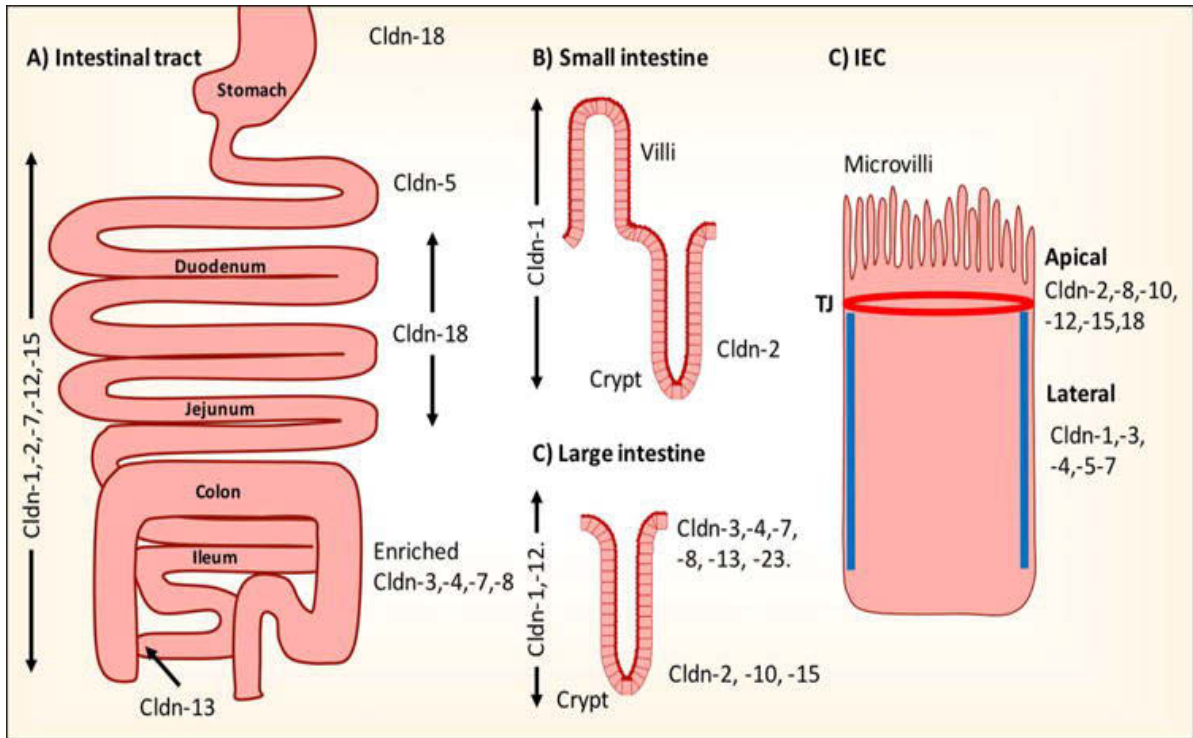


Figure 10: Schematic intestinal claudins distribution

Claudins (Cldn) are present all along the gastrointestinal tract. Different claudins are expressed in different intestinal portions and are responsible for variations in paracellular permeability. IEC: Intestinal Epithelial cells. (Garcia-Hernandez *et al.*, 2017)

b) Occludin

Occludin was the first integral TJ protein to be identified. It was discovered by Furuse *et al.*, and was assigned the name “occludin” from the Latin word "*occludere*" which means restricted passage (Furuse *et al.*, 1993; Feldman *et al.*, 2005). As mentioned above, occludin is a protein characterized by four transmembrane domains (tetraspan transmembrane), presenting one intracellular and two extracellular loops, as well as intracellular N-terminal and C-terminal sequences (Figure 11). The homophilic interaction of extracellular loops of occludin with adjacent cells seems to create a barrier for macromolecules, but not against small ions and seems to be involved in the regulation of paracellular permeability and cell adhesion (Traweger *et al.*, 2002). The long C-terminal domain has been found to interact with several cytoplasmic proteins of the junctional plaque mainly to the guanylate kinase (GuK)-like domain present in ZO proteins, which are required for binding of occludin to the actin cytoskeleton (Cummins 2012).

Many studies have demonstrated that occludin endocytosis is a common feature of tight junction injury and is closely associated with physiological barrier disruption (Yu and Turner 2008; Marchiando *et al.*, 2010; Cummins 2012). There is significant evidence that occludin is a component of detergent-resistant membrane microdomains, although it remains unclear whether occludin plays an active or passive role in organizing this membrane microdomain. However, some studies reported that occludin affects the location and lipid raft partitioning of caveolin-1, a component of caveolae with an important function of complex signaling regulator (Caserta *et al.*, 2008; Van Itallie *et al.*, 2010). Occludin enables caveolin function at the tight junction, where it participates in signaling pathways involved in barrier remodeling. The importance of such cholesterol-rich membrane domains in tight junction function has been also reported in some studies (Van Itallie *et al.*, 2010).

Although occludin knockout mice do not exhibit an increased intestinal permeability, these animals present a complex phenotype, including male sterility, an inability to nurse, and brain calcification. Phosphorylation appears to be a key mechanism for regulating the biological function of occludin, affecting barrier function and transepithelial and transendothelial transport. Occludin dephosphorylation is associated with decreased TEER. Specific occludin phosphosites also regulate occludin's interaction with other TJ proteins, suggesting additional points of the regulation (Bolinger *et al.*, 2016).

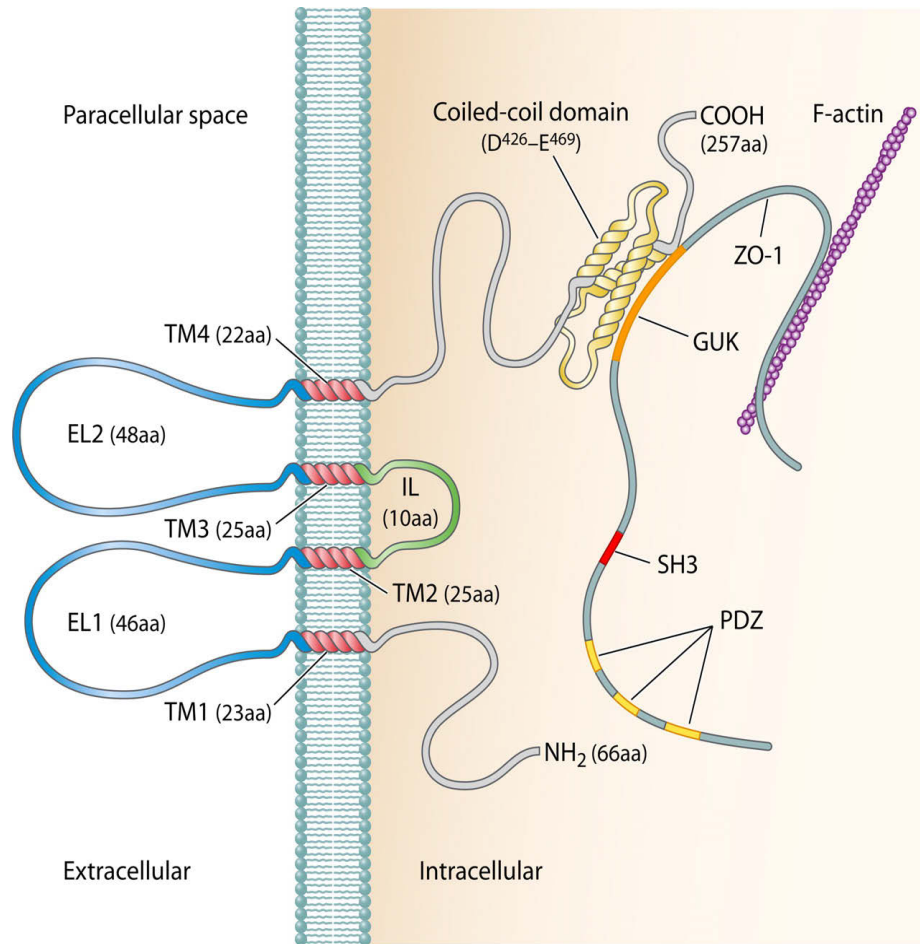


Figure 11: Occludin protein structure.

Plasma membrane-associated human occludin. Individual occludin domains (and amino acid lengths) are indicated. Occludin is a tetraspan transmembrane protein (four transmembrane domains), presenting with one intracellular and two extracellular loops, an intracellular N-terminal sequence and an internal C-terminus. The homophilic interaction of extracellular loops of occludin with adjacent cells seems to create a barrier for macromolecules. The C-terminal domain interact with several cytoplasmic proteins of the junctional plaque mainly to the GuK domain presents in the ZO proteins. GuK EL1/2, extracellular loops 1 and 2; GuK, guanylate kinase domain; IL, intracellular loop; SH3, Src homology 3 domain; TM1 to -4, transmembrane domains 1 to 4. (Cummins 2012)

c) Tricellulin

In contrast to other tight junction proteins implicated in cell-cell contacts between two adjacent cells (bTJs), tricellulin is preferentially localized at the cell-cell contacts of 3 adjacent cells, tricellular junctions (tTJs) although in some conditions it can also be observed at bicellular junctions along with occludin and claudins. Tricellulin is a tetratranspan membrane protein with one intracellular and two extracellular loops, and C- and N-terminal cytoplasmic domains. Studies suggest that the C-terminal domain of tricellulin is important for the lateral translocation of the protein, whereas the N-terminal domain appears to be involved in directing tricellulin to tricellular contacts (Figure 12) (Suzuki 2013).

Recent studies indicated that tricellulin plays an important role in epithelial TJ barrier regulation at both tricellular and bicellular junctions, although different permselective properties exist. When tricellulin is exogenously expressed at low levels in MDCK cells, it is localized at the tTJs, but not at the bTJs. This tricellulin localization is associated with decreased paracellular permeability to macromolecules, but not with small ions. Whereas, when tricellulin is overexpressed, tricellulin localizes at all TJs (bicellular and tricellular) and it decreases the permeability to both macromolecules and small ions. These data suggest that tricellulin forms an effective barrier to macromolecules at the tricellular junctions and to all solutes at the bicellular junctions (Ikenouchi *et al.*, 2005; Oda *et al.*, 2014). Moreover, some studies have demonstrated that occludin and tricellulin seem to affect each other's cellular localization, although the mechanism is unknown (Ikenouchi *et al.*, 2008).

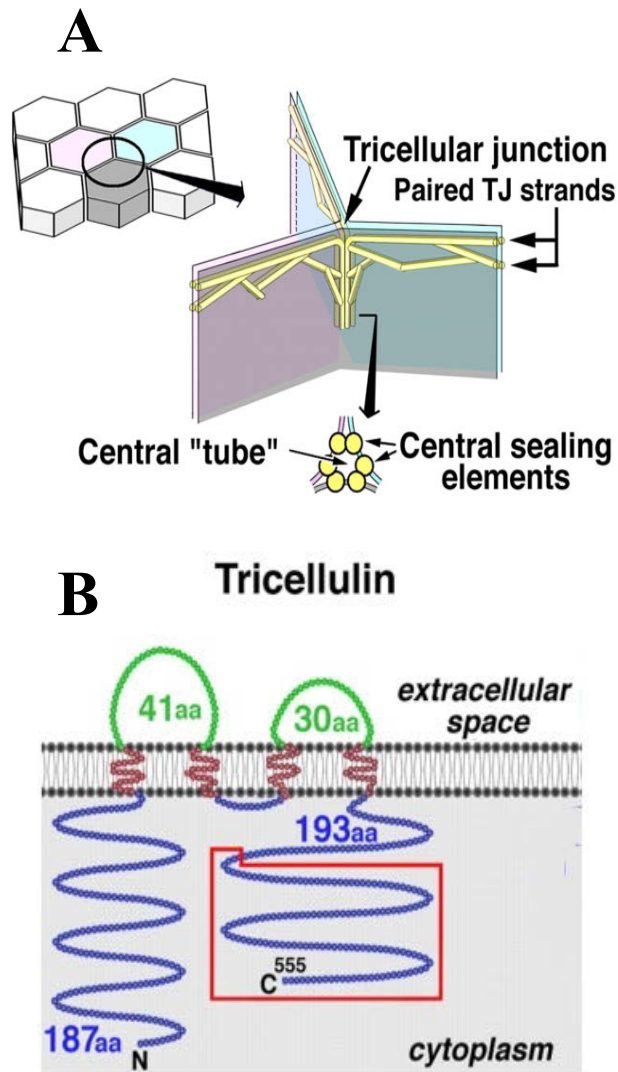


Figure 12: Tricellulin protein structure.

A. Schematic organization of tricellular TJs. One tricellular contact (left drawing) is enlarged in the right drawing.

B. Structure of mouse tricellulin. Tricellulin, as occludin, is a tetra-transpan membrane protein with one intracellular and two extracellular loops, and C- and N-terminal cytoplasmic domains. The first extracellular loop has a high content of tyrosine and glycine residues. The COOH-terminal (C) ~130 amino acids are 32% identical to occludin (boxed in red). However, tricellulin bears a longer NH₂-terminal (N) cytoplasmic domain (187 amino acids) as compared with occludin (61 amino acids). (Ikenouchi *et al.*, 2005)

d) Zonula Occludens

The ZO proteins were the first TJ-specific proteins identified and three ZO proteins, ZO-1, -2, and -3, have been inventoried to date. These ZO proteins are categorized as members of the membrane-associated guanylate kinase homolog family due to their sequence analysis. They are multi-domain proteins carrying three PDZ domains, a Src homology-3 (SH3) domain and a region of homology to guanylate kinase (GuK) (Figure 13). The N-terminal half region of ZO proteins binds to several TJ proteins (claudins, JAMA-A and occludin), while the C-terminal region interacts with the actin cytoskeleton and cytoskeleton-associated proteins. Besides, the ZO family has a second PDZ domain, which is used for interactions with other ZO proteins (Figure 13) (Suzuki 2013; Lee 2015). These multi-domain structures provide an intracellular scaffold in the TJs and are required for regulation and maintenance of TJ structure (Suzuki 2013; Lee 2015).

Interestingly, ZO-1 deficient cells are still able to maintain normal TJ structures and show normal permeability; however, an evident delay in the organization of other TJ proteins, such as occludin and claudins, is observed, indicating that ZO proteins play an important role in the regulation of this protein pool (Lee 2015).

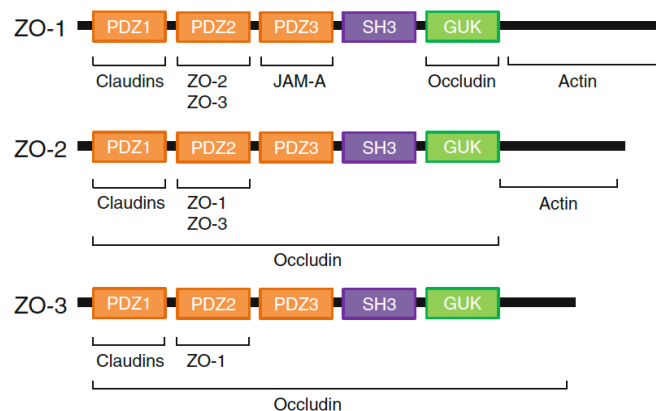


Figure 13: Zonula occludens (ZO) structure and interaction with others tight junction (TJ) proteins.

ZO proteins carry 3 post-synaptic density 95/Drosophila disc large/zona-occludens 1 (PDZ) domains, a Src homology-3 (SH3) domain, and a region of homology to guanylate kinase (GuK). The N-terminal half region of ZO proteins admits the binding of some TJ proteins, while the C-terminal region interacts with the actin cytoskeleton and cytoskeleton-associated proteins. Claudins are known to bind to the first PDZ domains of ZO-1, -2, and -3; JAM-A binds to the third PDZ domain of ZO-1, while occludin is able to bind to the GuK domain of ZO-1. The ZO family has a second PDZ domain, which is used for interactions with others ZO proteins. (Suzuki 2013)

e) Pore and leak pathway

The main function of tight junction proteins is to control the paracellular permeability. Some studies have even allowed more accurate modeling of paracellular permeability, which would have two physiological components. The first one is a passage through the pores, known as pore pathway, a high-capacity route that is size and charge-selective, with the maximal diameters of transported molecules ranging from approximately $\sim 5 \text{ \AA}$ to $\sim 10 \text{ \AA}$ (Shen *et al.*, 2011; France and Turner 2017). The second one, the leak pathway, is a path created by broad discontinuities of the barrier and allowing the passage of larger molecules independently of their charge; it supports the paracellular flux of molecules with diameters up to 125 \AA (Anderson and Van Itallie 2009). The pore pathway is influenced by the expression of a family of tight junction proteins, the claudins, while the leak pathway is rather controlled by cytoskeletal proteins, the tight junction proteins ZO-1 and occludin, and also by the state of the adherent junctions (Figure 14).

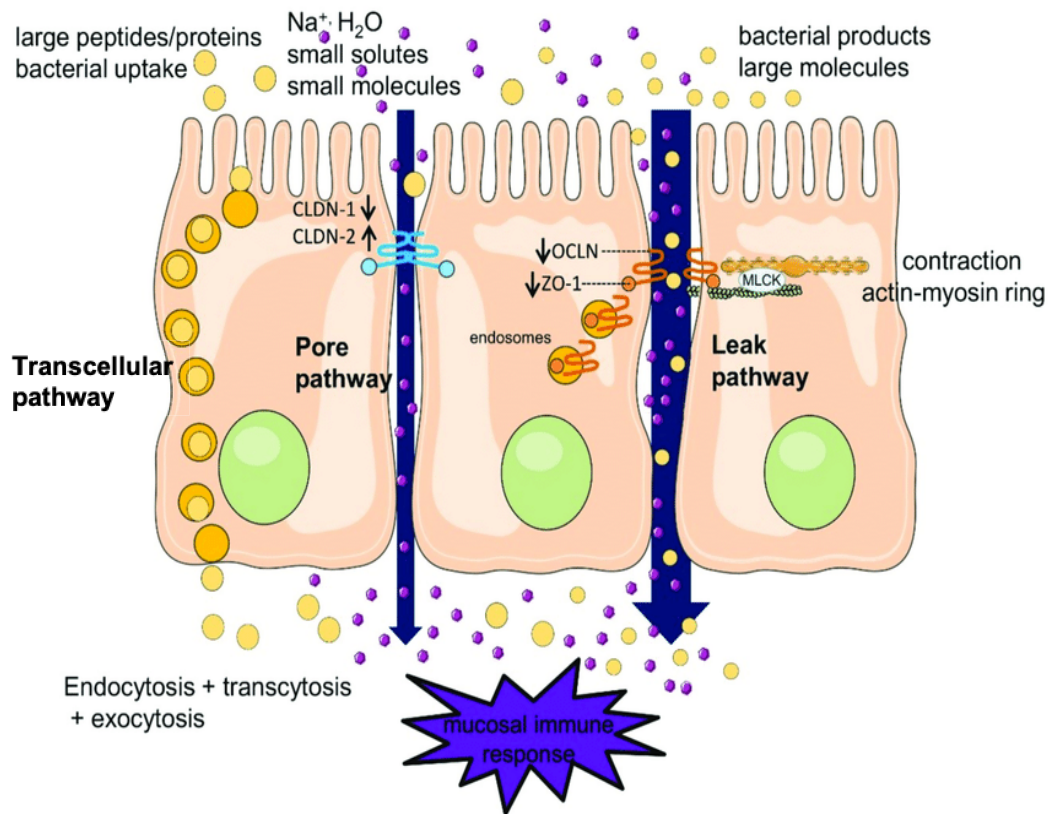


Figure 14: Pore and leak pathway controlling.

The enterocytes form a polarized single cell layer which allow two different paracellular pathways facilitating the transport from the apical to the basolateral side. The pore pathway, a high-capacity route that is size and charge-selective and the leak pathway, allowing the passage of larger molecules independently of their charge. The apical side, characterized by the presence of microvilli, is in contact with the intestinal lumen. The epithelial cells are tied together by several intercellular junctions, especially the tight junction proteins (claudins, occludin and ZO). In parallel, a transcellular pathway is involved in the transport of large proteins and nutrients as well as bacteria uptake. MLCK: Myosin light chain kinase; CLDN: Claudin; OCLN: Occludin; ZO: Zonula occludens.

(Meijers *et al.*, 2018)

2.4.1.2) Adherens junctions

The adherens junctions (AJs) are closely associated with establishing and maintaining the morphology of adjacent cells. They are the first junctions to be put in place following contact between two cells. As the name suggests, the AJs have important roles in cell-cell adhesion and signaling. They are located under the tight junctions and, together with desmosomes, they make up the rest of the components of the paracellular complex. They provide strong adhesive bonds that maintain cellular proximity and are also a site of intercellular communication. Loss of AJs results in disruption of cell-cell and cell-matrix contacts, ineffective epithelial cell polarization, differentiation, and early apoptosis of intestinal epithelial cells (Turner 2009; Lee *et al.*, 2018). As for tight junctions, adherens junctions are signaling platforms involved in multiple cellular processes.

Adherens junctions are assembled by the interaction of transmembrane proteins, intracellular adapter proteins and the cytoskeleton. They are composed of two families of transmembrane spanning, adhesive receptors: the cadherins and the nectins (Figure 15). The extracellular regions of these proteins mediate adhesion of cells to their neighbors while the intracellular regions interact with an array of proteins. These intracellular proteins control the assembly and dynamics of adherens junctions by modulating connections with the actin cytoskeleton and stimulating signaling pathways (Niessen 2007; Meng and Takeichi 2009; Campbell *et al.*, 2017).

The main AJs complex is formed by cadherin-catenin interactions. E-cadherin (also known as cadherin-1), which belongs to the family of classical cadherins, is the major classical cadherin in epithelia. E-cadherin is involved in the setting up of the complex of epithelial junctional complexes. This protein is a type I glycoprotein possessing a transmembrane segment, the C-terminus being intracellular and the N-terminus extracellular (Turner 2009; Campbell *et al.*, 2017). The extracellular domain of E-cadherin binds to calcium ions and forms homotypic interactions with the E-cadherin of neighboring cells. The cytoplasmic tail of the protein interacts directly with catenin δ 1 (also known as p120 catenin) and β -catenin. In turn, β -catenin binds to α -catenin 1, which regulates local actin assembly and contributes to the development of the perijunctional actinomyosin ring (Figure 15) (Niessen and Gottardi 2008). α -catenin binds the adherent junction to the cytoskeletal network

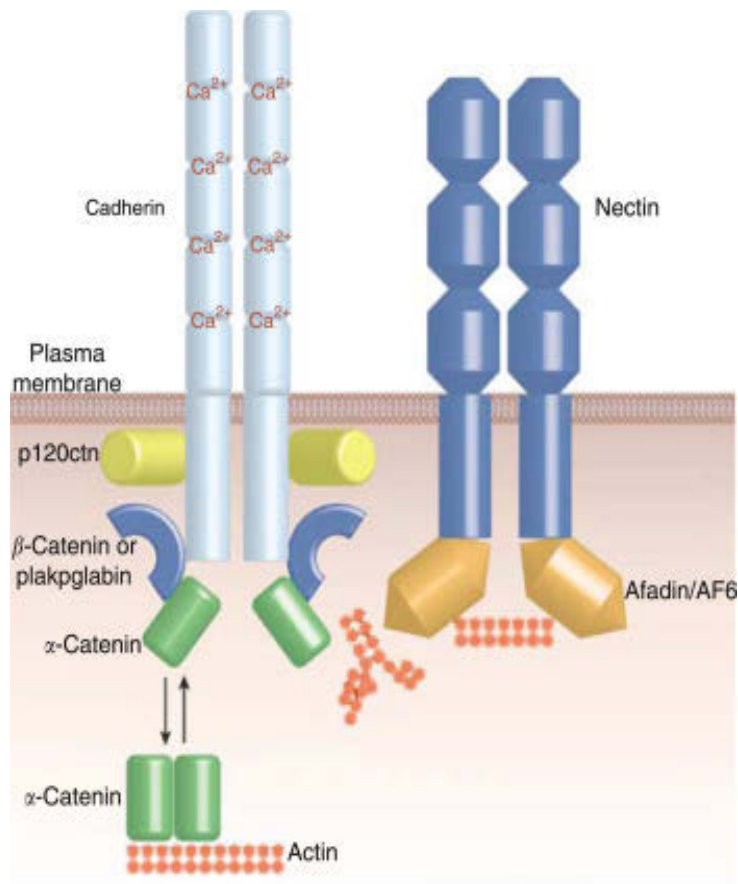


Figure 15: Schematic representation of the basic adherens junctions structure.

They are composed of two families of transmembrane spanning, adhesive receptors: the cadherins and the nectins. Shown are the cadherin–catenin complex and the nectin–afadin complex and their potential interactions with actin.

Classical cadherins (clear blue), which mediate calcium-dependent (Ca^{2+}) intercellular adhesion, are composed by an extracellular domain, a transmembrane domain and a cytoplasmic domain. The cytoplasmic tail interacts directly with catenin $\delta 1$ (also known as p120ctn) and β -catenin (blue). In turn, β -catenin binds to α -catenin (green), which regulates local actin assembly, contributes to development of the perijunctional actinomyosin ring and establishes a direct link between the cadherin-catenin complex and the actin cytoskeleton (orange points). Ctn: catenin.

(Niessen, 2007)

through direct binding of the C-terminal domain of F-actin, or indirectly via other adapter proteins such as afadine/AF6 (Ikeda *et al.*, 1999; Pokutta and Weis 2002; Pokutta and Weis 2007). Cadherin-catenin complexes are important not only for cell-cell adhesion but also for maintaining cell polarity, for regulating proliferation and epithelial migration, and for the formation of other adhesion complexes, such as desmosomes and tight junctions. Invalidation of E-cadherin has been shown to be lethal early in embryonic development (Larue *et al.*, 1994; Campbell *et al.*, 2017). Intestine-targeted invalidation of E-cadherin can lead to an increased susceptibility to experimental colitis in mice and seems to be implicated in the maintenance of the intestinal barrier function (Hermiston and Gordon 1995; Schlegel *et al.*, 2010; Bondow *et al.*, 2012).

β -Catenin is also an essential component of adherens junctions but also participates in the Wnt signaling pathway. It has been shown that the dissociation of the adherens junctions leads to an internalization of E-cadherin and β -catenin and to the translocation of β -catenin in the nucleus, in parallel with an activation of the Wnt / β -catenin pathway (Kam and Quaranta 2009)

There is a second complex of the adherent junction, the Nectin-afadine interactions. Nectins belong to the family of IgG-like adhesion receptors and are transmembrane proteins capable of establishing homophilic or heterophilic interactions. Nectins interact with the cytoskeleton via afadine or other F-actin binding proteins (Figure 16) (Miyoshi and Takai 2007; Niessen 2007; Takai *et al.*, 2008).

2.4.1.2) Desmosomes

In conjunction with AJs, desmosomes have been known for many years to ensure the mechanical strength of tissues. They have mainly been studied in the skin and the heart, which are tissues subjected to high mechanical stress and for which there are many pathologies related to the loss of function of desmosomal proteins (Dusek *et al.*, 2007; Waschke 2008). Unlike tight and adherens junctions, which are localized on the most apical part of the lateral membrane, desmosomes are found all along the lateral membrane.

Desmosomes form structures that attach to the intermediate filaments of the cytoskeleton, providing strong adhesive bonds that maintain cell proximity and are also sites of intercellular communications. They are composed of various protein subunits including desmoglein, desmocolin, plakoglobin, plakophilin and desmoplakin (Holthofer *et al.*, 2007;

Garrod and Chidgey 2008). Their role in maintaining barrier function may implicate its cellular prion protein (PrP^C) component (Holthofer *et al.*, 2007; Garrod and Chidgey 2008; Petit *et al.*, 2013).

2.4.2) Control of intestinal permeability.

Cell-cell junctions are central to the complex information network that maintains tissue organization. They receive multiple signals that guide them through the process of establishing contact with neighboring cells and installing the appropriate scaffolding in order to initiate cytoskeletal connections. They also behave as sensors that send back to the nucleus signals about both the location of cells within tissues and their immediate environment. The intestinal barrier is sensitive to changes in the microenvironment of the intestinal lumen (nutrients, microbiota), blood compartment (cytokines, hormones) and changes in the number or function of mucosal immune cells (immune cell recruitment from the mucosa to epithelium) (Konig *et al.*, 2016).

Deregulation and abnormalities related to intestinal barrier function are associated with intestinal pathologies such as IBD, irritable bowel syndrome, digestive cancers or celiac disease (Konig *et al.*, 2016). The alteration of the intestinal epithelial barrier has also been described in extra-intestinal pathologies such as in type 1 diabetes, cystic fibrosis and autism (De Lisle *et al.*, 2011; Sanctuary *et al.*, 2018). Since the intestinal barrier provides protection against the intrusion of foreign and potentially pathogenic elements, the deterioration of its integrity observed in certain pathologies raises the question of its role in the etiology of these diseases. The possible contribution of the intestinal barrier to metabolic diseases such as obesity has also been highlighted, particularly through the work of our team (Monteiro-Sepulveda *et al.*, 2015; Genser *et al.*, 2018). All of these studies gave birth to the so-called "leaky gut syndrome" concept, which proposes that defects in the barrier leading to a disturbance of intestinal permeability contribute to the development of different pathologies.

The effects of some different disruptors and different molecular pathways that are involved on intestinal barrier integrity are discussed below. Although the impact of these different mechanisms is presented here in separate chapters, a very close link exists between these different elements as modulators of intestinal barrier integrity.

2.4.2.1) Cytokines

Immune-induced intestinal barrier dysfunction is thought to be critical in the predisposition to numerous autoimmune diseases (IBD, food allergy, celiac disease and diabetes) and their exacerbation. Among the inflammatory molecules that modulate the integrity of the intestinal barrier, pro- and anti-inflammatory cytokines are the most studied. The impact of cytokines on the intestinal barrier may vary according to the study model considered, which sometimes leads to contradictory observations in the literature. Among the pro-inflammatory cytokines, interferon- γ (IFN γ), tumor necrosis factor- α (TNF α) and interleukin IL-1 β are well-known for their role in the regulation of barrier integrity by modulating tight junctions. These cytokines are central mediators of intestinal inflammatory diseases (Al-Sadi *et al.*, 2008; Chelakkot *et al.*, 2018).

I will present here only a few examples showing the involvement of cytokines and the molecular mechanisms involved in their deleterious or beneficial effects on the intestinal barrier.

TNF α is capable of increase intestinal permeability *in vivo* in mice (Al-Sadi *et al.*, 2016) and in Caco-2 cells, a model of intestinal epithelial cells (Ma *et al.*, 2004). In Caco-2 cells, it has been shown that TNF- α activates a signaling cascade leading to the destabilization of tight junctions and consequently to an increase in the permeability of the cell monolayer (Ma *et al.*, 2004; Al-Sadi *et al.*, 2016). In humans, high levels of TNF- α are found in the intestinal mucosa or lamina propria of patients with IBD or obesity (Lee 2015; Monteiro-Sepulveda *et al.*, 2015). TNF- α appears to have a major role in Crohn's disease, since the treatment of these patients suffering from this pathology with anti-TNF- α antibodies is an effective therapeutic strategy that leads in particular to an improvement of the intestinal barrier function (Levin *et al.*, 2016). After the binding on its receptor, present on the surface of enterocytic cells, TNF- α activates the MAP3 kinase pathway, which leads to the activation of the NF- κ B pathway. NF- κ B signal transduction pathway is a central mechanism involved in tight junction regulation. NF- κ B inhibition protected mice from severe water loss and diarrhea, which indicated its role in the regulation of the barrier property of IECs. Besides, NF- κ B increases the expression of myosin light chain kinase (MLCK). MLCK regulates the TJs stability and is important in the destabilization of TJs in response to TNF- α (Shen *et al.*, 2006). By phosphorylating the myosin light chain (MLC), MLCK controls the contraction of

actin-myosin belt, which is necessary for the stabilization of TJs. However, an excessive contraction and tension in this complex leads to TJs destabilization, resulting in the opening of the paracellular pathways (Cunningham and Turner 2012) (Figure 16). MLC phosphorylation is predominantly affected by MLCK, but also by the Rho kinase (ROCK). ROCK can either directly phosphorylate MLC or inhibit the action of myosin phosphatase by phosphorylating it (Murthy 2006; Guan *et al.*, 2013). It appears that destabilization of tight junctions following MLCK and contraction of the actin cytoskeleton is due to disruption of the junctional localization of ZO-1 and occludin (Shen *et al.*, 2006; Du *et al.*, 2016). TNF- α acts also in combination with INF- γ on the reduction of occludin expression (Mankertz *et al.*, 2000).

The pro-inflammatory cytokine IL-1 β plays an important role in Crohn's disease. IL-1 β leads to the phosphorylation of MLC by MLCK, which then causes a contraction of the cytoskeleton of actin, opening the tight junction complex and therefore increasing permeability. Besides, IL-1 β triggers ERK1/2 signaling pathway, which activates the nuclear transcription factor Elk-1. Activated Elk-1 is translocated into the nucleus where it allows the expression of the MLCK coding gene and by consequence the opening of TJs (Al-Sadi *et al.*, 2008).

Other cytokines are also able to disrupt the intestinal barrier, such as IL-6. The mechanisms of action involved in the increased intestinal permeability by these cytokines are partially described. They are involved in the activation of signaling pathways involving numerous kinases (PKA, PKC, ROCK), the Rho and Rac GTPases and the NF- κ B pathway. The current data do not make it possible to determine whether the activation of a precise signaling cascade is actually specific for a cytokine (Andrews *et al.*, 2018).

Some cytokines, such as IL-10, IL-22 and IL-17 have a beneficial effect on the intestinal barrier (Lee *et al.*, 2015; Onyiah and Colgan 2016; Andrews *et al.*, 2018). IL-22, for example, contributes to maintaining the integrity of the intestinal barrier. It is described as playing a protective role on the intestinal barrier by increasing the secretion of mucus and antimicrobial peptides by intestinal epithelial cells via the action of the STAT3 kinase (Sovran *et al.*, 2015). Nonetheless, recent work also showed that IL-22 could be involved on intestinal barrier defects via increased expression of claudin 2 (Tsai *et al.*, 2017; Wang *et al.*, 2017). Thus, the data acquired on IL-22 (Zenewicz 2018) illustrate the complexity of the mode of action of cytokines on the intestinal barrier.

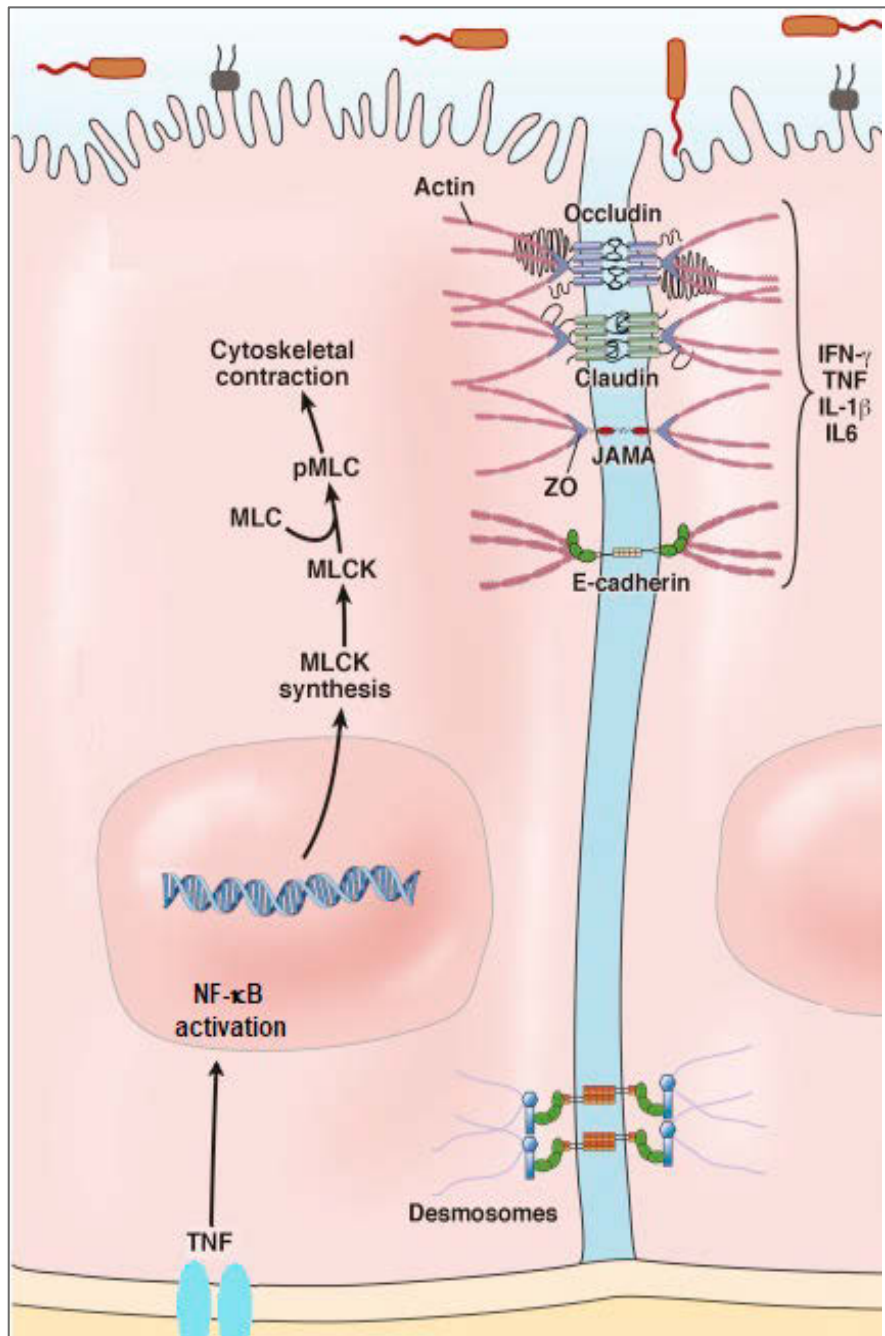


Figure 16: MLCK regulates the tight junctions stability in response to TNF- α .

After the binding on its receptor, present on the surface of enterocyte cells, TNF- α activates the MAP3 kinase pathway, which leads to the activation of the NF- κ B pathway. NF- κ B increases the expression of myosin light chain kinase (MLCK). By phosphorylation of the myosin light chain (MLC), MLCK controls the contraction of actin-myosin belt. An excessive contraction and tension in this complex lead to TJs destabilization, resulting in the opening of the paracellular pathways. pMLC, phosphorylated MLC.

(Shawki and McCole, 2017).

2.4.2.2) Calcium depletion

Calcium depletion provokes extensive endocytosis at the plasma membrane of mammary epithelial cells in culture, and some of the endocytic vesicles carry segments of junctional strands. Calcium is also involved in the assembly and sealing of newly formed TJs. When calcium is not present, junctional components are nevertheless synthesized but seem to accumulate at the outermost cisternae of the Golgi, or even beyond this point, but without reaching the outer surface. Freeze-fracture replicas show that when calcium is added to monolayers under such conditions (“Calcium switch”), junctional strands reappear as early as 15 min, and the whole structure of the TJ is essentially completed in 4-5 h, coinciding with the increase of TEER (Gonzalez-Mariscal *et al.*, 1990; Rothen-Rutishauser *et al.*, 2002; Ivanov *et al.*, 2004; Shen and Turner 2006).

When cell monolayers are exposed to EGTA, a well-known calcium chelator, an increase of paracellular permeability occurs and occludin and ZO-1 are localized predominantly in intracellular vesicular structures (Nunbhakdi-Craig *et al.*, 2002; Seth *et al.*, 2007), indicating that calcium chelation seems to be involved in disruption of tight junctions by the internalization of TJ proteins. However, in each case, tight junction protein internalization was defined at time points associated with or following maximal barrier loss, making it impossible to determine whether internalization is directly related to loss of barrier function or is a secondary event.

2.4.2.3) Phosphorylation of tight junction proteins

Significant evidences indicate that the activities of some protein kinases regulate the integrity of TJs. Indeed, AJ and TJ proteins are regulated by post-translational phosphorylation, which drastically alters their membrane distribution and turnover. Phosphorylation can either promote TJs formation and barrier function or alternatively promote TJ proteins redistribution and complex destabilization.

Among the TJ proteins, occludin phosphorylation has been widely studied and is responsible for both opening and sealing TJs. Several various intracellular signaling molecules regulate the phosphorylation status of occludin. Some studies have suggested that serine/threonine phosphorylation is the predominant phosphorylation modification of occludin; nevertheless, recent works emphasized the importance of phosphorylation of

occludin on tyrosine (Tyr) residues, which may result in the loss of its interaction with ZO family proteins. As a consequence, kinases such as atypical protein kinase C (PKC) and c-Src and phosphatases such as protein phosphatases 2A (PP2A), which surround area of TJs and are able to phosphorylate and dephosphorylate occludin, have a crucial role to play in intestinal barrier integrity (Figure 17) (Seth *et al.*, 2007; Chelakkot *et al.*, 2018)

In intact epithelium, occludin presents hyperphosphorylation on Ser/Thr residues, mediated by atypical PKC, however, the significance of this phosphorylation in TJs assembly is unclear. Occludin is shown to undergo dephosphorylation on Ser/Thr residues during the disruption of TJs by calcium depletion, phorbol esters, or bacterial infection (Basuroy *et al.*, 2006). In addition, a study indicated that PP2A, a Ser/Thr-phosphatase, interacts with TJ protein complex and suggested that it may influence the integrity of TJs in MDCK cells overexpressing PP2A (Seth *et al.*, 2007). Therefore, the balance between atypical PKC and PP2A may determine the Ser/Thr phosphorylation status of occludin (Jain *et al.*, 2011; Manda *et al.*, 2018).

The c-Src kinase seems to play a role in the regulation of tight junction integrity in Caco-2 and MDCK cell monolayers. Studies have demonstrated that hydrogen peroxide can rapidly activate c-Src. Src kinase inhibitor or a kinase-inactive c-Src mutant appeared to attenuate the hydrogen peroxide-induced disruption of tight junction, while overexpression of wild-type c-Src exacerbates the hydrogen peroxide effect (Rao 2009; Chelakkot *et al.*, 2018).

2.4.2.4) Autophagy

Interestingly, a number of polymorphisms in a wide range of genes implicated in autophagy have been identified as risk factors predisposing an individual to the development of IBD. Recent genome-wide association scanning (GWAS) studies have identified several genes contributing to the development of Crohn's disease (CD), including the autophagy-related gene 16-like (*ATG16L1*), intracellular bacterial sensing (*NOD2*), endoplasmic reticulum (ER) stress and claudin-2. Two of the most prominent associations with CD have been found in the genes *ATG16L1* and *NOD2* (Randall-Demllo *et al.*, 2013; Hu *et al.*, 2015). Moreover, gut microbiota and autophagy have also been linked and this relationship appears to be bidirectional (Haq *et al.*, 2019).

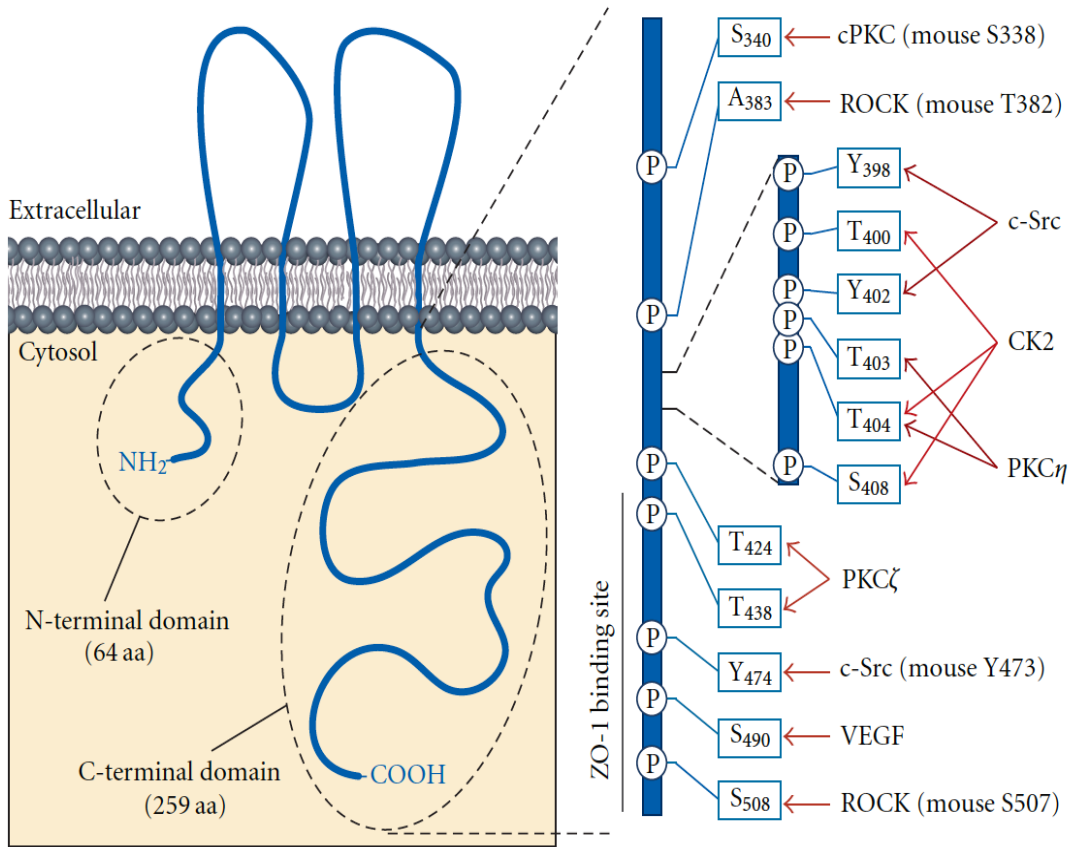


Figure 17: Schematic presentation of occludin amino acid residues targeted by specific kinases.

Serine/threonine phosphorylation is the predominant phosphorylation modification of occludin. The phosphorylation of occludin on tyrosine (Tyr) residues may result in the loss of its interaction with ZO family proteins. Phosphatases or kinases such as atypical protein kinase C (PKC) and c-Src, which surround area of TJ, are able to dephosphorylate and phosphorylate occludin residues. (Dörfel and Huber, 2012)

Some studies showed that autophagy reinforced barrier function resulting in increased TEER and reduced paracellular permeability of small solutes and ions. This mechanism seems to be mediated in part by lysosome (autophagy)-mediated degradation of the tight junction claudin-2. Besides, works have shown that the inflamed intestinal mucosa in patients with active IBD presents increased claudin-2 expression and a claudin-2-dependent increase in TJ permeability (Hu *et al.*, 2015).

In the intestine, a sensitive balance between tolerance and defense is required to maintain homeostasis. The conserved process of autophagy seems to play a critical role in maintaining this balance by regulating the invasion and dissemination of pathogens, maintaining barrier integrity and preserving intestinal homeostasis (Hu *et al.*, 2015; Haq *et al.*, 2019).

2.4.2.5) Diet and lipids

Numerous components in the diet can affect the regulation and organization of TJ proteins and in turn the permeability of the intestinal barrier. Several studies have been investigated the impact of diet components on epithelial barrier function. The food abundance or scarcity and also the nature of the food, may influences the intestinal barrier differently by increasing or decreasing TJ permeability. Some amino acids, vitamins, polyphenols and peptides were described to decrease paracellular permeability, while alcohol and some fatty acids, for example, were shown to increase paracellular permeability and to decrease the TEER, mainly through TJs impairment (De Santis *et al.*, 2015). Taken alone, many diet components are able to modulate the intestinal barrier via different mechanisms.

I will focus in this chapter on the impact of certain lipid classes on intestinal barrier function and on the effect of a high-fat diet on barrier integrity, which has been the subject of many studies.

As noted above (see chapter 2.2.1), the short chain fatty acids (SCFAs) are produced by the microbiota primarily in the colon. These SCFAs have been described to have a protective effect on the intestinal barrier. In cultured epithelial cells, butyrate increases the TJ protein expression (occludin, ZO-1 and claudin-1) and restores the alteration of permeability induced by a change in calcium availability (Wang *et al.*, 2012; Cheng *et al.*, 2018). These effects are thought to be dependent on the increased expression of claudin-1 by butyrate

(Wang *et al.*, 2012). *In vivo*, in diabetic mice (following a mutation on the leptin receptor, *db/db* model), butyrate restores the integrity of the intestinal barrier via the activation of GPR43 receptor-dependent signaling pathway and involving the protein NLRC3 (a member of the NOD-like receptor family) (Cheng *et al.*, 2018).

Bile acids, whether primary or secondary (deconjugated by the microbiota), are capable of disrupting the epithelial barrier integrity. Their actions on the intestinal permeability and the secretion of antimicrobial peptides depend on the nature of the bile acid considered, the exposure time and the cell model used, some bile acids exerting deleterious effects and others no or few effects (Sarathy *et al.*, 2017; Tremblay *et al.*, 2017). There are only a few data related to the molecular mechanisms involved, however a decrease in occludin and an induction of oxidative stress are observed in intestinal epithelial cells in culture (Sarathy *et al.*, 2017).

The role of cholesterol on the intestinal barrier has been reported. Indeed, it has been shown in the Caco-2 intestinal epithelial monolayer, that the depletion of cholesterol membranes causes an increase in permeability to ions and macromolecules, and a decrease in TJ protein localization at intercellular junctions (Lambert *et al.*, 2005). A role of cholesterol in the maintenance of TJ proteins in cholesterol-rich membrane domains has been suggested (Lambert *et al.*, 2005).

Also, phospholipids, and in particular phosphatidylcholine, participate in maintaining the integrity of the intestinal barrier. They are constituents of the mucus layer and they contribute to the maintenance of the integrity of the epithelial cell membranes (Lichtenberger 2013). Although the mechanisms involved have not been described, phosphatidylcholine has been shown to exert a protective role against the invasion of pathogens such as *Clostridium difficile* (Olson *et al.*, 2015). An intake of phosphatidylcholine is currently used to treat patients with ulcerative colitis (Stremmel *et al.*, 2005; Schneider *et al.*, 2010).

Many studies whose purpose was to establish or reproduce the characteristics of human pathologies such as diabetes or obesity have used animals submitted to high-fat diets, generally over long-term treatments. In these models, perturbations induced by lipid intake such as alteration of the intestinal barrier, microbiota change, and inflammation are intimately linked. A high fat diet in rats is associated with the elevation of intestinal inflammatory markers such as fecal calprotectin, but also with a reduction of claudin-4 expression at villi

and at Peyer's patches (Aqilah Zainal Abidin 2015) leading to the disruption of intercellular junctions and by consequence to an increased transport of antigens across these patches (Khan 2017). Moreover, after 4 weeks of high-fat diet, mice display increased intestinal permeability and reduced expression and membrane location of chloride ion transporter (CFTR). These defects lead to a disruption of ion secretions by the epithelial cells, in particular, the chloride ion, which massively alters the mucus layer (Tomas *et al.*, 2016).

A link between lipid intake, change in the composition of gut microbiota, alteration of the barrier and metabolic alterations was identified for the first time by the team of P. Cani and N. Delzenne. This team observed high plasma LPS level in obese mice. This increase in blood LPS has been proposed as a consequence of an intestinal barrier defect, as evidenced by the decrease in the expression of occludin and ZO-1 (Cani *et al.*, 2007; Cani *et al.*, 2008). A single infusion of LPS for 4 weeks in mice on a standard diet reproduces metabolic disorders equivalent to those observed during high-fat obesogenic treatment, giving rise to the concept of metabolic endotoxemia (Cani *et al.*, 2007). Since these pioneering studies, several other studies have shown in mice that are genetically obese or made obese by a diet rich in lipids, that the expression and localization of tight junction proteins are altered, possibly contributing to the translocation of LPS into the circulation (Cani and Jordan 2018). Lipoproteins present in the circulation are involved in LPS clearance. Several evidences showed the importance of the phospholipid transfer protein (PLTP) in the transfer of circulating LPS to lipoproteins leading to the neutralization of LPS toxicity (Gautier *et al.*, 2008; Gautier and Lagrost 2011). Moreover, the administration of recombinant human PLTP reduced endotoxemia and prevented inflammation in LPS-treated mice along with anti-bacterial properties of this protein, making PLTP a promising therapeutic target (Deckert *et al.*, 2017). These results suggest a role for PLTP in gut barrier function.

Several reports showed that inflammatory status works in conjunction with lipid intake to modulate the intestinal barrier. It has been shown that IFN- γ *-/-* mice fed with a high-fat diet rich presents a reduced intestinal permeability compared to wild mice receiving the same diet. This work suggested that IFN- γ acts are involved in the lipid-dependent alteration of intestinal permeability (Luck *et al.*, 2015). In addition, in humans studies, our team recently showed that after a lipid load, the jejunal permeability (analyzed *ex vivo*) is twice as high in obese than in non-obese subjects, whereas no statistical difference was observed between these two types of subjects in basal conditions (Genser *et al.*, 2018). This

increased permeability in response to a lipid load is correlated with systemic and intestinal inflammation (Genser *et al.*, 2018).

The modification of intestinal microbiota plays a critical role in the dysfunction of the epithelial barrier since antibiotic treatment to high-fat diet treated mice prevents the increase in intestinal permeability due to the diet (Cani *et al.*, 2008). Moreover, the modulation of microbiota composition by the administration of prebiotics in genetically obese mice improves barrier function *via* a decrease in intestinal permeability and ameliorates of the inflammatory status of treated-mice (Cani *et al.*, 2009).

Taken together all these data show that dietary lipids impaired intestinal barrier function (Cani *et al.*, 2008; De Santis *et al.*, 2015; Johnson *et al.*, 2015; Araujo *et al.*, 2017; Llewellyn *et al.*, 2018). It is proposed that excessive passage of macromolecules could contribute to local activation of immune cells, thereby contributing to tissue and systemic low-grade inflammation (Le Gall *et al.*, 2018).

3) Aryl Hydrocarbon Receptor

In addition to genetic susceptibility factors, diet and commensal flora, environmental changes also play a role in epithelial barrier disruption in several pathologies. Recent studies showed that the transcription factor aryl hydrocarbon receptor (AhR) is an environmental sensor. AhR is activated by endogenous physiological ligands some of them being produced by commensal flora, or by environmental ligands present in pollutants and food. AhR is an important regulator of the immune cells differentiation and it provides a pathway by which endogenous and environmental signals could control multiple immunological processes and inflammatory responses (Esser *et al.*, 2009; Quintana and Sherr 2013; Esser 2016).

The aryl hydrocarbon receptor is a member of the basic helix–loop–helix (bHLH)–periodic circadian protein (PER)–AhR nuclear translocator (ARNT)–single-minded protein (SIM) superfamily of transcription factors. The PER–ARNT–SIM (PAS) is a subgroup of the bHLH superfamily of transcription factors, in which PAS domain senses environmental alterations of both endogenous and exogenous factors. The AhR is a highly conserved nuclear receptor that regulates gene expression and is the only member of this bHLH subgroup known to be activated by ligands (Hankinson 1995; Esser *et al.*, 2009; Barouki *et al.*, 2012; Murray *et al.*, 2014; Rothhammer and Quintana 2019).

AhR is widely expressed in the body and evolutionarily conserved from invertebrates. Its activity is tightly controlled. As a nuclear receptor, AhR is bound in an inactive cochaperones complex in the absence of a ligand, (Figure 18) (Stockinger *et al.*, 2014; Esser and Rannug 2015). This complex formed by a dimer of the 90-kDa heat shock protein (HSP90), the AhR-interacting protein (AIP, also known as XAP2 or Ara9), the cochaperone p23 protein and the c-SRC protein kinase (Figure 18), maintain the AhR inactive and its localization in the cytoplasm (Okey *et al.*, 1994; Barouki *et al.*, 2012; Rothhammer and Quintana 2019). HSP90 stabilizes AhR in a high affinity for its ligands conformation. In addition, AIP prevents AhR ubiquitination and degradation, maintaining AhR steady-state cellular levels. Upon ligand binding, AIP is released from the complex, which triggers conformational changes in AhR that expose its nuclear localization signal (NLS), leading to AhR translocation to the nucleus (Hankinson 1995; Barouki *et al.*, 2012; Tsuji *et al.*, 2014). These conformational changes also expose a protein kinase C target site that when phosphorylated interferes with AhR nuclear translocation, constituting one of several mechanisms to control AhR (Chen and Tukey 1996; Gutierrez-Vazquez and Quintana 2018). Once translocated into the nucleus, the ligand-activated AhR complex is dissociated and AhR forms a heterodimer with ARNT already present in the nucleus. This heterodimer subsequently binds to a partially characterized set of co-activators and/or co-repressors and the resulting complex interacts with consensus regulatory sequences (AhR-, dioxin- or xenobiotic- responsive elements: AHRE, DRE or XRE) located in the promoter of target genes, leading to their transcriptional control. These genes include, for example, several xenobiotic metabolizing enzymes such as the microsomal cytochrome P450-dependent monooxygenases including cytochrome P450 family-1 subfamily-A polypeptide-1 (CYP1A1), cytochrome P450 family-1 subfamily-A Polypeptide-2 (CYP1A2) and cytochrome P450 family-1 subfamily-B polypeptide-1 (CYP1B1) (Figure 18) (Jones *et al.*, 1985; Reyes *et al.*, 1992; Yao and Denison 1992; Probst *et al.*, 1993; Barouki *et al.*, 2007; Stockinger *et al.*, 2014). Moreover, the AhR-ARNT heterodimer triggers the transcription of AhR-regulating antagonistic AhR repressor (AhRR) protein. The AhRR closely resembles AhR, but lacks the transactivation domain and may initiate a negative feedback loop potentially competing with AhR for heterodimerization with ARNT as well as for binding to

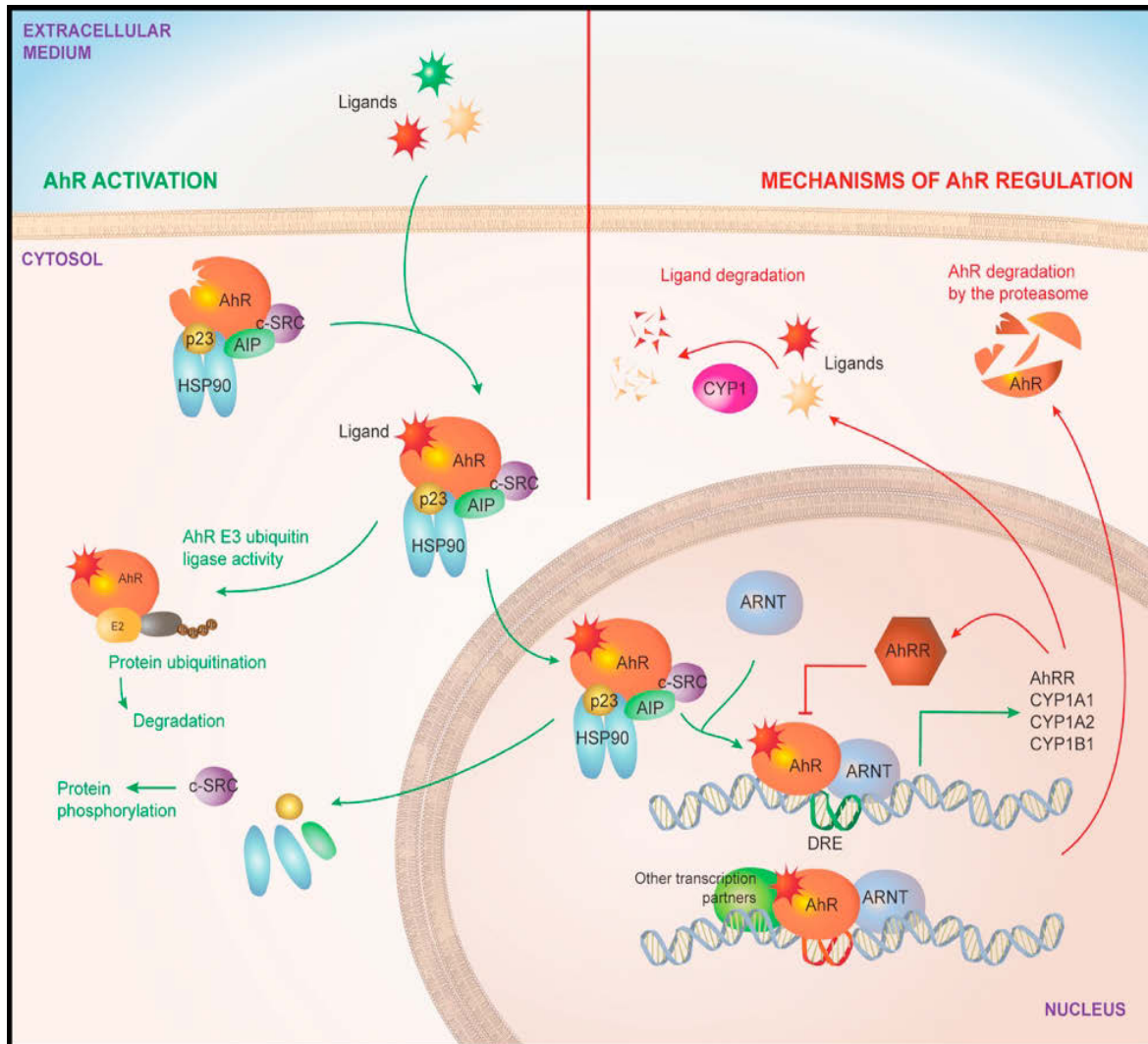


Figure 18: AhR Signaling Pathway

Inactive AhR is localized in the cytosol forming a complex with HSP90, AIP, p23, and c-SRC. After agonist binding to AhR, conformational changes result in the translocation of the complex to the nucleus, dissociation of the cytoplasmic complex and the interaction of AhR with ARNT. The AhR-ARNT heterodimer controls the transcription of DRE-containing AhR target genes such as CYPs 1A1, 1A2 and 1B1. AhR activation is limited by regulatory mechanisms, some of which are actually triggered by AhR activation itself such as CYP enzymes, which degrade AhR ligands and by its repressor AhRR. AhRR inhibits the formation of AhR/ARNT complex required for AhR signaling. AIP: AhR-interacting protein; p23: p23 co-chaperon protein; ARNT: AhR nuclear translocator; DRE: dioxin response element; HSP90: 90-kDa heat shock protein. (Gutierrez-Vazquez and Quintana, 2018)

XRE sequence and therefore mediates a negative feedback mechanism of AhR activation (Figure 18) (Mimura *et al.*, 1999; Stange and Veldhoen 2013).

A non-canonical AhR signaling pathway also exists, and involves binding of the ligand-bound AhR to the nuclear factor- κ B (NF- κ B) family member, RelB, which subsequently activates transcription of genes controlled by both AhR and NF- κ B (Tian *et al.*, 1999; Tian *et al.*, 2002; Vogel and Matsumura 2009). Therefore, both of these AhR signaling pathways have the potential to elicit immunomodulatory effects following AhR activation.

3.1) AhR ligands

The AhR ligands are in general very lipophilic and diffuse through the plasma membrane of the cells. To date, there is no known AhR ligand transporter system. Numerous chemicals exhibit high-affinity binding to AhR, altering its activity in a ligand-dependent manner. The first AhR ligands described and well-studied are the environmental chemicals such as xenobiotic 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and synthetic and halogenated polycyclic aromatic hydrocarbons (PAHs) (e.g., benzoflavones and dioxins) (Esser 2016). However, interest in physiological exogenous and endogenous ligands has increased in the last years (Guyot *et al.*, 2013). Identification of many natural AhR ligands generated by cells and microbiota or derived from dietary sources have been reported. Among them, an important group of AhR ligands is indoles, which can be generated by bacterial metabolism of tryptophan and are also derived from the metabolism of nutrients.

One of the dietary sources of AhR ligands is a cruciferous vegetable, such as Brassica, including broccoli, cauliflower, Brussel sprouts and cabbages (Denison and Nagy 2003; Nguyen and Bradfield 2008), which are rich in the glucosinolate glucobrassicin, an indole precursor. It is enzymatically degraded into indole-3-carbinol (I3C), which at the acidic environment of the stomach undergo dimerization and generate diindolylmethane (DIM) and further converted to the high-affinity AhR ligands indolo-[3,2-b]-carbazole (ICZ). Other described dietary ligands of AhR are natural flavonoids present in fruits and vegetables as well as resveratrol, which is abundant in red wine (Zhang *et al.*, 2003). However, it is still unclear which, if any, dietary or naturally occurring ligands of AhR have a direct agonistic activity or could mediate an antagonist effect.

Other endogenous AhR ligands are derivatives of the essential amino acid tryptophan that were discovered during exposure of L-tryptophan to UVB radiation. UV light-mediated degradation of tryptophan generates 6-formylindolo-[3,2-b]-carbazole (FICZ), by photolysis, which can occur with both visible and UV light. FICZ present in humans has a high affinity for AhR (at picomolar concentrations) (Rannug *et al.*, 1995; Cella and Colonna 2015) and is thus considered as an essential endogenous and physiologic AhR ligand (Hubbard *et al.*, 2015; Hubbard *et al.*, 2015; Kiyomatsu-Oda *et al.*, 2018). Moreover, FICZ has the particularity to trigger a transient activation of AhR signaling in cell systems. Its capacity to be a perfect substrate for mammalian CYP1A enzymes results in efficient auto-regulatory feedback of its action since FICZ is degraded by these enzymes (Wincent *et al.*, 2009; Wincent *et al.*, 2016). Interestingly, bacteria, including commensals, and fungi, can produce and metabolize tryptophan into AhR ligands and also regulate tryptophan synthesis by sensing tryptophan concentrations due to dietary intake (Sarsero *et al.*, 2000). *Malassezia furfur*, a fungus common causative agent of pityriasis versicolor of the skin, secretes several ligands including malassezin, ICZ and FICZ that engage AhR (Gaitanis *et al.*, 2008). Several species of lactobacilli, including *Lactobacillus bulgaricus* (Takamura *et al.*, 2011) and *Lactobacillus reuteri* (Zelante *et al.*, 2013) produce AhR ligands, such as indole-3-aldehyde (IAId), and modulate the mucosal immune response. Moreover, pathogens such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* produce pigmented virulence factors that activate AhR (Moura-Alves *et al.*, 2014). These findings highlight the role of AhR as a central node for chemical communication between the environment and the host.

In this context, AhR has been characterized as a promiscuous receptor because it is able to bind to a variety of exogenous and endogenous ligands with different structures and physiochemical characteristics (Table 1). Recently the classical AhR mechanism of activation has been questioned by different studies. The AhR activation pathway has been generally well characterized and implies that several AhR agonist ligands would trigger similar AhR-mediated biological responses. However, ligand-specific biological responses have been observed in some instances of AhR-dependent transcription (Gouédard *et al.*, 2004; Quintana *et al.*, 2008; Zhang *et al.*, 2008; Murray *et al.*, 2010; Murray *et al.*, 2010). To explain these ligand-specific differences, studies have been suggested some modifications of the classical pathway (Huang and Elferink 2012). These different mechanisms seem to imply ligand-

dependent differences in the AhR complex conformation, and that different ligand-specific mechanisms of ligands binding to AhR would exist (Guyot *et al.*, 2013). Indeed, different AhR ligands may bind in distinct sites within the AhR complex ligand-binding domain (LBD) (Petkov *et al.*, 2010). Thus, AhR can be directly bound and be activated by structurally diverse chemicals, which could result in different biological responses (Soshilov and Denison 2014; Shinde and McGaha 2018).

Class	Compounds	Origin	
AhR agonist	Endogenous		
	<ul style="list-style-type: none"> • Indole-3-carbinol (I3C) • 3,3'-diindolylmethane (DIM) • Indolo [3,2-b]carbazole (ICZ) • 2-(Indol-3-ylmethyl)-3,39-diindolylmethane (Ltr-1) • Indole-3-acetonitrile (I3ACN) • Curcumin • Diosmin 	Dietary	
	<ul style="list-style-type: none"> • Indole • Indole-3-acetic acid (IAA) • Indole-3-aldehyde (IAld) • Tryptamine • Indoxyl-3-sulfate (I3S) • 3-Methyl-indole (skatole) 	Microbiome	
	<ul style="list-style-type: none"> • Kynurenine (Kyn) • Kynurenic acid (KA) • Xanthurenic acid • Cinnabarinic acid (CA) • 2-(19H-indole-39-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) • 6-Formylindolo [3,2-b] carbazole (FICZ) • 5-hydroxy-tryptophan (5HTP) • Bilirubin • Biliverdin • Lipoxin A4 • Prostaglandin 	Host metabolism	
	<ul style="list-style-type: none"> • Indigo • Indirubin 	Plant/mammalian enzymes	
	<ul style="list-style-type: none"> • Trypthantrin • Malassezin 	Yeast/fungi	
	Exogenous		
	<ul style="list-style-type: none"> • 2,3,7,8-Tetrachlorodibenzop-dioxin (TCDD) • 3-Methylcholanthrene • Beta-naphthoflavone 	Polycyclic/halogenated aryl hydrocarbon (PAH/HAH) pollutants	
	<ul style="list-style-type: none"> • Omeprazole • VAF347 • 4-hydroxy-tamoxifen (4OHT) • 6-Methyl-1,3,8-trichlorodibenzofuran (6-MCDF) 	Synthetic	
	AhR antagonist	<ul style="list-style-type: none"> • Resveratrol • Quercetin 	Dietary
		<ul style="list-style-type: none"> • CH223191 • StemRegenin 1 (SR1) • GNF351 • MNF (3'-methoxy-4'-nitroflavone) • 3',4'-Dimethoxyflavone (DMF) 	Synthetic

Table 1: Major AhR agonists and antagonists studied. (Shinde and McGaha 2018)

3.2) AhR and physiological functions

AhR was originally discovered through its key role in mitigating the toxic effects of environmental pollutants, by binding of anthropogenic xenobiotic substances as for example TCDD or certain PAHs. However, studies conducted in AhR knock out models have revealed its physiological roles as recently reviewed by Larigot and colleagues (Larigot *et al.*, 2018). Indeed, AhR null mice strain displayed a range of physiological defects, including growth retardation, reduced liver size, abnormalities in vascular structure, portal tract fibrosis, and decreased fertility, arguing for endogenous functions of AhR, especially during development (Hao and Whitelaw 2013).

Several studies showed that AhR plays a critical significant role in the regulation of a wide range of immune functions such as the maintenance of innate and adaptive cell populations at mucosal barrier sites, and the control of inflammation nodes (Esser *et al.*, 2009; Quintana 2013; Stockinger *et al.*, 2014; Esser 2016; Cervantes-Barragan and Colonna 2018; Gutierrez-Vazquez and Quintana 2018; Neavin and Liu 2018; Rothhammer and Quintana 2019). In particular, it can affect the differentiation of B-cells (De Abrew *et al.*, 2011) and T-cells (Li *et al.*, 2017) and immune interactions between mucosal tissues and microbiome (Lawrence and Vorderstrasse 2013; Bessede *et al.*, 2014; Korecka *et al.*, 2016).

Moreover AhR seems to also be involved in other physiological functions on the body such as on nervous system regulation (Rothhammer *et al.*, 2016; Lee *et al.*, 2017; Juricek and Coumoul 2018), cell apoptosis (Stolpmann *et al.*, 2012; Esser *et al.*, 2013), skin differentiation (van den Bogaard *et al.*, 2015) and endocrine signaling (Reen *et al.*, 2002; Ohtake *et al.*, 2003). Recent studies suggested its role on pathogenesis and therapy in IBD (Lamas *et al.*, 2016), cardiovascular diseases (Yi *et al.*, 2018) and metabolic diseases such as obesity (Natividad *et al.*, 2018).

In the following subchapter, I focus on the role of AhR in intestinal homeostasis and gut inflammation as well as in metabolic diseases.

3.2.1) AhR and intestinal homeostasis and gut inflammation

In intestinal mucosa, AhR appears to play an important role in maintaining the intestinal homeostasis, mainly on epithelial cell renewal and turnover (Rannug and Rannug 2018). In particular, it seems to be involved in the proliferation of colonic crypt stem cells (Metidji *et al.*, 2018). Moreover, mice with intestinal epithelium-specific AhR deficiency had

enhanced apoptosis of epithelial cells, reinforcing the intrinsic role of AhR in intestinal epithelial cells integrity (Chinen *et al.*, 2015).

Some effects of AhR on epithelium integrity have been attributed to lymphocyte T cell response and altered interleukin and cytokine secretions (Monteleone *et al.*, 2012; Megna *et al.*, 2016). AhR influences different aspects of intestinal barrier function. For example, AhR is required for the development of intraepithelial lymphocytes (IEL) and of innate lymphoid cells (ILC) producing IL-22 (Li *et al.*, 2017), which as previously mentioned (see chapter 2.2.3) contribute to the immunological barrier. Interestingly CYP1A1 deficiency in intestinal epithelial cells (IEC) has been shown to increase the availability of AhR ligands to immune cells, suggesting that IEC serve as gatekeepers for the supply of AhR ligands to the host (Schiering *et al.*, 2017). Moreover, as bacteria produce AhR ligands, AhR plays an important role in the reciprocal relationship between gut microbiota and cellular host defense including gut immune system (Gao *et al.*, 2018) (Figure 19).

According to these functions, most of the data concerning the role of AhR in the gut are related to inflammatory diseases. Several research groups have shown that AhR is involved in the regulation of the immune response to Crohn's disease (CD) and ulcerative colitis (UC), which are the two main subtypes of IBD, which present with important gut inflammation. Studies have demonstrated that lack of AhR could intensify the pathological process in IBD and suggest that AhR activation could have a protective role in the gut (Islam *et al.* 2017; Lamas *et al.* 2016; Monteleone *et al.* 2013). Interestingly, AhR expression is low in inflamed tissue from CD patients, but not UC patients, compared with CD patients in remission or with healthy subjects suggesting specificities linked to the intestinal pathology or intestinal segment injured (Qiu and Zhou 2013). In experimental animal models, AhR deficient mice are more susceptible to chemically and bacterially induced colitis (Stockinger *et al.*, 2014; Lamas *et al.*, 2018). Conversely, the administration of AhR agonists decreased the Trinitrobenzene Sulfonic Acid (TNBS)-, Dextran Sodium Sulfate (DSS)- and T-cell-transfer induced colitis severity, by down-regulation of pro-inflammatory cytokines, such as tumor necrosis factor TNF- α , IL-6, and IL-1 β (Furumatsu *et al.*, 2011; Li *et al.*, 2011; Monteleone *et al.*, 2011; Qiu and Zhou 2013; Lamas *et al.*, 2016; Long *et al.*, 2016). In these models as well as in the intestinal epithelial cell line Caco-2 treated with cytokines or LPS, an impairment of intestinal epithelium permeability and cell-cell junctions can be rescued by AhR agonist (Yu *et al.*, 2018; Yu *et al.*, 2018).

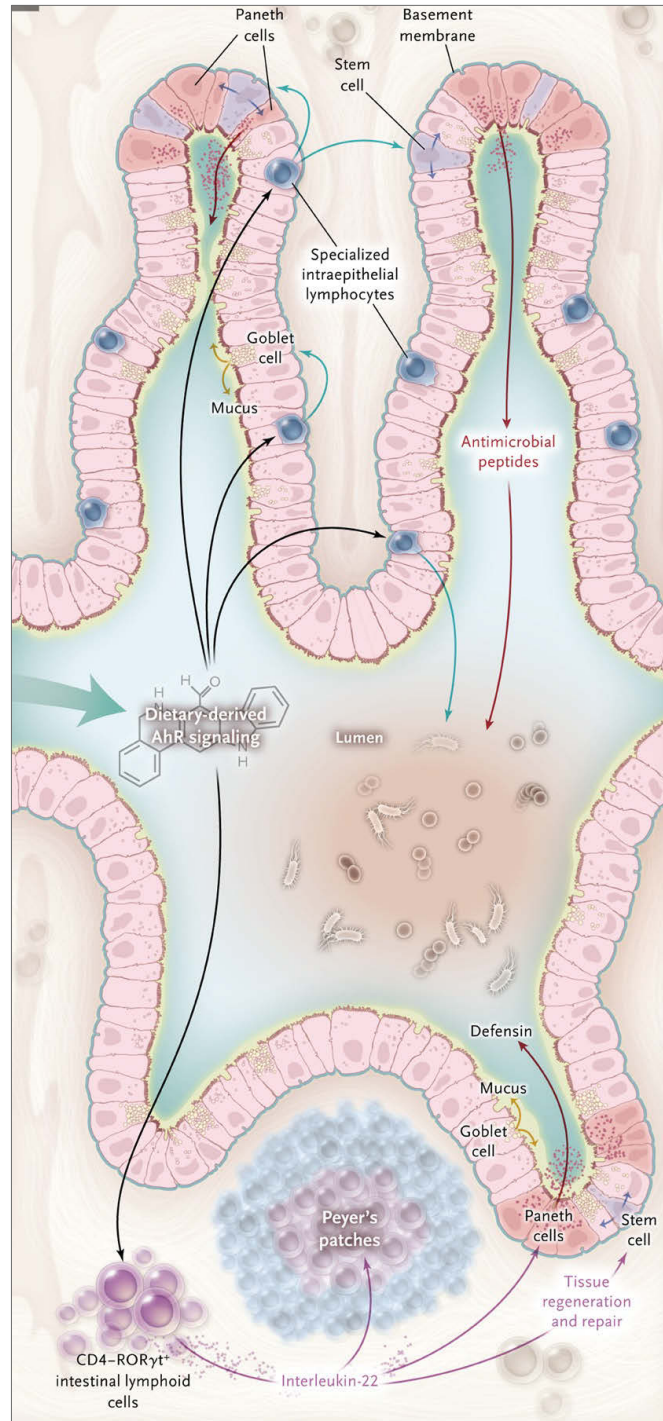


Figure 19: AhR and intestinal homeostasis

Two major cell types are critically dependent on dietary derived AhR signals: specialized intraepithelial lymphocytes (e.g., intraepithelial T-cell receptor $\gamma\delta$ cells) and CD4-ROR γ t⁺ intestinal lymphoid cells within lymphoid tissue (e.g., Peyer's patches). Studies have shown that mice lacking AhR signals (obtained genetically or through dietary deprivation) presents a deficiency in these cell types, which leads to reduced epithelial turnover, reduced expression of antimicrobial peptides, altered microbiota and increased susceptibility to intestinal inflammation. (Tilg 2012)

3.2.2) AhR and metabolic disorders

Some studies have investigated the possible role of AhR in metabolic syndrome by comparing the evolution of diet-induced metabolic impairments in AhR null mice or by treating mice with AhR agonists and antagonists. However, the reported results were contradictory.

Indeed, several reports showed that AhR deficient mice are protected from diet-induced obesity and associated metabolic disorders such as insulin resistance, and hepatic steatosis through mechanisms related to lipid and energy metabolisms (Xu *et al.*, 2015; Moyer *et al.*, 2016; Moyer *et al.*, 2017). Conversely, the activation of AhR using genetic mouse models or agonists in particular TCDD promoted hepatic steatosis (Lee *et al.*, 2010; He *et al.*, 2013). The study of Lee *et al.* (Lee *et al.*, 2010) revealed that the steatotic effect of AhR activity is due to the upregulation of the fatty acid transporter CD36, to the inhibition of hepatic export of triglycerides, and to the suppression of fatty acid oxidation associated with an increased peripheral fat mobilization. A study using two mice strains which naturally encoding for two different alleles of *AhR* gene, one strain displaying an high-affinity *AhR* allele and the other one a low-affinity *AhR* allele, showed that mice with the high-affinity *AhR* allele are more susceptibility to diet induced-obesity than the other one (Kerley-Hamilton *et al.*, 2012).

The deleterious effects of AhR activation/activity reported above are in contrast with other works showing a protective role of AhR towards liver steatosis in mice (Wada 2016, Natividad 2018). Moreover, in human low levels of AhR ligands were quantified in feces of obese subjects and were associated with metabolic syndrome, type 2 diabetes, high BMI and high blood pressure (Natividad 2018).

The discrepancy concerning the role of AhR in metabolic diseases illustrated by these two sets of reports may be due to the different mouse models used, (in particular regarding the complex phenotype of AhR KO or constitutively activated mice) as well as the diversity of AhR functions in different organs or cell types in the body.

Taken together, the various examples mentioned below show that AhR plays an important role in intestinal inflammation, obesity and metabolic disorders. However, the mechanisms and the contradictory results driving AhR effects remains unclear so far.

OBJECTIVES

Objectives

The involvement of the intestine in the etiology of the low-grade systemic inflammation observed during obesity has been put forward over the last decade by several studies in humans and in murine models submitted to a high-fat diet. In the case of established obesity, the coexistence of metabolic disturbances, the alteration of the intestinal barrier, the changes in gut microbiota composition and the presence of tissue inflammation makes it difficult to identify the initiating factors. Several evidences implicate the role of diet, especially lipid as well as environmental factors or microbiota-derived metabolites influencing the activity of the aryl hydrocarbon receptor, on the onset of gut inflammation and intestinal barrier damages during obesity. One hypothesis is that initiation of gut inflammation was linked to the alteration of intestinal barrier integrity, promoting the translocation of bacteria fragments through the intestinal mucosa that would activate gut immune response contributing to the establishment of a vicious circle leading to systemic inflammation. This hypothesis implies that part of these events such as intestinal barrier impairment and gut inflammation occurs before the onset of obesity as suggested by several studies (Cani *et al.*, 2007; Erridge *et al.*, 2007; Cani *et al.*, 2008; Ghoshal *et al.*, 2009; Laugerette *et al.*, 2011; Vors *et al.*, 2015; Genser *et al.*, 2018).

In this context, my thesis work was to study **the role of two environmental factors on intestinal barrier dysfunctions reported in obesity**. I focused my research on the impact of lipid and of the activation of the transcription factor AhR. My purpose was to identify cause-effect relationships and the molecular mechanisms involved.

In a first work (article 1, Ghezzal *et al.*), of which I am co-first author, we sought to determine if single or few repeated lipid or fatty acid intake is sufficient to cause barrier disruption and initiate gut inflammation. I studied the effect of palmitic acid, the main fatty acid present in the human diet. This work was carried out on wild-type mice and on a human model of intestinal epithelial cells in culture, the Caco-2/TC7 cell line.

In a second work (article 2, Postal *et al.*), I evaluated the AhR tone in the jejunum of obese subjects presenting with small intestine inflammation and I investigated the consequences of AhR activation in mice and in Caco-2/TC7 cells. I studied the effect of AhR activation by its agonist, β -naphthoflavone, on intestinal epithelium whose integrity was perturbed by factors affecting paracellular permeability and cell junctional complexes.

RESULTS

Results

Effect of palmitic acid on intestinal barrier function

The results of this study are presented at the end of this chapter in the form of an article in review in BBA Molecular and Cell Biology of Lipids journal, and entitled: "Palmitic acid damages gut epithelium integrity and initiates production of inflammatory cytokines", which I am the co-first author (article 1). I participated directly and actively in this work during the first two years of my thesis to establish the effects of palmitic acid on intestinal barrier function. However, I had a less important participation in the determination of the mechanisms involved via the ceramides pathway.

It has been observed that the nature of fatty acids in the diet could differently affect metabolic inflammation. Indeed in mice, a greater increase in inflammation was caused by an isocaloric diet enriched in palm oil compared to other oils such as sunflower or rapeseed oil (Laugerette *et al.*, 2012; Ravussin *et al.*, 2012). These studies suggested that a high-fat diet triggers intestinal barrier dysfunction and emphasized the potential role of saturated fatty acids in this effect. Moreover, several studies showed in rodent models of high fat diet-induced obesity that alteration of intestinal barrier function, especially of paracellular permeability, occurred as soon as the first week of the diet and thus before the onset of obesity (Johnson *et al.*, 2015). Altogether these data conduct us to hypothesize that an intestinal barrier disruption triggered by lipids would be an early event involved in the initiation of intestinal barrier or even systemic inflammation.

Thus, the aim of this work was to study the short-term effects of a saturated fatty acid, palmitic acid, on intestinal barrier and inflammation. This work was carried out on a mouse model and on Caco-2/TC7 cells.

Palm oil and palmitic acid treatments perturbed intestinal epithelial barrier.

We studied the effect of a unique or repeated supply of palm oil or palmitic acid on the intestinal barrier integrity by different complementary approaches: the measurement of intestinal permeability to macromolecules using a fluorescent tracer (FITC-dextran 4kD) and the evaluation of paracellular permeability to ions using the transepithelial electrical resistance (TEER) as well as the location and the expression of junctions proteins by immunofluorescence, western blot and RT-qPCR analyses.

In vivo measurement of intestinal permeability to macromolecules performed in wild – type mice, has revealed that a single gavage of palm oil is sufficient to increase intestinal permeability, however, repeated gavages of palm oil for 5 consecutive days does not seem to exacerbate this effect (Figure 1A in article 1). As cell-cell junctions control intestinal permeability we performed immunofluorescence analyses on mouse jejunum sections to visualize the location of proteins involved in cell junctions. We specifically studied tight junction proteins (ZO-1, occludin and tricellulin) and adherent junction protein (E-cadherin). We observed that a single intake of palm oil altered the location of occludin and tricellulin at the cell membrane, while the repeated supply provoked the mislocalization of all junction proteins studied (Figure 1B-C in article 1).

We then assessed intestinal inflammation, in different segments of the intestine (jejunum, ileum and colon), in mice (Figure 2 in article 1). We quantified the expression of genes and proteins known to be modulated in inflammatory conditions. We observed that repeated palm oil gavages provoked a mild inflammatory response characterized by the decrease of the intestinal expression of GATA3 (a master gene controlling the function of T lymphocytes and innate lymphoid cells), of Reg3 γ (coding for an antimicrobial peptide) and the increase of pro-inflammatory cytokine IL-1 β . Our results indicated that short-term treatment with palm oil modified the expression of genes involved in host defense and immunity, leading to an imbalance of intestinal defense and immune response towards a proinflammatory profile.

Links between changes in microbiota composition, obesity and inflammation have been reported (Chassaing and Gewirtz 2014; Sanz and Moya-Perez 2014; Boulangé *et al.*, 2016; Sun *et al.*, 2018; Aron-Wisnewsky *et al.*, 2019; Cuevas-Sierra *et al.*, 2019). Since, microbiota composition can change rapidly after a dietary transition (David *et al.*, 2014), we investigated whether repeated intake of palm oil for 4 days is able to modify this composition. We chose to study selected microbiota species known to be modulated in inflammatory bowel diseases and obesity. Ours analyze performed by qPCR showed that species known to exert protective effects against pathogens and beneficial role to host cells, such as *Clostridium leptum*, *Akkermensia muciniphila* and Segmented Filamentous Bacteria (Marchix *et al.*, 2018), are reduced after palm oil treatment compared to the oil-free control group. Conversely, the genus *Bacteroides*, increased in animals subjected to an obesogenic diet

enriched with saturated fatty acids (Devkota *et al.*, 2012), was higher in palm oil-treated mice (Figure 3 in article 1).

Thus, the mouse experiments demonstrate that a single intake of palm oil is sufficient to trigger damages to the intestinal barrier and its repetition exacerbates these deleterious effects. The repeated supply of palm oil, even though in short-term treatment, leads to a proinflammatory response in the intestine and a change in the abundance of fecal bacterial species.

To conduct the *in vitro* studies, we choose a cellular model of intestinal epithelial barrier, the Caco-2/TC7 cell line. Indeed, Caco-2 cell line and especially its clone TC7, is one of the most potent *in vitro* models to study intestinal functions (Chantret *et al.*, 1994). This cell line spontaneously differentiated when the cells reach the confluence and express a normal human enterocyte-like phenotype (Chantret *et al.*, 1994). The Caco-2/TC7 cell monolayer is used to mimic *in vitro* the epithelial barrier that exists *in vivo* between the gut lumen and the underlying tissues. Intestinal epithelial cells are able to produce cytokines (Stadnyk 2002; Miron and Cristea 2012) among which CXCL8 (IL-8), TGFB1 and IL1B genes are particularly well expressed in Caco-2/TC7 cells.

Palm oil being composed palmitic and oleic acids, both fatty acids might exert specific impact on intestinal cells. We investigated the effects of these two fatty acids, delivered as complex micelles, on paracellular permeability of Caco-2/TC7 cell line monolayer and cytokine expression. Our results showed that a unique palmitic acid supply triggers an increase in paracellular permeability (Figure 4 in article 1) and a decreased expression and mislocalization of junctional proteins (ZO-1, occludin, tricellulin and E-cadherin) at cell-cell contacts (Figure 5 in article 1). The repeated palmitic acid intake over 4 days exacerbates these effects. Moreover, a 24 hours-treatment with palmitic increased the expression of IL-8 and induced the secretion of this pro-inflammatory cytokine (Figure 6, article 1). A longer palmitic acid supply (for 4 consecutive days) accentuated these effects. Repeated palmitic acid supply reduced also the mRNA level of TGF- β , an anti-inflammatory cytokine (Figure 6 in article 1). In addition, we did not observe any effect of oleic acid in all studied parameters (paracellular permeability and cytokine expression).

We were also interested whether the deleterious effects on the epithelial barrier and the initiation of an inflammatory response mediated by palmitic acid are transitory effects. For

that, we treated the cells with palmitic acid for 4 days followed by 24h or 48h without any treatment. Our results show that the impact of the 4 days treatment, although it is attenuated after 48 hours, persists over time (Figure 7 in article 1).

Identification of molecular mechanisms involved in intestinal barrier dysfunction and cytokine expression triggered by palmitic acid.

We intended to determine the molecular mechanisms responsible for the deleterious effects of palmitic acid on the intestinal barrier and the inflammatory response in Caco-2/TC7 cells. In our experiments, palmitic acid is supplied as “postprandial” lipid micelles to mimic the complex physiological form of fatty acids present in the intestinal lumen after a meal. These lipid micelles are containing phospholipid, cholesterol, biliary acid and monooleoylglycerol. Palmitic acid is known to activate endoplasmic reticulum (ER) stress and *de novo* synthesis of ceramides, both pathways described to disrupt cellular functions (Chaurasia and Summers 2015; Lindholm *et al.*, 2017). So we investigated the implication of these two pathways.

The 4 days treatment with palmitic acid showed a slight increase in the expression of some ER stress marker genes, compared to untreated cells. However, similar effects were observed after oleic acid treatment (Figure 8 in article 1). Thus, the activation of the ER stress pathway does not seem to explain the specific impact of palmitic acid vs oleic acid on the epithelial barrier and cytokine expression in Caco-2/TC7 cell line.

Palmitic acid, but not oleic acid, is a precursor of *de novo* ceramide synthesis (Castro *et al.*, 2014), which contributes not only to membrane structure but also to signaling and metabolic dysfunctions in various cell types, including Caco-2/TC7 cells (Chavez and Summers 2012; Tran *et al.*, 2016). We first investigated whether ceramide supply to Caco-2/TC7 cells could perturb paracellular permeability and increases cytokine expression. Cells were incubated with a cell-permeable short-chain ceramide, C2-ceramide, which will then be elongated in the cell (Hajdich *et al.*, 2001; Uchida 2014; Tran *et al.*, 2016). We have observed that the addition of C2-ceramide produced similar deleterious effects than palmitic acid, on the epithelial barrier and on inflammatory cytokines expression (Figure 9 in article 1). Moreover, in order to determine the implication of the ceramide pathway on palmitic acid deleterious effect, we used a specific inhibitor (L-cycloserine) of the first enzyme of *de novo* ceramide synthesis. Cells were treated with palmitic acid in the presence or absence of L-

cycloserine (Figure 10 in article 1). Our results demonstrated that the addition of an inhibitor of *de novo* ceramide synthesis prevented the increase of IL-8 expression following treatment with palmitic acid. However, it did not block the increase of paracellular permeability induced by palmitic acid suggesting the implication of other mechanisms to explain all the palmitic acid effects.

Taken together, our results obtained in mice and in a human cell line of the intestinal epithelial barrier showed that palmitic acid administered acutely is able to induce epithelial barrier dysfunctions, to trigger an inflammatory response and to modify microbiota composition. Studies performed in the Caco-2/TC7 cell line showed that part of these effects is ceramides synthesis dependent. Deleterious effects of palmitic acid occurring rapidly in the intestine may lead to the stimulation of systemic inflammation that in turn may affect other organs. The repetition of such deleterious effects associated with a lower capacity of the intestinal epithelial barrier to ensure an appropriate defense against pathogens, may initiate or contribute to low-grade tissue and systemic inflammation observed in obesity.

ARTICLE 1

Palmitic acid damages gut epithelium integrity and initiates production of inflammatory cytokines

Sara Ghezzal^{a,1}, Barbara Graziela Postal^{a,1}, Elodie Quevrain^c, Loic Brot^d, Philippe Seksik^{d,e}, Armelle Leturque^a, Sophie Thenet^{a,b,2}, Véronique Carrière^{a,2,*}

^{1,2}These authors contributed equally to this work

^a Sorbonne Université, INSERM, Université Paris Descartes Paris 5, CNRS, Centre de Recherche des Cordeliers, F-75006 Paris, France

^b EPHE, PSL Research University, F-75006 Paris, France

^c Laboratoire des Biomolécules, LBM, Département de chimie, Ecole Normale Supérieure, ENS, PSL University, Sorbonne Université, CNRS, F-75005 Paris, France.

^d Sorbonne Université, Centre de Recherche de Saint Antoine INSERM, UMRS 938, F-75012 Paris, France

^e Service de Gastroentérologie & Nutrition, Hôpital Saint-Antoine, APHP, F-75012 Paris, France

*To whom correspondence should be addressed (present address): Dr Véronique Carrière, UMRS 938, Centre de Recherche de Saint-Antoine, 27 rue de Chaligny, 75012, Paris, France, Phone: 33140011389, Email: veronique.carriere@sorbonne-universite.fr

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Abbreviations:

DAPI: 4',6-diamidino-2-phenylindole

DDIT3: DNA Damage Inducible Transcript 3

DSS : dextran sodium sulfate

DTT: dithiothreitol

EGTA: ethylene glycol tetraacetic acid

ELISA : enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

EtOH : ethanol

HSPA5: Heat Shock Protein Family A (Hsp70) Member 5

OA: oleic acid

PA: palmitic acid

TEER: Transepithelial electrical resistance

XBP1: X-box binding protein-1

ZO-1: zonula occludens-1

Abstract

The mechanisms involved in low-grade inflammation observed in human obesity and in rodent submitted to high-fat diet, are not fully understood yet. We tested the hypothesis that the intestine could be damaged by the repetition of lipid supplies and thus participates in inflammation. In mice, one to five palm oil gavages damaged intestinal permeability via decreased expression and mislocalization of junctional proteins at cell-cell contacts, changed the intestinal bacterial species by decreasing the abundance of *Akkermansia muciniphila*, Segmented Filamentous Bacteria and *Clostridium leptum*, and increased inflammatory cytokine expression. This was further studied in human intestinal epithelial cells Caco-2/TC7, using the two main components of palm oil i.e palmitic and oleic acids. Saturated palmitic acid, but not unsaturated oleic acid, impaired paracellular permeability and junctional protein location and induced inflammatory cytokine expression in Caco-2/TC7 cells. Part of these effects was prevented by inhibition of *de novo* ceramide synthesis.

Taken together, our data show that exposure to palm oil or palmitic acid, induces intestinal dysfunctions on barrier integrity and inflammation. Excessive consumption of palm oil could be an early player in gut alterations observed in metabolic diseases.

Highlights

- Palm oil gavages damage the intestinal barrier.
- Bacterial species in mice are changed after 4 gavages with palm oil.
- Palmitic acid impairs paracellular permeability and cell-cell junctions.
- Palmitic acid modifies cytokine expression profile in intestinal epithelial cells.
- Inhibition of ceramide synthesis blocks part of palmitic acid effects.

1. Introduction

Obesity is the result of an energy imbalance between calories consumed and calories expended and its rising incidence is associated with changes in eating habits. Obesity is associated with systemic and tissue low-grade inflammation, which, contributes to the development of insulin resistance and increased risk of cardiovascular diseases (Pereira and Alvarez-Leite 2014; Monteiro-Sepulveda *et al.*, 2015). While adipose tissue inflammation has been described as an important actor in the maintenance of the inflammatory state once obesity is established (Pereira and Alvarez-Leite 2014), the origin of this inflammation remains undetermined.

The subclinical systemic inflammation observed during obesity has been attributed to the immune response to increased levels of lipopolysaccharides (LPS) from Gram-negative bacteria cell wall, then called metabolic endotoxemia (Cani *et al.*, 2007). The passage of bacterial fragments into the blood through the intestinal mucosa emphasized the importance of intestinal epithelial barrier in this process (Cani *et al.*, 2007; Neves *et al.*, 2013; Boutagy *et al.*, 2016). In a recent study, we showed that obese subjects present subtle impairments of the intestinal barrier function that is exacerbated after a lipid challenge, this feature being correlated with systemic and intestinal inflammation (Genser *et al.*, 2018). Thus, loss of intestinal barrier integrity is probably part of the mechanisms driving subclinical chronic inflammation.

Studies in rodents under long term high-fat diet (HFD) also highlighted the role of lipids on endotoxemia but only few have analyzed the HFD impact on intestinal barrier integrity (for recent reviews (Boutagy *et al.*, 2016; Araujo *et al.*, 2017)). Decreased mRNA levels of the tight junction proteins ZO-1 and occludin were observed in the intestine of mice after 4 weeks of HFD (Cani *et al.*, 2008). Gulhane *et al.* (Gulhane *et al.*, 2016) showed that prolonged HFD in mice leads to an increase in colonic inflammation, which is associated with endotoxemia and a decreased expression of the tight junction protein claudin-1 in this tissue. Some studies reported that the composition of HFD could influence microbiota changes, endotoxemia and immune response. Laugerette *et al.* (Laugerette *et al.*, 2012) compared, in mice, the effects of 8-week diets enriched in oils and showed that a diet enriched

in palm oil results in the most active transport of LPS toward tissues and the highest plasma level of the pro-inflammatory cytokine IL-6. More recently, Lam et al (Lam *et al.*, 2015) showed that mice fed for 8 weeks with high-fat diets enriched in either saturated fatty acids or in n-6 polyunsaturated fatty acids displayed similar weight gain and adiposity, but only mice fed with saturated HFD exhibited an increased paracellular permeability to ions evaluated by a decreased transepithelial resistance (TEER) and macrophage infiltration in the colon. Palmitic acid, one of the main saturated fatty acid present in human diet might exert an important impact on gut permeability and inflammation, and should therefore be further investigated.

Several studies suggest that the effects of HFD on intestinal permeability occur before the onset of obesity. An increase of albumin in feces, reflecting higher intestinal permeability, was observed as early as after 1 day of HFD and an increase of the passage of FITC-dextran molecule through the intestinal epithelium was shown after 7 days (Johnson *et al.*, 2015). Moreover, studies conducted in lean humans or in rodents under chow diet showed that a single lipid meal led to a moderate and transient increase in endotoxemia (Erridge *et al.*, 2007; Ghoshal *et al.*, 2009; Laugerette *et al.*, 2011) as well as proinflammatory cytokine IL-6 plasma level (Laugerette *et al.*, 2011). In these studies, neither the importance of fatty acids composition of the meal (especially the effects of palmitic acid), nor the integrity of intestinal epithelial barrier was documented. Nevertheless, these results suggested that an acute supply of lipids was sufficient to provoke a transient alteration of intestinal epithelial barrier.

It can be hypothesized that the repetition of lipid supplies could amplify or maintain a defect of intestinal epithelial barrier, which in turn could initiate or contribute to low-grade inflammation. The purpose of this study was to explore the impact of an acute single or repeated supply of palm oil and palmitic acid on intestinal epithelial barrier integrity and its consequence on the expression of intestinal genes involved in inflammatory response. Mechanistic investigations were conducted on ER stress and ceramide synthesis.

2. Materials and Methods

2.1 Mouse treatments, induction and assessment of colitis, and intestinal permeability

Three-month-old male C57BL/6J mice (Janviers labs, Le Genest-Saint-Isle, France) were fed *ad libidum* a standard chow diet (A04, SAFE, Augy, France). Mice were kept with the artificial light-dark cycle 12:12h with lights on 07:00h. Mice were gaved with palm oil (Sigma-Aldrich, Saint Quentin-Fallavier, France) whose fatty acid composition is rich in saturated palmitic acid (about 45%) and unsaturated oleic acid (about 35%). Mice were gaved with 0.2ml of palm oil for one or 5 consecutive days at 18:00h i.e. just before the feeding period. Mice submitted to the lipid loads had a 20% reduction of their food consumption but the calorie intake was preserved (9kcal/mice/24h and 8.8kcal/mice/24h for control and palm oil treated-mice, respectively). The last day of the experiment, mice were successively gaved with 0.2ml palm oil and with 0.2ml of 4kDa FITC-dextran (Sigma-Aldrich) solution (0.5mg/g of mice in water) at 9:00h. Mice were anesthetized and then euthanized 1 hour after the last gavage. Blood, small intestine and colon were then collected. Duodenum was removed and the rest of the small intestine was divided in two equal parts named here jejunum and ileum. For *in vivo* measurement of intestinal permeability, FITC-dextran concentrations were determined in the plasma by fluorometry (FLUOstar Omega; BMG Labtech, Champigny-sur-Marne, France). Colitis was induced in mice by 5 days of 3.5% dextran sodium sulfate (DSS) (MP Biomedicals, Illkirch-Graffenstaden, France) in drinking water, followed by a 3-day recovery period without DSS. All experiments involving mice were approved by the French Minister of Education and Research and by the Animal Care and Use Committee N°5 (agreement number: APAFIS#2710-201510301447819).

2.2 Fecal bacterial species analysis

Mice received one gavage with 0.2ml palm oil or water each day for 4 consecutive days. Fresh feces were collected 16h after the last gavage and immediately stored at -80°C until analysis. DNA was extracted from 100mg of feces obtained from pools of fecal pellets from 1 to 3 mice, as previously

described (Sokol *et al.*, 2009). The primers used for group and species-specific 16S rRNA-targeted are described in Table 1. PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR Systems (ThermoFischer Scientific, Illkirch, France). Determination of bacteria count in fecal samples was achieved using DNA extracted from cultured bacterial strains (see Table 1) and analyzed as described previously (Sokol *et al.*, 2009). For the two species, Segmented Filamentous Bacteria and *Akkermansia muciniphila*, abundance was determined by relative quantification to DNA extracted from feces of untreated mice.

2.3 Cell culture and cell treatments

Caco-2/TC7 cell line is a clonal population of the human colon carcinoma-derived Caco-2 cells, which reproduces to a high degree most of the morphological and functional characteristics of enterocytes (Chantret *et al.*, 1994). Cells were checked for absence of mycoplasma contamination. In all experiments, cells were cultured on 6-well Transwell® filters (ThermoFischer Scientific) for 3 weeks to obtain fully differentiated enterocyte-like cells as previously described (Beaslas *et al.*, 2009; Morel *et al.*, 2018).

Palmitic acid or oleic acid (Sigma-Aldrich) was supplied as complex micelles to mimic the physiological form in which these fatty acids are present in the intestinal lumen *in vivo*. Complex micelles (2 mM sodium taurocholate, 0.6 mM oleic acid or 0.6 mM palmitic acid, 0.2 mM lysophosphatidylcholine, 0.05 mM cholesterol, and 0.2 mM monoacylglycerol) were prepared in serum-free medium as previously described (Chateau *et al.*, 2005; Beaslas *et al.*, 2009) and added to the upper compartment for the indicated times. In some experiments, palmitic acid concentration was changed to 0.3 mM or 0.1 mM. Micelles were removed and replaced by fresh apical medium during the analysis of epithelium integrity. The release of LDH cytoplasmic enzyme in the medium, used as toxicity test, remained below 1% of total LDH activity of the cell lysate when palmitic or oleic acids were added to cultured cells. In some experiments, cells were treated with 4.5mM EGTA (Sigma-Aldrich) added in the upper compartment or with 2mM dithiothreitol (Sigma-Aldrich), 20mM L-

cycloserin (Sigma-Aldrich), 100 μ M C₂-ceramide (Cayman Chemical Compagny, Montigny-le-Bretonneux, France) added in both upper and lower compartments. C₂-ceramide was dissolved in 0.5% ethanol as previously described (Tran *et al.*, 2016). The duration of treatments is indicated in the legend of figures.

2.4 Permeability measurements in Caco-2/TC7 cells

To assess paracellular permeability, 1mg/ml of 4 kDa FITC-dextran (TdB Consultancy AB, Uppsala, Sweden) was added to the apical medium the last day of treatment. Samples of basal medium were collected after 4h, and fluorescence was determined with a microplate fluorometer (FLUOstar Omega; BMG Labtech). Transepithelial electrical resistance (TEER), which is inversely proportional to permeability to small ions, was measured before and after treatments using a Volt-Ohm Meter (Millipore, Guyancourt, France).

2.5 Total RNA extraction and RT-PCR analysis

Total RNA from Caco-2/TC7 cells and from intestinal segments was extracted with TRI Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. Reverse transcription (RT) was performed with 1 μ g of RNA using high-capacity cDNA reverse transcriptase kit (Applied Biosystem, ThermoFisher Scientific, Illkirch France). Semi-quantitative real-time polymerase chain reactions were performed with the Mx3000P Stratagen system using SYBR Green (Agilent, Les Ulis, France) according to the manufacturer's procedures. The list of human and mouse primers sequences are displayed in Table 2. The primer sequences for the specific quantification of the spliced XBP1 mRNA have been previously described in Van Schadewijk *et al.* (van Schadewijk *et al.*, 2012).

2.6 Cytokine content and secretion

Cytokine IL-8 and IL-1 β protein levels were quantified by enzyme-linked immunosorbent assay (ELISA) using kits from R&D System (Lille, France). For cytokine quantification in mouse intestine, whole intestinal segments were homogenized in PBS buffer using FastPrep® instrument (MP-Biosciences, Illkirch-Graffenstaden, France), submitted to sonication (Bioruptor®, Diagenode, Seraing, Belgium) and then centrifuged at 10,000g for 10 min at 4°C. The supernatant (100 μ g of proteins) was used for ELISA. For experiments in Caco-2/TC7 cells, 0.2ml of basal medium was used for ELISA.

2.7 Analysis of junctional proteins by immunofluorescence

Immunofluorescence analyses were performed as previously described (Petit *et al.*, 2012). Briefly, Caco-2/TC7 cells were fixed and permeabilized by incubation for 5 minutes in methanol at -20°C. Jejunum cryosections were fixed for 30 minutes with 4% paraformaldehyde at 4°C, and permeabilized for 30 minutes in 0.1% Triton X-100 at 4°C. For immunostaining of mouse jejunum cryosections, we used primary antibodies for tricellulin (1:10; Tric2469; kindly provided by Dr Furuse (Ikenouchi *et al.*, 2005; Ikenouchi *et al.*, 2008)), ZO-1 (1:200; 617300; Thermofischer scientific), occludin (1:10; Moc-37; kindly provided by Dr Furuse (Saitou *et al.*, 1997)) and for E-cadherin (1:500; ECCD2 M108; from Takara Bio Europe, Saint-Germain-en-Laye, France). For immunostaining on Caco-2/TC7 cells, we used primary antibodies for tricellulin (1:200; MARVELD2 700191; Thermofischer scientific), ZO-1 (1:200; ZO1-1A12; 33-9100 Thermofischer scientific), occludin (1:200; 71-1500; Thermofischer scientific) and for E-cadherin (1:500; ECCD2 M108; Takara Bio Europe). Alexa 488 and Alexa 546-conjugated anti-immunoglobulin G were used as secondary antibodies (1/400; Molecular Probes, Life Technologies, Saint-Aubin, France). Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI) to assess the monolayer integrity. Cells were examined by microscopy using an Axio Imager 2 microscope equipped with an apotome.2, allowing optical sectioning (Zeiss,

Oberkochen, Germany). Images were acquired by ZEN 2011® software (Zeiss,) and analyzed by Image J 1.46c.

2.8 Simple Westerns™

Caco-2/TC7 cells were lysed as previously described (Beaslas *et al.*, 2009). Protein levels were detected in cell lysates using a Wes™ capillary electrophoresis system (ProteinSimple, San José, CA, USA) according to the manufacturer's instructions. Reconstituted images and quantification were performed using Compass for Simple Western software (ProteinSimple). Primary antibodies were rabbit anti-tricellulin (1:2000; MARVELD2 700191; Thermofischer scientific), rabbit anti-ZO-1 (1:200; ZO1-1A12; 33-9100 Thermofischer scientific), rabbit anti-occludin (1:25; 71-1500; Thermofischer scientific) and rabbit anti-E-cadherin (1:1000; clone 36, 610181; BD Biosciences, Rungis, France). Secondary antibodies and reagents used were provided in the separation and detection module kits (ProteinSimple). Junctional proteins levels were normalized to Hsc70 (1:100; sc7298 Santa Cruz, Clinisciences Nanterre, France) or to actin (1:1000; NB600-532SS Novus Biologicals, Bio-techne Ltd. Lille, France).

2.9 Statistical analysis

Values were expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Two group comparisons were performed using Student's T-test. Comparisons involving multiple groups were done using one-way analysis of variance (ANOVA). A level of $p < 0.05$ was considered as significant.

3. Results

3.1 Palm oil gavage in mice increases intestinal permeability, perturbs cell-cell junction and modifies the expression of genes involved in inflammation

We first analyzed in mice the effects of palm oil administration on intestinal barrier integrity by measuring intestinal permeability to FITC-dextran 4kDa. We compared the effects of a single gavage of palm oil to daily gavages for 5 consecutive days. We calculated that a lipid gavage of 180 mg represents, in one taking, the daily lipid consumption (210 mg) of a mouse fed control diet containing 8 % w/w fat. A group of mice treated with dextran sodium sulfate (DSS) was used as a model of chemically induced barrier defect (for review (Eichele and Kharbanda 2017)). As previously described (Yan *et al.*, 2009), DSS treatment induced a 2-fold increase in intestinal permeability as compared to control group ($p=0.0008$) (Fig. 1A). The permeability to 4 kDa molecules after palm oil gavage was increased in the same range as in our positive control, DSS. Mice that received one palm oil gavage displayed higher intestinal permeability ($p<0.0001$) than the control group. Surprisingly, no further increase followed multiple gavages.

As an efficient epithelial barrier is dependent on cell-cell junction integrity, we analyzed by immunofluorescence in the jejunum the localization of three tight junction proteins (ZO-1, occludin and tricellulin) and of the adherens junction protein E-cadherin. One palm oil gavage resulted in reduced occludin and tricellulin labeling at apico-lateral membranes. ZO-1 labeling was more diffuse while E-cadherin was not affected (Fig.1B). After 5 consecutive palm oil gavages, the distribution of all these proteins was perturbed. In addition to the reduction of occludin and tricellulin labeling, we observed a shift of E-cadherin and ZO-1 labeling toward the basal region of the lateral membrane (Fig.1B, red arrows). A decreased occludin mRNA level was observed after 1x and 5x palm oil gavages (Fig.1C) while the mRNA levels of the other junction proteins remained unmodified (data not shown).

We then analyzed the expression of several genes linked to inflammation in intestine (Fig. 2). Gata3 is known as a master gene controlling the functions of T cells and innate lymphoid cells (Wan 2014; Zhu 2017). When compared to control mice, Gata3 mRNA level in jejunum was decreased in mice from the DSS ($p=0.0003$) or 5 palm oil gavages ($p=0.0024$) groups but not yet after a single palm oil gavage (Fig. 2A). This suggests that repeated palm oil gavages altered immune response in

intestine. Reg3g is a major antimicrobial peptide gene involved in host defense against bacterial invasion (Loonen *et al.*, 2014; Wang *et al.*, 2016). One palm oil gavage increased the mRNA level of Reg3g ($p=0.0118$) in ileum as compared to the control group (Fig. 2B) suggesting that antimicrobial host defense is engaged, but this vanished in mice submitted further to 5 palm oil gavages or to DSS. One can hypothesize that repeated palm oil treatment impaired antimicrobial host defense that could participate to intestinal barrier defects. This result suggests also that changes in microbiota composition could occur after palm oil treatment. The protein level of pro-inflammatory IL-1 β tended to increase ($p=0.053$) in colon of mice submitted to one palm oil gavage and a 2.4-fold increase ($p=0.0027$) was observed after 5 gavages (Fig. 2C).

Altogether, we show *in vivo* in mice that a short-term treatment with palm oil is sufficient to increase intestinal permeability, to provoke the mislocalization of several proteins involved in cell-cell junctions and to modify immune-related gene expression profile in intestine suggesting tissue inflammation.

3.2 Palm oil gavages in mice modify fecal bacterial species

Previous studies report major changes in the relative abundance of bacteria species during high-fat feeding (for review (Murphy *et al.*, 2015)). Palm oil might affect bacterial abundance via bacterial growth or via modification of genes involved in antimicrobial response (such as Reg3g) as previously observed after long-term high-fat diet (Everard *et al.*, 2014). We measured the abundance of selected fecal bacteria after 4 palm oil gavages. We found that *Clostridium leptum* (Fig. 3A, $p=0.0273$), *Akkermansia muciniphila* (Fig. 3B, $p=0.0274$) and Segmented Filamentous Bacteria (Fig. 3C, $p=0.0133$) abundances were significantly decreased after 4 palm oil gavages, while *Bacteroides* sp. abundance was increased (Fig. 3D, $p=0.0230$). The abundance of *Lactobacillus* sp. (Fig. 3E) and *Clostridium coccooides* (Fig. 3F) as well as total bacteria count (Fig. 3G) were unaffected. Thus, these results indicate that the gut bacterial species composition is modified rapidly after 4 palm oil gavages.

3.3 Palmitic acid perturbs permeability and cytokine expression in human intestinal epithelial cells

Palm oil being composed of palmitic and oleic acids, both fatty acids might exert specific impact on intestinal cells. We investigated the effects of these two fatty acids, delivered as complex micelles, on paracellular permeability of a monolayer of the human enterocytic-like cell line Caco-2/TC7. Cells were incubated for 24h or daily during 4 consecutive days with micelles containing either palmitic or oleic acid. A 24h-treatment with 4.5mM EGTA, a calcium chelator, a treatment known to increase epithelial permeability (Artursson 1990; Boulenc *et al.*, 1993), was used as positive control. As expected, EGTA treatment increased the permeability of Caco-2/TC7 cell monolayer to 4kD FITC-dextran ($p < 0.0001$) (Fig. 4A) and to ions (decreased TEER, $p < 0.0001$) (Fig. 4C). Interestingly, 1 day-treatment with palmitic acid provoked a significant increase of 4kD FITC dextran flux (Fig. 4A) without significant changes permeability assessed by TEER (Fig. 4D). A repeated supply for 4 days of palmitic acid markedly enhanced the permeability of the cell monolayer to 4kD FITC-dextran ($p = 0.0152$). TEER was decreased as soon as the first 48h of treatment ($p = 0.038$). These effects were not observed when oleic acid was supplied instead of palmitic acid. A dose-response effect of palmitic acid concentration increased 4kD FITC-dextran flux (Fig. 4B) and decreased TEER (Fig. 4E). This suggests that in mice gaved with palm oil (composed of 45% palmitic and 35% oleic acids) gavage in mice, the observed effects on intestinal permeability are probably due to palmitic rather than oleic acid in oil.

We analyzed by immunofluorescence the localization of the tight junction proteins ZO-1, occludin and tricellulin and of the adherens junction protein, E-cadherin, in cells treated with palmitic acid- or oleic acid-containing micelles (Fig. 5A). In cells incubated for 24h with palmitic acid we observed a decrease in the fluorescence intensity of all junction proteins analyzed, without modification of their localization at cell-cell contact. However, repeated supply of palmitic acid micelles during 4 days provoked the internalization of E-cadherin, ZO-1 and occludin as well as the loss of tricellulin at tricellular junction. In accordance with the absence of effects on paracellular permeability, the distribution of junction proteins was not altered by oleic acid micelles. The

determination of junctional proteins amount in total lysates (Fig. 5B-F), showed a decrease of E-cadherin, occludin and tricellulin protein levels but not for ZO-1, in cells treated for 4 days with palmitic acid. Any modification of junctional protein levels was observed in total cell lysates from cells treated for 24h with palmitic or oleic acids (data not shown). The mRNA level of occludin is decreased only after 4-days palmitic acid treatment (Fig. 5G) but the mRNA levels of the other junctional proteins studied were unchanged (data not shown). Altogether these results showed that 4-days palmitic acid treatment impaired both the localization of junction proteins at cell membrane and their expression.

Intestinal epithelial cells are able to produce cytokines (Stadnyk 2002; Miron and Cristea 2012) among which CXCL8 (IL-8), TGFB1 and IL1B genes are particularly well expressed in Caco-2/TC7 cells. We then determined whether palmitic acid could induce the production of these cytokines. Cells were treated for 24h or 4 days with palmitic acid- or oleic acid-containing micelles (Fig. 6). Palmitic acid increased the expression of IL-8 (as soon as after 24h of treatment ($p=0.0214$), Fig. 6A) and its secretion in basal medium (after 4-days treatment, Fig. 6B), while oleic acid did not exert any effect. A dose-dependent increase of IL-8 mRNA expression (Fig. 6E) as well as a trend for IL-8 secretion (Fig. 6G) were observed upon palmitic acid treatment. A small decreased of TGFB1 mRNA level ($p=0.0022$) was observed after a repeated supply of palmitic acid (Fig. 6C), whereas oleic acid did not modify its expression. A lower concentration of palmitic acid (0.1 or 0.3 mM instead of 0.6mM) is not sufficient to modulate TGFB1 expression (Fig. 6F). Neither oleic acid nor palmitic acid modified IL-1 β mRNA level whatever the duration of the treatments (Fig. 6D). Interestingly, we showed that EGTA increased IL-8 ($p<0.0001$) and IL-1 β mRNA levels ($p<0.0001$) as well as IL-8 secretion ($p=0.0042$), indicating that the destabilization of cell-cell junctions was sufficient to induce cytokine production.

We then determined whether the effects of palmitic acid could rapidly vanish after its removal from medium culture (Fig.7). Cells were treated daily during 4 days with palmitic-acid containing micelles and then cultured in control medium for 1 or 2 more days. We observed a time-dependent

decrease of 4kDa FITC-dextran passage through the cell monolayer after the removal of palmitic acid. However, 2 days after the medium change, the paracellular permeability of the monolayer remained 3.5-fold higher in cells previously treated with palmitic acid than in untreated cells (Fig. 7A, $p=0.0008$). The removal of palmitic acid did not restore the basal level of TEER, which remained between 10 to 15% lower than control cells (Fig. 7B, $p=0.007$). Immunofluorescence analyses of junctional proteins were performed after 4 days of palmitic acid treatment and 1 and 2 days after its removal (Fig. 7C). A gradual recovery of occludin and tricellulin distribution was observed during the time-course after palmitic acid removal. The distribution of E-cadherin and ZO-1 remained largely altered even 2 days after the cells were cultured back with control medium. Western blots experiment showed that tricellulin (Fig.7D) as well as E-cadherin and ZO-1 (data not shown) protein amounts were recovered 2 days after palmitic acid removal. While occludin mRNA level returned to control value 2 days after palmitic acid removal (Fig. 7E), occludin protein amount remained lower than in control cells (Fig. 7D). We also observed that the effects of palmitic acid on IL-8 (Fig. 7F) and TGFB1 (Fig. 7G) mRNA levels were abolished 2 days and 1 day, respectively, after its withdrawal from culture medium. Altogether these results show that the effects of palmitic acid are slowly attenuated after the arrest of treatment.

3.4 Palmitic acid induces a moderate endoplasmic reticulum stress

We sought to analyze the molecular mechanisms involved in the effects of palmitic acid on barrier integrity and cytokine production in Caco-2/TC7 cells. Palmitic acid is known to be a more potent inducer of endoplasmic reticulum (ER) stress than oleic acid (Caviglia *et al.*, 2011; Deguil *et al.*, 2011; Danino *et al.*, 2015; Pardo *et al.*, 2015). Moreover, it has been shown that the induction of ER stress by various stimuli, including fatty acids, is able to increase the expression of cytokines in several cell types (Willy *et al.*, 2015; Tang *et al.*, 2017) and may induce dysfunction of epithelial barriers (for review (Ma *et al.*, 2017)). We thus wanted to determine whether the observed effects of palmitic acid were due to an induction of ER stress. Caco-2/TC7 cells were then treated for 24h or

daily during 4 consecutive days with palmitic acid- or oleic acid-containing micelles. A treatment with 2mM DTT was used as a known inducer of ER stress (Osowski and Urano 2011; Beriault and Werstuck 2013). As expected, DTT treatment increased paracellular permeability (Fig. 8A) and IL-8 mRNA level (Fig. 8B). The induction of ER stress by DTT treatment was assessed through the increased expression of GRP78/HSPA5, GADD153/DDIT3 and of the spliced form of XBP1 (Fig. 8C-E), three gene markers of unfolded protein response. Oleic acid treatment provoked a moderate induction of GRP78/HSPA5 expression, detectable only after 24h treatment ($p=0.0085$) but not after 4 days (Fig. 8C), while GADD153/DDIT3 and spliced XBP1 mRNA levels were unchanged (Fig. 8D-E). Palmitic acid increased, but to a limited extent as compared to DTT, the expression of all these three gene markers of ER stress. This effect was mainly observed after 24h treatment (Fig. 8C-E). As palmitic acid exerts only a moderate induction of ER stress while its impact on epithelial barrier integrity and IL-8 expression was important, we thus hypothesized that other molecular mechanisms may also be involved in the deleterious effect of palmitic acid.

3.5 Ceramide mediates the deleterious effect of palmitic acid on epithelial barrier and immune response.

Palmitic acid, but not oleic acid, is a precursor of *de novo* ceramide synthesis (for review (Castro *et al.*, 2014)), which contributes not only to membrane structure but also to signaling and metabolic dysfunctions in various cell types, including Caco-2/TC7 cells (Chavez and Summers 2012; Tran *et al.*, 2016). We thus wanted to determine whether ceramide could provoke epithelial barrier damages and induce cytokine production. Caco-2/TC7 cells were treated with C₂-ceramide, a short chain cell-permeable biologically active analogue of ceramide. We observed that a 24h-treatment with C₂-ceramide provoked a marked increase of paracellular permeability ($p<0.0001$) (Fig. 9A) along with a decrease of TEER ($p<0.0001$) (Fig. 9B). These effects were associated with a decreased expression of all junctional proteins (Fig. 9C). Immunofluorescence analysis showed that the localization at cell-cell contacts of tight junction proteins (ZO-1, occludin and tricellulin) and of the adherens junction

protein E-cadherin was altered (Fig. 9D). C2-ceramide treatment increased the expression and the secretion of IL-8 ($p=0.0201$) (Fig. 9E-F), decreased TGFB1 mRNA level ($p=0.038$) (Fig. 9G) and had no effect on IL-1 β mRNA level (Fig. 9H). For all these parameters, no effect of ethanol used as the vehicle for C2-ceramide was observed. Altogether, these results show that C2-ceramide treatment induces effects similar to palmitic acid on intestinal epithelial barrier integrity and cytokine production.

We then determined whether the inhibition of *de novo* ceramide synthesis could attenuate the deleterious effect of palmitic acid. Caco-2/TC7 cells were treated with L-cycloserine, an irreversible inhibitor of serine palmitoyltransferase, the first enzyme of *de novo* ceramide synthesis (Kang *et al.*, 2010; Lowther *et al.*, 2010). The treatment with L-cycloserine did not modify the paracellular permeability of Caco-2/TC7 cell monolayer at the basal state or in presence of palmitic acid-containing micelles (Fig. 10A). However, L-cycloserine treatment abolished the palmitic acid-dependent increase in IL-8 mRNA level (Fig. 10B). These results suggested that inflammatory but not permeability effects of palmitic acid on enterocytes were dependent on *de novo* ceramide synthesis.

4. Discussion

In this study, we showed *in vivo* that treatment with palm oil gavages for 5 days is sufficient to provoke a defect in intestinal epithelial barrier integrity and an alteration of the expression of genes involved in the immune response in intestine. Using a model of human enterocytes, we demonstrated that palmitic acid exerted direct deleterious effects on intestinal epithelial cells, part of which were linked to *de novo* ceramide synthesis. Our results highlight direct links between the repeated consumption of palmitic acid and intestinal barrier defects as well as proinflammatory cytokine expression. These alterations, occurring before the onset of diet-induced obesity, may thus represent an early event in the initiation of low-grade inflammation.

We observed that a single supply of palm oil *in vivo* in mice provoked an increase in intestinal epithelium permeability accompanied by the loss of several tight junction proteins at cell-cell contacts, whereas a global mislocalization of tight and adherens junctional proteins was evidenced only after repeated gavages. This rapid intestinal barrier defect is in accordance with the increase of albumin fecal content, used as a marker of intestinal permeability alteration, observed in mice one day after the beginning of a high-fat diet (Johnson *et al.*, 2015). Similar effects on epithelium permeability without marked changes in the localization of cell-cell junction proteins at cell-cell contacts were showed in Caco-2/TC7 cell monolayer incubated for 24h with palmitic acid-containing micelles. These results indicate that a single supply of saturated lipids is sufficient to affect intestinal permeability. Interestingly, the increased expression of the gene coding for antimicrobial peptide Reg3 γ , as soon as after one palm oil gavage, suggests that intestine has engaged mucosal defense. Moreover, in Caco-2/TC7 cells, an increase in pro-inflammatory cytokine IL-8 mRNA level was observed after only 24h treatment with palmitic acid-containing micelles. Altogether our results show that a single supply of saturated fatty acids is sufficient to initiate an immune response by intestinal epithelial cells.

After repeated supplies of palm oil *in vivo* or palmitic acid in Caco-2/TC7 cells, we observed a decreased expression of junctional proteins and a marked mislocalization at cell-cell contacts, which were associated with enhanced paracellular permeability as well as additional modifications of immune gene expression profile suggesting tissue inflammation. The deleterious effects of palmitic acid vanished more slowly than they appeared.

IL-8 is a chemokine produced by many cell types, including intestinal epithelial cells, and it is known to participate to the acute phase response of inflammation (Gruys *et al.*, 2005). In intestine, IL-8 is involved in the neutrophil activation and infiltration in intestinal mucosa where it participates to host defense against bacteria invasion. The level of expression of IL-8 was enhanced with the duration of lipid challenge in palmitic acid-treated human enterocytic Caco-2/TC7 suggesting tissue inflammation. IL-8 expression is absent in mice (Nomiya *et al.*, 2010), IL-1 β is an important pro-inflammatory cytokines in this species. In colon of mice, the protein IL-1 β level increased

significantly in parallel with the duration of lipid challenge, highlighting a tissue inflammatory response to lipid gavages. An increased expression of IL-1 β expression in intestine was frequently reported in high-fat-diet models and in human obesity (for review (Winer *et al.*, 2016)). Both IL-1 β and IL-8 are known to perturb tight junction proteins (Al-Sadi *et al.*, 2013; Yu *et al.*, 2013), their time-dependent increase of expression with lipid challenge may contribute to exacerbate the mislocalization of junction protein and thus to amplify permeability defects.

We observed that the expression of Gata3 mRNA level is decreased in proximal intestine only after repeated palm oil gavages. Gata3 is a key transcription factor involved in the differentiation and the maturation of innate lymphoid cells controlling both the expression of pro-and anti-inflammatory cytokines (Wan 2014). A link between Gata3 activity and visceral fat inflammation during obesity was recently reported (Qiang *et al.*, 2016). However, its exact role in intestinal inflammation remains to be characterized. Interestingly the increase of Reg3g mRNA level observed after one palm oil gavage was no longer observed after repeated gavages. As Reg3g is involved in host defense against bacteria, this suggests that saturated fat overload results in a decreased efficiency of intestinal defense response.

Associated with changes in immune-related gene expression and intestinal barrier defects, we observed that repeated palm oil gavages provoked changes in microbiota composition. We studied some bacteria species previously reported as modified by high-fat diet or obesity. We showed a decreased fecal abundance of *Clostridium leptum*, *Akkermansia muciniphila* and segmented filamentous bacteria in palm oil-treated mice as compared to control. These three bacteria species are known to exert a protective role against pathogen proliferation and to contribute to host defense by promoting maturation of immune cells (Thursby and Juge 2017). Their decreased abundances were in relevance with inflammatory status of the tissue. The anti-inflammatory properties of *Akkermansia muciniphila* has gained attention and highlight its interest as a therapeutic target to treat intestinal inflammation (Ottman *et al.*, 2017). In particular, gut *Akkermansia muciniphila* abundance is reduced in human obesity (Karlsson *et al.*, 2012; Dao *et al.*, 2016) and in high-fat diet-induced obesity in mice (Everard *et al.*, 2013). The decreased of abundance segmented filamentous bacteria observed after

palm oil gavages is in accordance with its reduction in mice submitted to long-term high-fat diet (Garidou *et al.*, 2015), but its role in inflammation is still questioned (Ericsson *et al.*, 2014). We observed an increase in *Bacteroides* sp. in palm oil-treated mice, in congruence with several reports showing an increase in the abundance of this species associated with the consumption of high-fat diet, rich in saturated fatty acids (Wu *et al.*, 2011; Yan *et al.*, 2013; Caesar *et al.*, 2015; Heinritz *et al.*, 2016). We can conclude that 4 gavages with palm oil are sufficient to promote changes in microbiota composition corresponding to the changes reported in obesity induced by high-fat diet and relevant to gut inflammation.

The studies of molecular mechanisms involved in the deleterious effects of palmitic acid on intestinal epithelial cells revealed the contribution of *de novo* synthesis of ceramides. In established obesity, elevation of circulating saturated fatty acids and inflammatory signals promote ceramide synthesis (Fucho *et al.*, 2017). In the present study, we observed that C2-ceramide acted on three parameters: increase in intestinal epithelial permeability, mislocalization of junctional proteins and modification of cytokine expression. Interestingly, intestinal barrier defect was shown recently to be associated with elevated C16-ceramide levels, (Kim *et al.*, 2017) and with increased ceramides content induced by sphingomyelinase addition (Bock *et al.*, 2007). Ceramides can increase permeability through their properties to perturb membrane raft domains (van Blitterswijk *et al.*, 2003; Zhang *et al.*, 2009; Bieberich 2018) where several junctional proteins are localized (Nusrat *et al.*, 2000; Dodelet-Devillers *et al.*, 2009). Ceramides may also alter membrane integrity by their capacities to form channels (Perera *et al.*, 2012). Nevertheless, we observed that the inhibition of *de novo* ceramide synthesis reduced pro-inflammatory cytokine expression but failed to restore cell monolayer permeability suggesting a role of *de novo* ceramide synthesis limited to the inflammatory process. Very few studies have analyzed the direct impact of ceramides on cytokine expression, however an induction of IL-6 by C2-ceramide was reported in human fibroblast (Laulederkind *et al.*, 1995) and in human astrocytoma cells (Fiebich *et al.*, 1995). We showed that the inhibition of *de novo* ceramide synthesis prevented the increase of IL-8 secretion in palmitic acid-treated cells. Thus, palmitic acid

engaged multiple mechanisms, including ceramide pathways, for exerting its deleterious effects on intestinal permeability and inflammation.

Recent advances suggest that fatty acids may act via their binding to membrane receptors (such as TLRs, G protein-coupled receptor) or nuclear receptors (such as PPARs, LXR or FXR) and the subsequent rapid activation of signaling cascades (for review (Suzuki 2013)). Our study showed a specific effect of palmitic acid since oleic acid showed no effect on intestinal barrier integrity and cytokine expression. However, several fatty acids, such as short and medium fatty acids, were reported to modulate intestinal barrier integrity (for review (Suzuki 2013)). Interestingly we observed in Caco-2/TC7 cells that a very short treatment (4 hours) with oleic acid under the form of mixed micelles might perturb the distribution of tricellulin and increase intestinal permeability (Genser *et al.*, 2018). Altogether, these data underline the differential effects of fatty acids according to their nature and length of exposure on intestinal barrier integrity. Beneficial or deleterious effects of fatty acids on intestinal barrier and inflammation were reviewed by Fritsche K.L. (Fritsche 2015). Interestingly the deleterious effect of palmitic acid on cellular functions can be counteracted or diminished in presence of oleic acid (Palomer *et al.*, 2018). Other lipids or nutrients are contributing to barrier dysfunctions (De Santis *et al.*, 2015).

In healthy humans, meals are constituted of mixed nutrients and intestinal barrier integrity is maintained and post-prandial inflammation is limited. Nevertheless, in pathological condition a chronicization of the alteration of barrier functions might occur. Indeed, we have previously observed that obese subjects presents only subtle intestinal barrier defect that can be revealed after a lipid load (Genser *et al.*, 2018) and hyperglycemia was recently described to disturb gut barrier (Thaiss *et al.*, 2018). Thus, excess of nutrients in pathological context might further aggravate barrier dysfunctions probably by exceeding regulatory capacities of the gut. Further studies are needed to identify other contributors of metabolic-associated gut barrier defects.

In conclusion, our results showed that limited repeated supply of saturated fatty acids and particularly palmitic acid are sufficient to deregulate intestinal inflammatory response, to induce an intestinal barrier defect and to change microbiota composition. Most of these alterations, which are features of obesity and metabolic diseases, stress that reiterated consumption of saturated fatty acids is an early player in gut alterations observed in these pathologies.

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6. Conflict of interest

No conflict of interest declared.

7. Authors contributions

S.G., B.G.P., S.T. and V.C. designed and conducted the experiments and analyzed the data. E.Q., L.B., P.S. managed the microbiota analysis. S.G. and V.C. wrote the paper. B.G.P., S.T., P.S., E.Q. and A.L. revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Figure legends

Figure 1: Palm oil gavage increases intestinal permeability *in vivo*. Mice were submitted to one (1x) or 5 gavages (5x) with 200µl palm oil or water. A control group was constituted of mice treated with DSS in drinking water to induce barrier damage. The intestinal permeability (A) was assessed after the gavage with oil or water by measuring plasma concentration of FITC-dextran one hour after an oral 4kDa FITC-dextran load. Results are expressed in µg/ml (mean ± SEM, n=5-10). **p<0.01, ***p<0.0001 as compared to water. The distribution of cell-cell junction proteins, E-cadherin, ZO-1, occludin and tricellulin (B) was analyzed by immunofluorescence on jejunum sections. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). An enlargement is shown for each condition. White arrows indicate the localization of the junctional proteins in control (water) conditions. Red arrows point out E-cadherin and ZO-1 mislocalization after 5x palm oil gavages. Scale bar= 20µm. The expression of occludin in jejunum (C) was determined by RT-QPCR. Cyclophilin (cyclo) was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin mRNA level (mean ± SEM, n=10). *p<0.05, ***p<0.001 as compared to water condition.

Figure 2: Repeated gavages with palm oil modify expression of genes involved in immune response. Mice were submitted to one (1x) or 5 (5x) gavages with 200µl palm oil or water. A control group was constituted of mice treated with DSS. Intestine was collected one hour after the last gavage. The expression of Gata3 in jejunum (A) and of Reg3g in ileum (B), were determined by RT-PCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level (mean ± SEM, n=5-10). The quantification of Il-1β

protein in the colon (C) was determined by ELISA. Results are expressed in pg/mg of protein as mean \pm SEM, n=5-9. *p<0.05, **p<0.01 as compared to water condition.

Figure 3: Repeated palm oil gavages modify gut bacterial species. Mice were submitted to daily gavages with water or palm oil for 4 consecutive days. Total DNA from feces was extracted and used for quantification of bacteria species content by qPCR. Results are expressed as mean \pm SEM, n=4-6, *p<0.05 as compared to water, ns: not statistically significant, a.u.: arbitrary unit. Fecal quantification of (A) *Clostridium leptum*, (B) *Akkermansia muciniphila*, (C) Segmented Filamentous bacteria, (D) *Bacteroides* sp., (E) *Lactobacillus* sp., and (F) *Clostridium coccooides*. (G) Total fecal bacterial counts.

Figure 4: Saturated palmitic acid but not unsaturated oleic acid increases paracellular permeability of an intestinal epithelial monolayer. Caco-2/TC7 cells were incubated with control medium (Ctrl) or treated with micelles containing either palmitic acid (PA) or oleic acid (OA) for 24h or daily for 4 consecutive days (4d). A 24h-treatment with 4.5mM EGTA was used as positive control known to display altered epithelial barrier integrity. Paracellular permeability across Caco-2/TC7 cell monolayer (A) was evaluated by measuring the accumulation during 4 h of 4kDa FITC-dextran in the basal compartment. Results are expressed as percentage of 4kDa FITC-dextran input (amount added in the apical compartment), mean \pm SEM, n=6-15. Fold-increase, as compared to control condition, is indicated at the top of the corresponding histogram. Dose-response to palmitic acid (B) was evaluated on cells incubated for 4 days with micelles containing 0.1mM or 0.3mM or 0.6mM of palmitic acid (PA). The accumulation of 4kDa FITC-dextran in the basal compartment was determined. Results are expressed as in (A). mean \pm SEM, n=6. **p<0.01, *** p<0.001 as compared to control (Ctrl) cells. TEER (inverse relationship to permeability) (C) was assessed in control and EGTA-treated cells. Results are expressed in percentage of TEER measured in control condition, as mean \pm SEM, n=6. The percentage of decrease compared to the control condition is indicated. Time-course of TEER (D) was measured before (0) and every 24h during the treatment with micelles containing palmitic acid (PA) or oleic acid (OA) or in untreated cells (control). The percentage of decrease in PA-treated cells as compared to control cells is indicated. Results are expressed in ohm.cm^2 as mean \pm SEM, n=6. *p<0.05, **p<0.01, *** p<0.001 as compared to untreated cells, unless otherwise indicated. Dose-response of TEER (E) was assessed in control and in cells treated for 4 days with micelles containing 0.1mM, or 0.3mM or 0.6mM palmitic acid. Results are expressed as in D, mean \pm SEM, n=6. *p<0.05, *** p<0.001 as compared to control cells.

Figure 5: Repeated supplies of palmitic acid, but not of oleic acid, alter the expression of junctional proteins and their localization at cell-cell contacts. Cells were incubated or not with

palmitic acid (PA)-containing micelles (for 24h or 4 days) or with oleic acid (OA)-containing micelles (for 4 days). Immunofluorescence analysis (A) was performed to study the localization of E-cadherin, ZO-1, occludin and tricellulin. Nuclei were stained DAPI. Bar=20 μ m Protein levels (B) were determined in cell lysates by Simple Western. Reconstituted images are shown. Hsc70 protein levels were used as loading control. Quantification of junctional protein levels normalized to Hsc70 protein levels (C-F). Results are expressed in arbitrary units (a. u.) as mean \pm SEM, n=4, *p<0.05, **<0.001 as compared to control (Ctrl). The mRNA level of occludin (G) was quantified by RT-PCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level (mean \pm SEM, n=6-15).

Figure 6: Palmitic acid modifies cytokine expression in Caco-2/TC7 cells. Caco-2/TC7 cells were cultured in the same conditions as in Figure 4. The mRNA levels of IL-8 (A), TGFB1 (C) IL-1 β (D), were quantified by RT-PCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level (mean \pm SEM, n=6-15). The concentration of IL-8 protein (B) in the basal compartment was quantified by ELISA. Results are expressed in pg/ml as mean \pm SEM, n=6. Fold-increase as compared to control condition is indicated at the top of histograms. Dose-response to palmitic acid on mRNA levels of IL-8 (E) and TGFB1 (F) was studied. Cells were incubated for 4 days in presence of micelles containing 0.1mM, 0.3mM or 0.6mM palmitic acid (PA). The mRNA levels were quantified by RT-PCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level (mean \pm SEM, n=4). Dose-response to palmitic acid on IL-8 secretion (G) was determined as in (B). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 as compared to control.

Figure 7: The removal of palmitic acid partially restores its deleterious effects on intestinal epithelial cell monolayer integrity and on expression of genes involved in immune response. Caco-2/TC7 cells were cultured in control medium (Ctrl) or with palmitic acid-containing micelles (PA) for 4 days followed or not by a period of one or 2 more days in control medium without PA (w/o PA). (A) Paracellular permeability was evaluated by measuring 4kDa FITC-dextran flux across Caco-2/TC7 monolayer, after a 4-day palmitic acid treatment and each day after cells were shifted to control medium. FITC-dextran 4 kilodaltons was added in the apical compartment and fluorescence values were determined in the basal compartment 4 hours later. Results are expressed in percentage of 4kDa FITC-dextran input in the apical compartment, mean \pm SEM, n=6-18. Fold-increase, as compared to control condition, is indicated at the top of histograms. *p<0.05, **p<0.01, *** p<0.001 as compared to control cells at the same day of the culture, unless otherwise indicated. (B) TEER was measured in control (white circles) and in palmitic acid-treated cells. The measure was performed before treatment

(day 0), at day 1 and 4 of palmitic acid treatment (black circles) and after the removal of palmitic acid-containing micelles (grey circles). Dashed line indicated the day when PA-treated cells were shifted to control medium. Results are expressed in $\text{ohm}\cdot\text{cm}^2$ as mean \pm SEM, n=6-18. The percentage of decrease in PA-treated cells as compared to control cells is indicated. **p<0.01, ***p<0.001 as compared to control cells. (C) Immunofluorescence analysis was performed to study the localization of E-cadherin, ZO-1, occludin and tricellulin. Nuclei were stained with DAPI. Bar=20 μm . Occludin and tricellulin protein levels (D) were measured by Simple Western. Reconstituted images are shown. Actin was used as loading control. The mRNA levels of occludin (E), IL-8 (F) and TGFB1 (G) were quantified by RT-PCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level (mean \pm SEM, n =6-18). *p<0.05, **p<0.01 as compared to control, unless otherwise indicated, ns: not statistically significant.

Figure 8: Palmitic acid provokes a moderate ER stress in Caco-2/TC7 cells. Caco-2/TC7 cells were incubated with control medium (Ctrl), or were treated for 24h or daily for 4 consecutive days (4d) with micelles containing either palmitic acid (PA) or oleic acid (OA). A 4h-treatment with 2mM DTT was used to induce an ER stress (positive control). (A) Paracellular permeability was evaluated by measuring FITC-dextran flux across Caco-2/TC7 cell monolayer. FITC-dextran 4 kilodaltons was added in the apical compartment the last day of the experiment and fluorescence values were determined in the basal compartment 4 hours later. Results are expressed in percentage of 4kDa FITC-dextran added in the apical compartment (input), mean \pm SEM, n=4-6. The mRNA levels of IL-8 (B) GRP78/HSPA5 (C), GADD153/DDIT3 (D) and XBP-1 spliced form (E) were quantified by RT-PCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level, (mean \pm SEM, n=6-15). Fold-increase as compared to control condition is indicated at the top of some histograms. *p<0.05, **p<0.01, ***p<0.01, ****p<0.0001 as compared to control.

Figure 9: C₂-ceramide addition provokes barrier defects and modulates cytokine expression. Caco-2/TC7 cells were culture in absence (Ctrl) or in presence for 24h of vehicle 0.5% ethanol (EtOH), or 100 μM C₂-ceramide (C₂-Cer). (A) Paracellular permeability was evaluated by measuring 4kDa FITC-dextran flux across Caco-2/TC7 cell monolayer as in Figure 4. Results are expressed as percentage of 4kDa FITC-dextran input in the apical compartment, (mean \pm SEM, n=6). (B) TEER was measured after 24h-treatments. Results are expressed in % of value obtained in untreated cells, mean \pm SEM, n=6. (C) Representative western blots of junction protein levels determined in cell lysates by Simple Western. Reconstituted images are shown. Hsc70 was used as loading control. (D) Caco-2/TC7 cells were analyzed by immunofluorescence for tight junction proteins ZO-1, occludin,

tricellulin and for E-cadherin an adherens junction protein. Nuclei are stained with DAPI. Bar= 20 μ m. The mRNA levels of IL-8 (**E**), TGF β 1 (**G**) and IL-1 β (**H**) were quantified by RT-PCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level (mean \pm SEM, n=6-15). (**F**) The concentration of IL-8 protein in the basal compartment was quantified by ELISA. Results are expressed in pg/ml as mean \pm SEM (n=6). *p<0.05, **p<0.01, ***p<0.01, ****p<0.0001 as compared to untreated cells.

Figure 10: Inhibition of *de novo* ceramide synthesis abolishes inflammatory effects of palmitic acid. Caco-2/TC7 cells were cultured for 24h in absence (Ctrl) or in presence of micelles containing palmitic acid (PA) or 20mM L-cycloserine (Lcyclo). In one condition, cells were pre-treated with L-cycloserine for 1 h before incubation for 24h with PA-containing micelles (PA+Lcyclo). (**A**) Paracellular permeability was evaluated by measuring 4kDa FITC-dextran flux across Caco-2/TC7 cell monolayer. Results are expressed in percentage of 4kDa FITC-dextran input in the apical compartment, mean \pm SEM, n=3-6. (**B**) The mRNA levels of IL-8 were quantified by RT-PCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of IL-8 gene mRNA level to cyclophilin (cyclo) mRNA level (mean \pm SEM, n=3-6). **p<0.01*** p<0.001 as compared to control cells unless indicated, ns: non statistically significant.

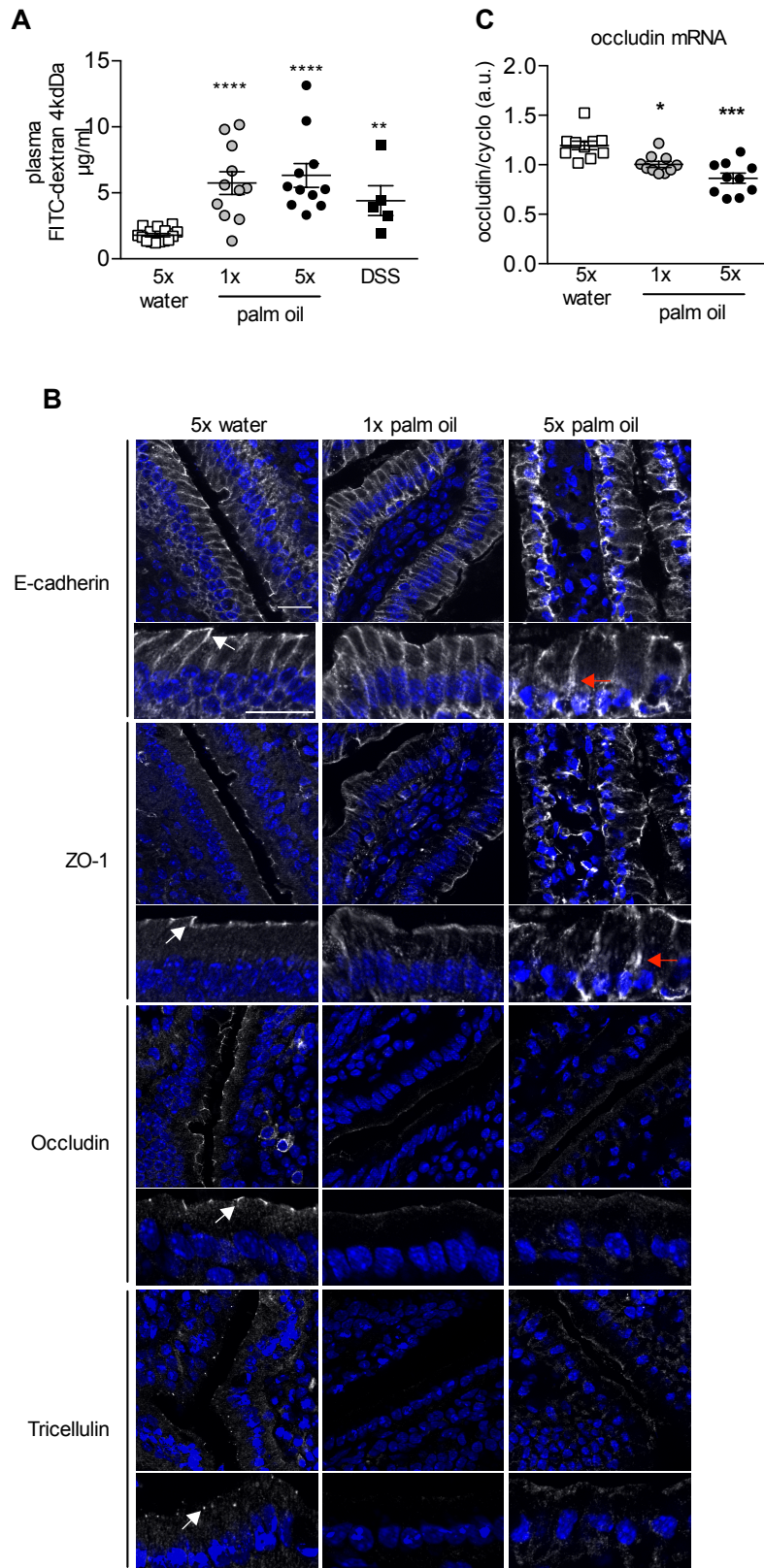


Figure 1

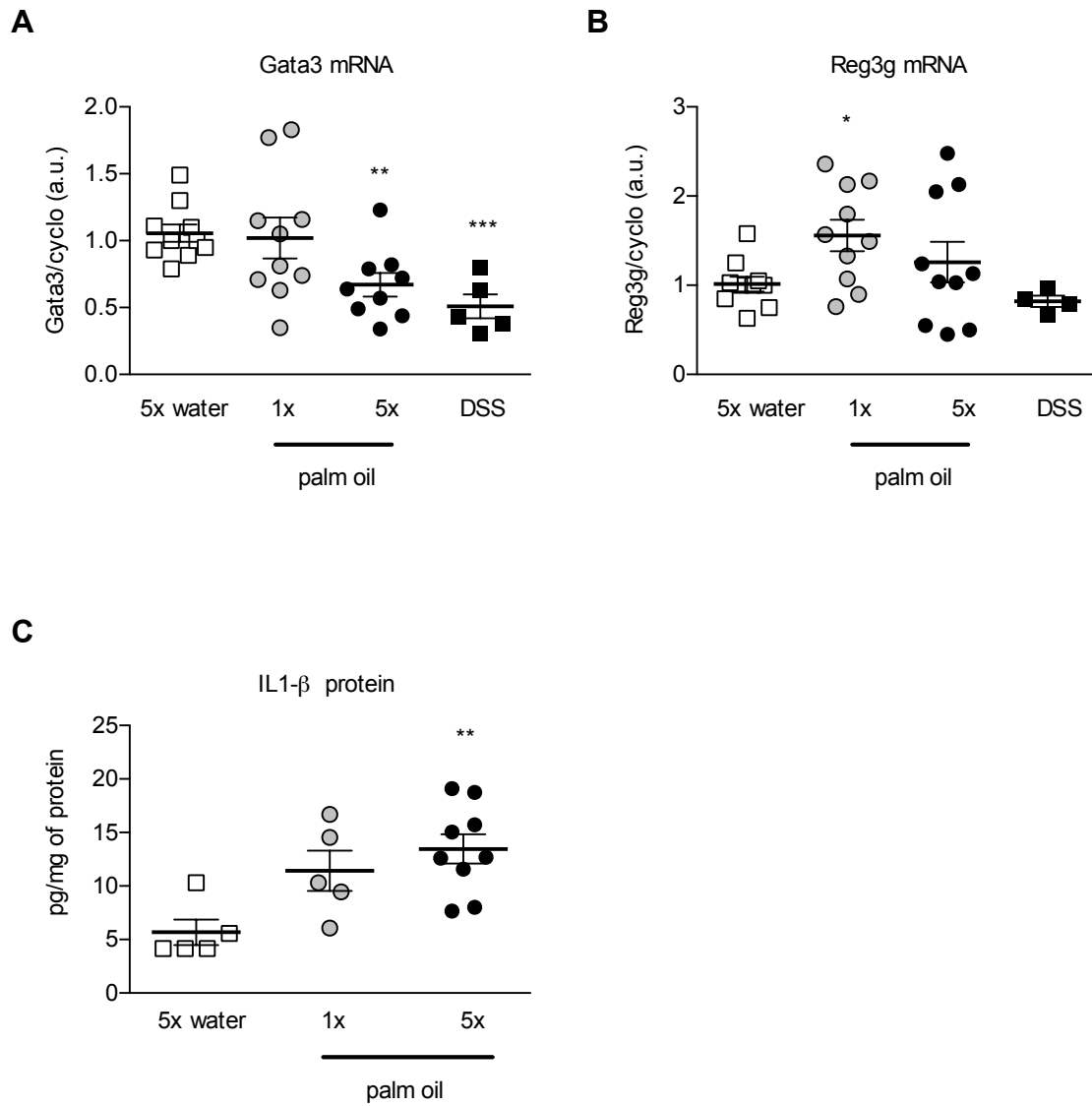


Figure 2

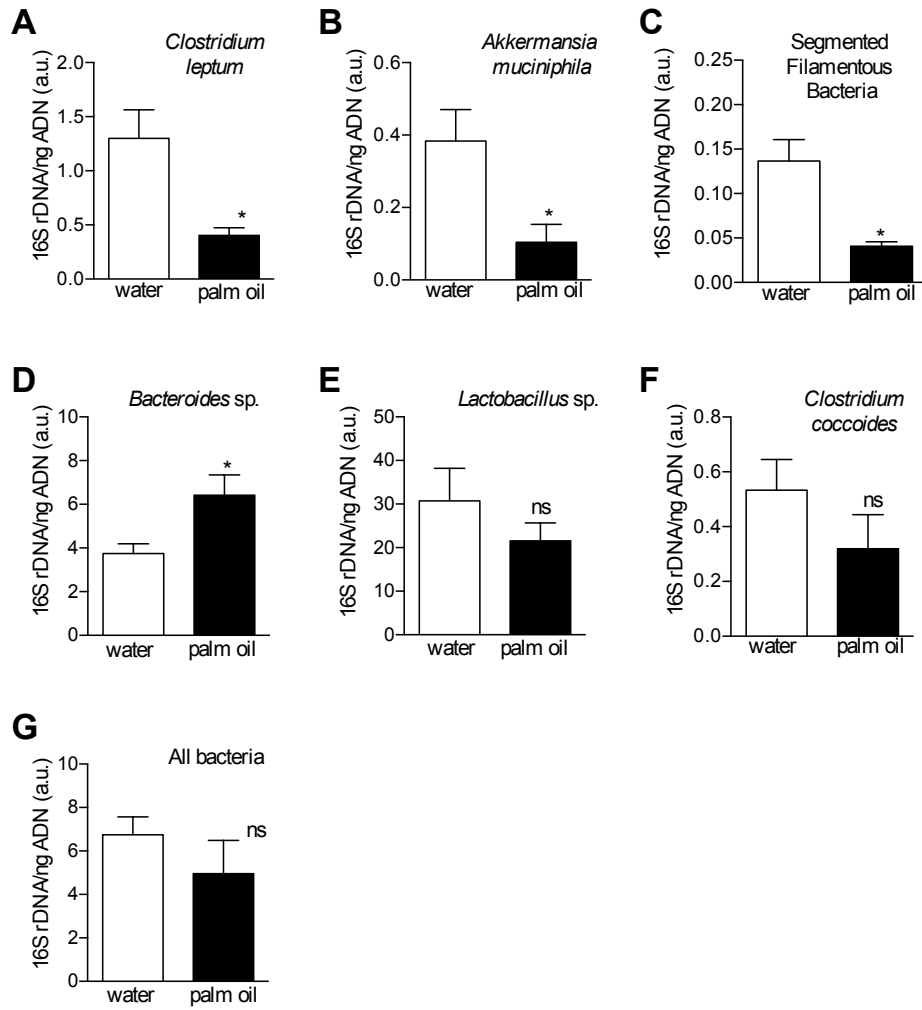


Figure 3

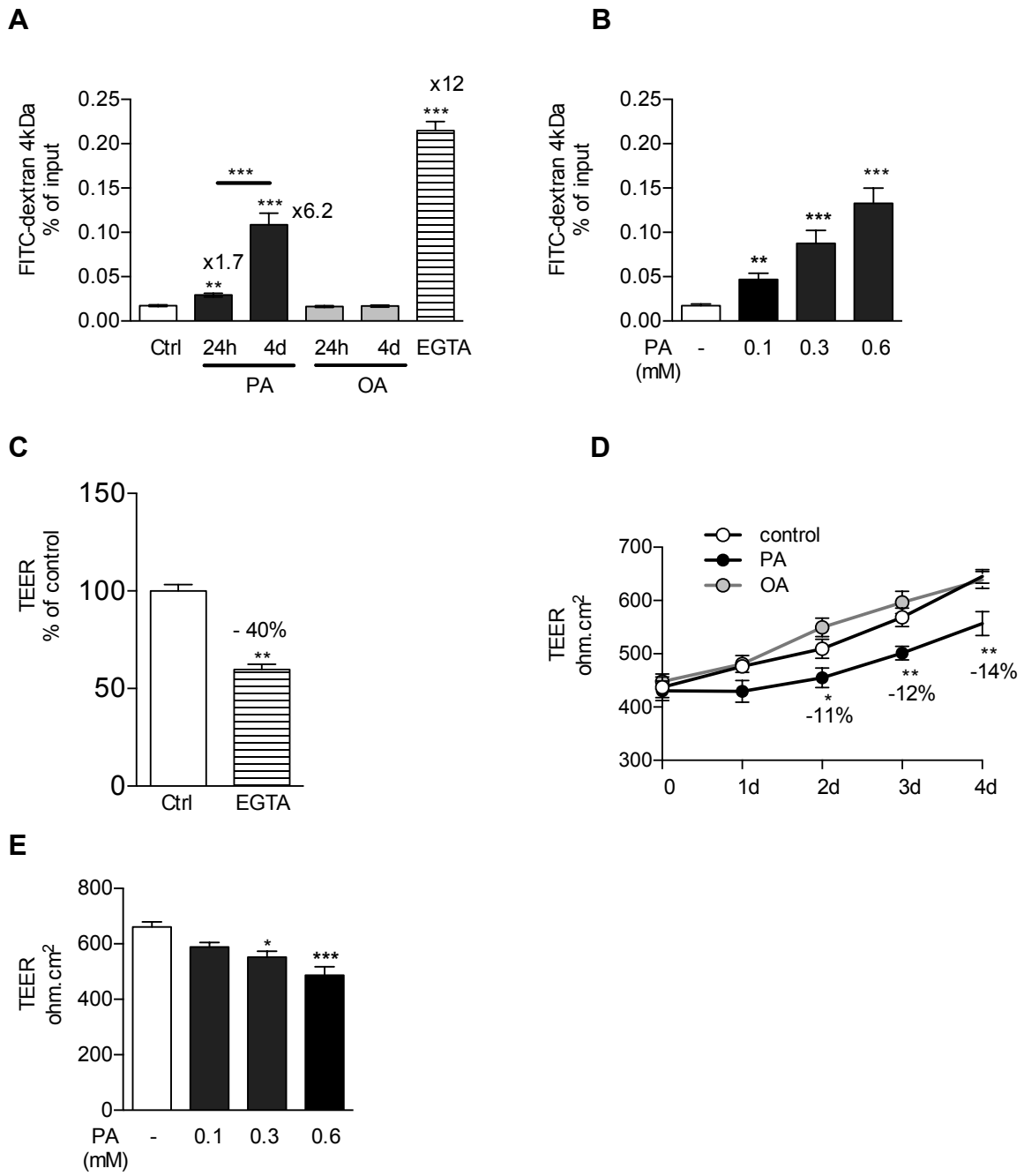


Figure 4

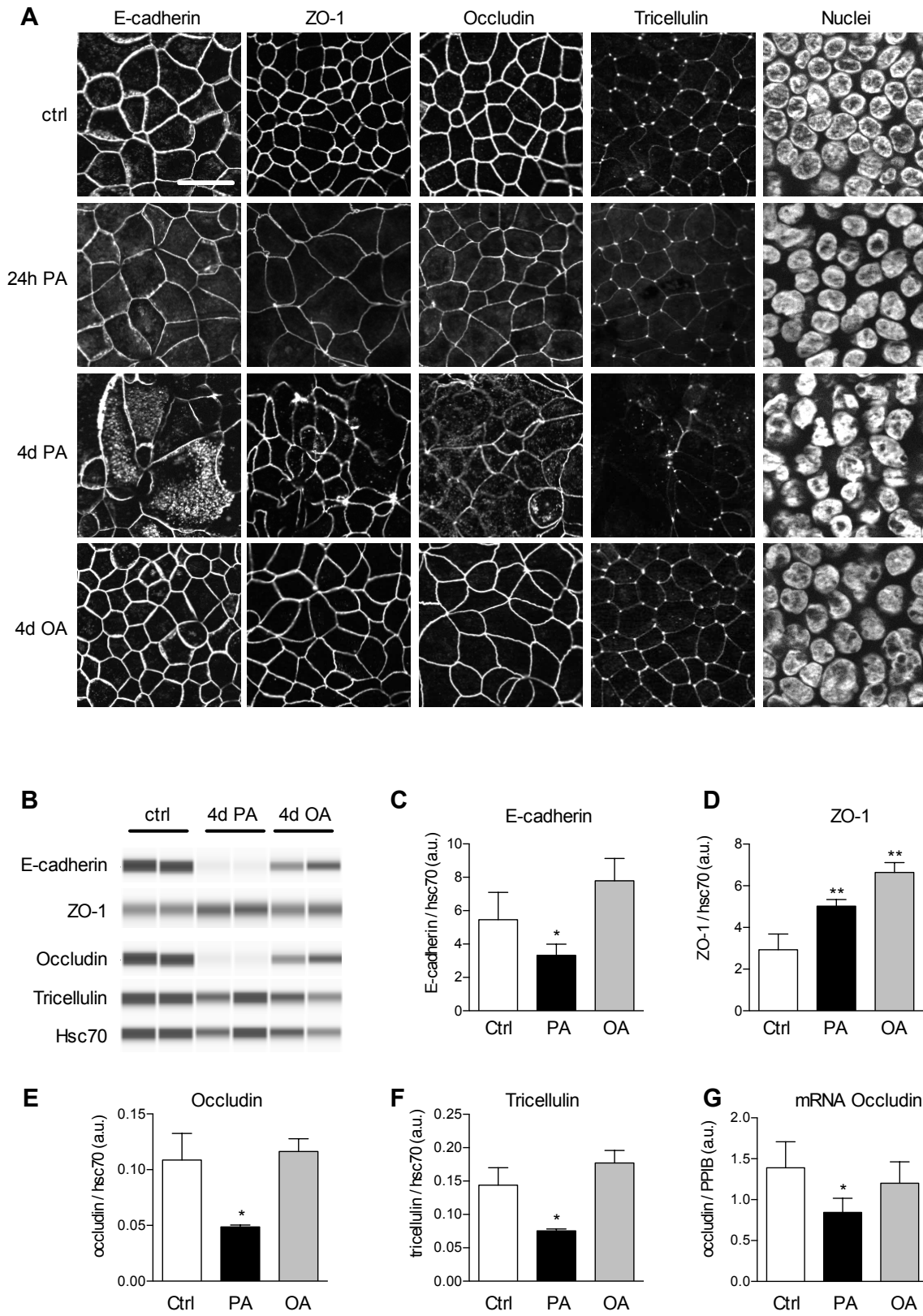


Figure 5

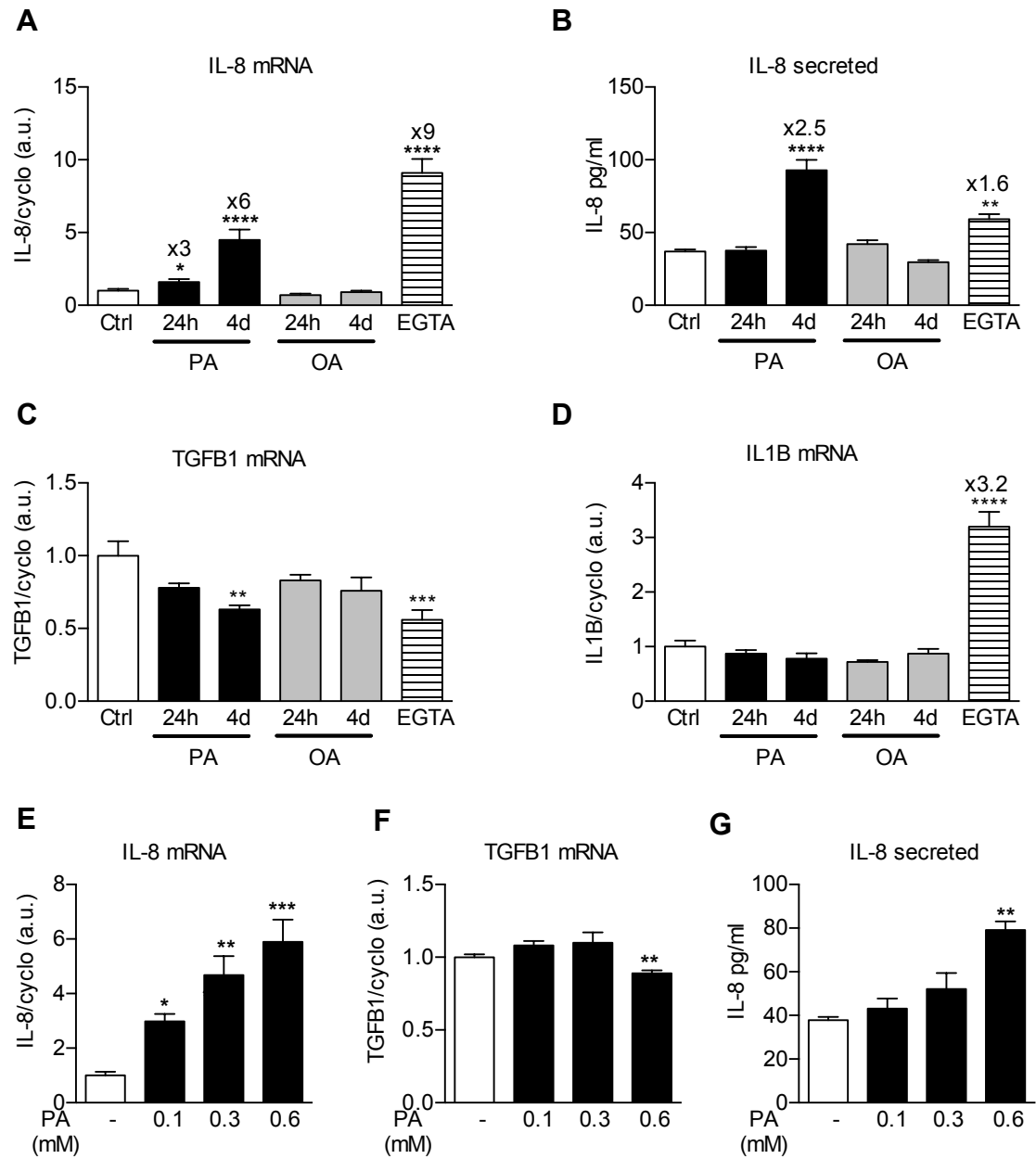


Figure 6

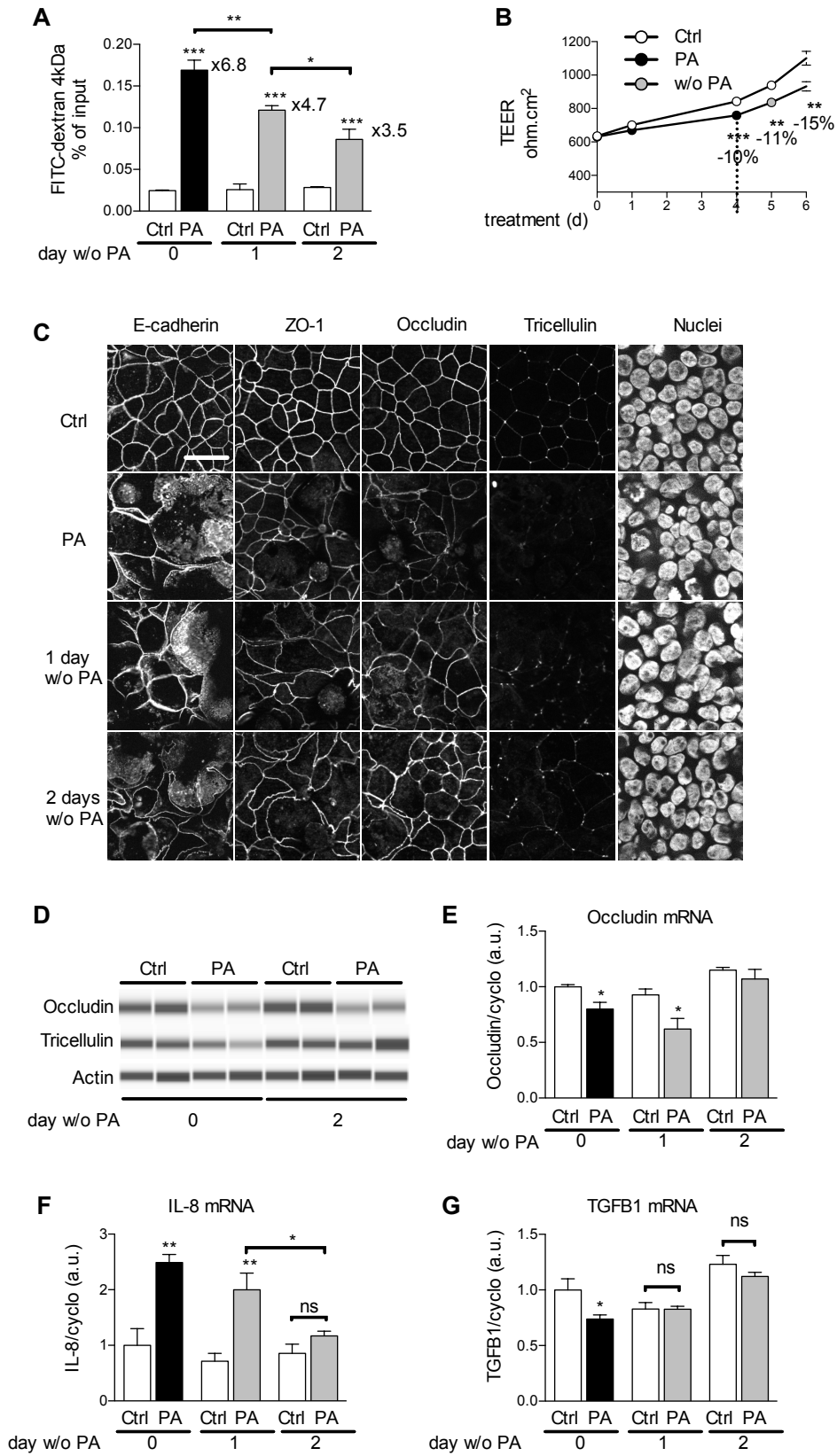


Figure 7

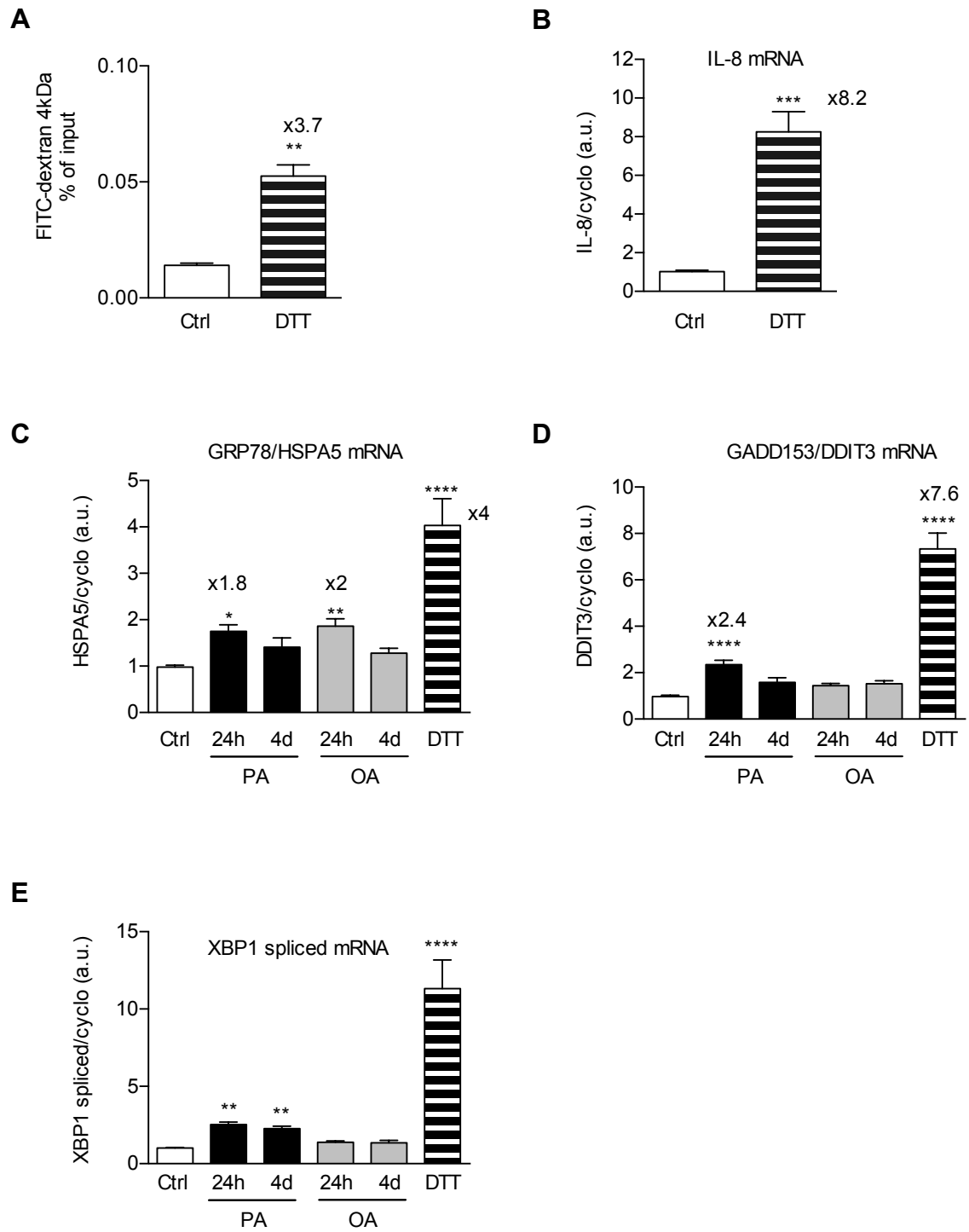


Figure 8

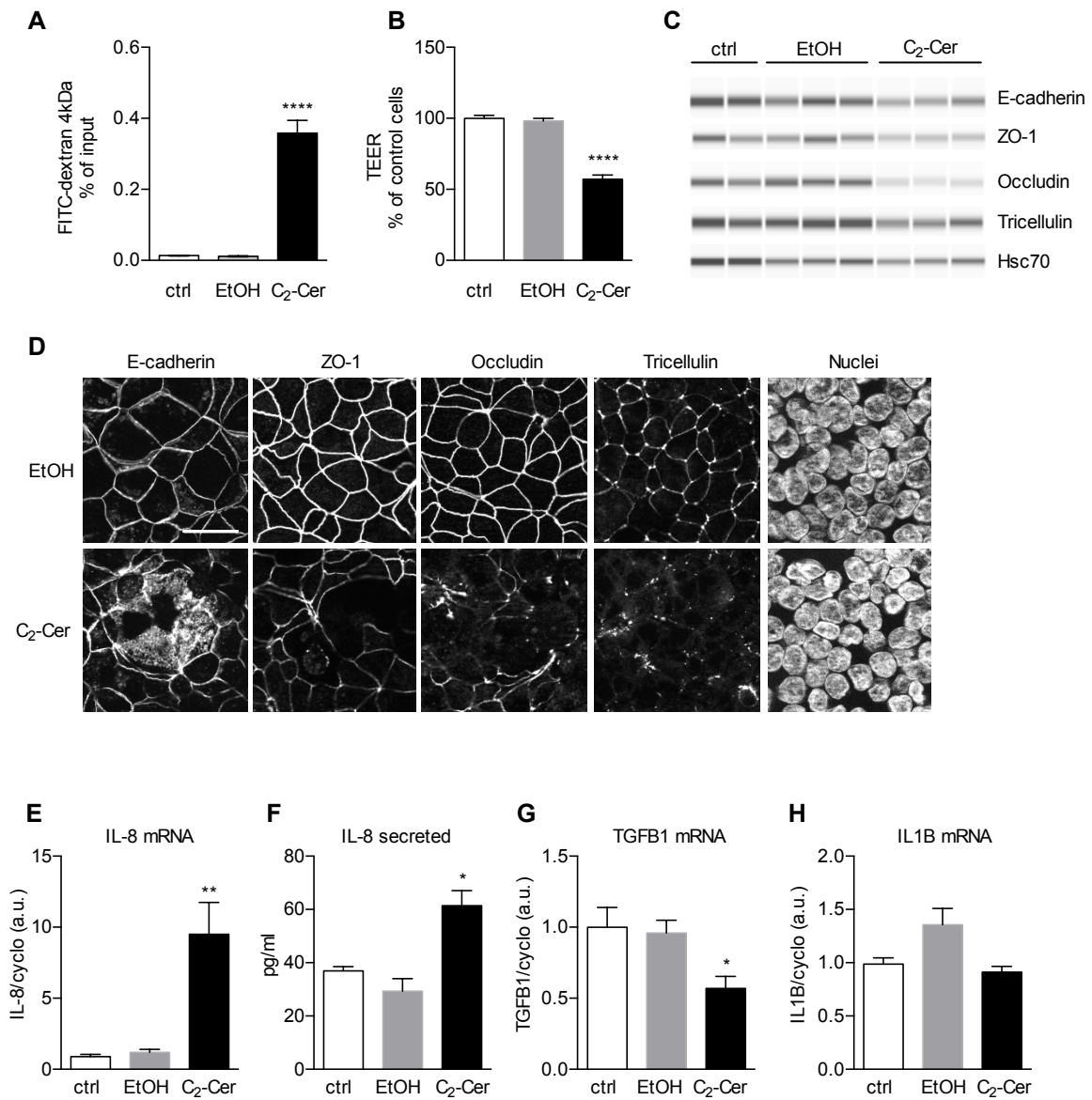


Figure 9

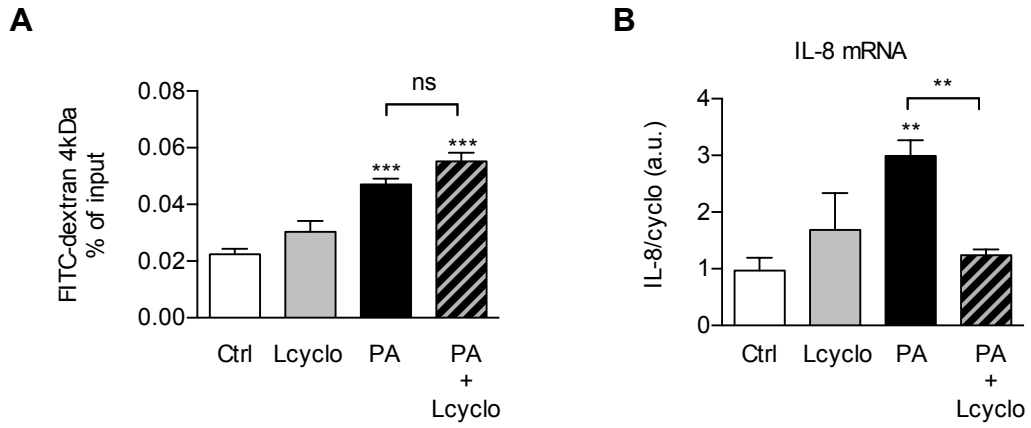


Figure 10

Table 1: Primer sequences and bacteria strains used used for the quantification of bacterai group or species

group or species	forward primer		reverse primer		bacteria strain used for the standard curve
	name	sequence	name	sequence	
<i>Clostridium leptum</i>	Clept 09	5'-CCTTCGGTCCGSAGTTA-3'	Clept 08	5'-GAATAAA CACAT CT CACTGCTT-3'	<i>Faecalibacterium prausnitzii</i>
<i>Akkermansia muciniphila</i>	AKK-F	5'-GGTAGCCGGTCTGAGAGGAT-3'	AKK-R	5'-TAGGTGTCTGGACCGTGTCTC-3'	see legend below
<i>Lactobacillus</i> sp.	Lacto 04	5'-CGCCACTGGTGTTCYTCCATA-3'	Lacto 05	5'-AGCAGTAGGGAATCTCCA-3'	<i>Lactobacillus acidophilus</i>
Segmented Filamentous Bacteria	SFB-F	5'-CACGGTCCATACTCCTACGG-3'	SFB-R	5'-AGGGTTTCCCCCATTGTG-3'	see legend below
<i>Bacteroides</i> sp.	Bacter 11	5'-CCTWCGATGGATAGGGGT-3'	Bacter 08	5'-CACGCTACTTGGCTGGTTCAG-3'	<i>Bacteroides thetaiotaomicron</i>
<i>Clostridium coccooides</i>	Ccoc 07	5'-GACGCCGCGTGAAGGA-3'	Ccoc 14	5'-AGCCCCAGCCTTTCACATC-3'	<i>Clostridium coccooides</i>
All bacteria	F_Bact 1369	5'-CGGTGAATACGTTCCCGG-3'	R_PROK 1492	5'-TACGGCTACCTTGTACGACTT-3'	<i>Escherichia coli</i>

The determination of *Akkermansia muciniphila* and Segmented Filamentous Bacteria abundance was determined by relative quantification to a control sample.

Table 2: List of primer sequences used for RT-PCR analyses

human genes (alias names)	forward primer	reverse primer
CXCL8 (IL-8)	5'-AGACAGCAGAGCACACAAGC-3'	5'-ATGGTTCCTTCCGGTGGT-3'
TGFB1 (TGF-Beta-1)	5'-GCAGCACGTGGAGCTGTA-3'	5'-CAGCCGGTTGCTGAGGTA-3'
IL1B (IL-1-Beta)	5'-CTGTCCTGCGTGTGAAAGA-3'	5'-TTGGGTAATTTTGGGATCTACA-3'
HSPA5 (GRP78, BIP)	5'-CTGGGTACATTTGATCTGACTGG-3'	5'-TCCTTGAGCTTTTGTCTTCCT-3'
DDIT3 (GADD153, CHOP)	5'-AGCTGTGCCACTTTCCTTTC-3'	5'-CAGAACCAGAGAGGTCACA-3'
XBP1 spliced form	5'-TGCTGAGTCCGCAGCAGGTG-3'	5'-GCTGGCAGGCTCTGGGGAAG-3'
PIIB (Cyclophilin B)*	5'-GCCTTAGCTACAGGAGAGAA-3'	5'-TTTCCTCCTGTGCCATCTC-3'
CDH1 (e-cadherin)	5'-GCCGAGAGCTACACGTTCA-3'	5'-GACCGGTGCAATCTCAAA-3'
TJP1 (ZO-1)	5'-CAGAGCCTTCTGATCATTCCA-3'	5'-CATCTCTACTCCGGAGACTGC-3'
OCLN (occludin)	5'-AGGAACCGAGAGCCAGGT-3'	5'-GGATGAGCAATGCCCTTAG-3'
MARVELD2 (tricellulin)	5'-CAGGCTGTCCTGAGGAAGTT-3'	5'-CCGAATGATGTGGCAATCT-3'
mouse genes	forward primer	reverse primer
Cdh1 (e-cadherin)	5'-TCCTTGTTCCGGCTATGTGTC-3'	GGCATGCACCTAAGAATCAG
Tjp1 (ZO-1)	5'-AGGACACCAAAGCATGTGAG-3'	5'-GGCATTCTGCTGGTTACA-3'
Ocln (occludin)	5'-TCCGTGAGGCCTTTTGAA-3'	5'-GGTGCATAATGATTGGGTTTG-3'
Marvel2 (tricellulin)	5'-AGGCTCCCACATCATTCTGA-3'	5'-TCCAGAAACGAAGGGTCATT-3'
Gata3	5'-TTATCAAGCCCAAGCGAAG-3'	5'-TGGTGGTGGTCTGACAGTTC-3'
Reg3g	5'-ACCATCACCATCATGTCTCTG-3'	5'-GGCATCTTCTTGGCAACTT-3'

* PPIB gene is used as reference gene and the same sequences are used for both human and mouse species.

ARTICLE 2

Role of AhR on intestinal barrier integrity and inflammation

The results of this study are presented at the end of this chapter in the form of a manuscript in preparation entitled: “AhR activation defends gut barrier integrity against damage occurring in obesity”, which I am the first author (article 2).

Our team described an inflammation in the small intestine of obese subjects leading to an increased susceptibility to epithelial cells dysfunctions (Monteiro-Sepulveda *et al.*, 2015). In a recent study, we showed that obese subjects present subtle intestinal barrier alterations in the basal state and increased jejunal permeability that was revealed after a lipid load. This susceptibility to lipid challenge was correlated to inflammatory and metabolic status (Genser *et al.*, 2018). In addition, studies showed that genetically and diet-induced obese mice present a higher intestinal permeability, higher endotoxemia and a higher level of proinflammatory cytokines compared to control mice (Brun *et al.*, 2007; Cani *et al.*, 2007; Cani *et al.*, 2008). Therefore, these findings led us to hypothesize possible links between environmental factors, lipids, obesity and low-grade intestinal inflammation, which can be correlated to intestinal barrier disruption.

Low AhR tone correlates with intestinal inflammation in obese patients.

For human studies, this project benefits from a well-characterized cohort of non-diabetic obese and lean subjects collected by the Research Center for Human Nutrition at the Pitié-Salpêtrière Hospital (Paris, France) through collaborative work with Christine Poitou and Karine Clément (Table 1 in article 2). This biobank includes blood samples and intestinal tissues obtained from bariatric surgery (gastric bypass) for obese subjects or after intestinal biopsy for non-obese subjects.

The obese patients underwent by-pass surgery meanwhile a piece of the small intestine, a surgical waste is collected. The jejunal fragment is dissociated into epithelium and lamina propria. Afterward, based on previous data obtained by our team characterizing low-grade intestinal inflammation in obese subjects (Monteiro-Sepulveda *et al.*, 2015), we classified the subjects according to their small intestine inflammation score, established by the densities of T lymphocytes using the CD3 marker. We used the epithelium/lamina propria (Epi/LP) CD3 ratio, as a parameter to evaluate intestinal inflammation quantifying infiltration of T lymphocytes within intestinal epithelium in jejunum samples. We analyzed the AhR tone

by measuring mRNA levels of AhR and of its target genes (CYP1A1, CYP1B1, IL-22, AhRR) by RT-qPCR (TaqMan LDA) in jejunum epithelium of obese subjects.

We observed that obese patients display a higher Epi/LP CD3 ratio in comparison to lean patients, indicating that the gut of obese patients presents a local inflammation. Moreover, we observed a more important heterogeneity in Epi/LP CD3 ratio in obese as compared to non-obese subjects (Figure 1A in article 2). We established a correlation between Epi/LP CD3 inflammation score and mRNA level of AhR target genes in jejunum epithelium (Figure 1B-F in article 2). Our results show that obese subjects presenting a high CD3 ratio display a lower AhR activity. This result suggests that obese patients with higher recruitment of T lymphocytes in the small intestine have an impaired AhR activation.

The obese patients studied in our work have well-establish obesity and inflammatory state. Thus, it is difficult to know whether the intestinal inflammation results from an impaired AhR tone or whether the decreased expression of their target genes was a consequence of the inflammation. Other factors can contribute to the modulation of AhR tone. Thus, we decided to use cellular and animal models to study the mechanistic implication of AhR activation on the intestinal barrier and the onset of gut inflammation.

AhR activation prevented damages on the intestinal epithelial barrier in mice and intestinal epithelial cells.

We assessed the induction of AhR activity in enterocytes presenting an intestinal epithelial barrier disturbance to evaluate the role of this transcription factor on intestinal inflammation and epithelial barrier disruption. For *in vivo* studies, we analyze the effect of AhR activation on intestinal inflammation in C57BL/6 wild-type mice submitted to the nutritional challenge.

Previous data of our team shows that mice fed with a long-term high fat diet exhibit low AhR activation in the intestine, which is reflected by the low expression of its target genes, CYP1A1 and CYP1A2 in the tissue (not published data). A low AhR activity may be due to the lower amount of dietary fibers present in the high-fat diet as compared to control chow diet. Indeed, fibers are known to be metabolized in the gut by microbiota fermentation resulting in the production of short chain fatty acids, such as acetate, propionate, and butyrate (Koh *et al.*, 2016) and a recent study have demonstrated that butyrate is able to activate AhR (Marinelli *et al.*, 2019). Thus, in order to limit the impact of fiber depletion in our

experiments, mice were gavaged daily with palm oil while being maintained on a standard diet (normal fiber composition) *ad libitum*. This model was chosen in article 1 to demonstrate a postprandial alteration of the intestinal barrier and a wild gut inflammation after palm oil intake.

Among the different exogenous or endogenous AhR ligands available, we chose to use the AhR agonist β -naphthoflavone (β NF) to activate AhR in mice and in Caco-2/TC7 cells. β NF is a synthetic derivative of a naturally occurring flavonoid that has been widely used as an AhR agonist although its capacity to induce AhR target genes is less potent than TCDD (Soshilov and Denison 2014). Some exogenous ligands such as TCDD (the most studied and powerful of them) are carcinogen and/or mutagen (Sugimura 2000). Contrary to these ligands, β NF displays no mutagenic activity and is thus less dangerous to manipulate (Yokouchi *et al.*, 2007; Allan 2008). Moreover, while the endogenous AhR agonist FICZ is considered to be as potent as TCDD, the effect of FICZ on the induction of AhR target genes, in our experimental conditions in Caco-2/TC7 cells, was lower than observed for β NF.

As observed in my previous work (see article 1), 5 days palm oil treatment increased paracellular permeability *in vivo*, perturbed the location of tight junction proteins ZO-1, occludin and tricellulin at cell-cell contact in the jejunum and induced a mild inflammation in the intestine. In mice submitted to the concomitant supply of palm oil and β NF (Figure 2 in article 2), I observed that AhR activation, confirmed by the induction of the AhR target genes CYP1A1 and CYP1B1 in the jejunum, partially prevented the mislocalization of three junction proteins caused by palm oil gavage. This partial restoration was not sufficient to counteract the defects induced by palm oil on paracellular permeability. However, in our experimental conditions, AhR activation seems to protect the cell-cell junctions' disruption induced by lipids.

To better understand the role of the AhR activation on cell-cell junction disruption, we carried out experiments in Caco-2/TC7 cell model. In this cellular model, we can study the role of AhR pathway directly on the intestinal epithelium without the interference of other mechanisms in which AhR is involved, such as microbiota, immune cell regulation, etc.

To investigate the consequences of an increase of AhR tone in enterocytes, we studied the effect of the AhR agonist β NF on intestinal epithelium whose functions are perturbed by factors affecting the intestinal epithelial barrier permeability and cell-cell junctional

complexes, i.e. in the presence of palmitic-acid containing micelles or of a calcium chelator (see article 1).

I showed in Caco-2/TC7 cells that the concomitant treatment with palmitic acid and β NF was able to improve the cell-cell junctions' disruption induced by palmitic acid (data not shown). Indeed, I observed that the tight junction proteins, occludin, ZO-1 and tricellulin were maintained at cell-cell contact upon β NF treatment. However, the AhR activation was not able to counteract the deleterious effect of palmitic acid on paracellular permeability and on inflammatory markers (IL-8, TGF β and TNF α) (data not shown). So, as in mice fed with palm oil, AhR activation seems to act on cell-cell junctions in Caco-2/TC7 cells treated with palmitic acid.

Since AhR agonist appears to play a role in the integrity of cellular junctions in the intestinal epithelium, we studied the effect of AhR activation on cell-cell junction integrity in a situation of chemically-induced barrier damage. We used EGTA, a calcium chelator known to provoke the disruption of junctional protein complexes and to prevent the return of junction proteins to the membrane, that resulted in the opening of the intercellular junction and in the increase of paracellular permeability (Tria *et al.*, 2013). We then analyzed the role of β NF on barrier integrity and cytokines expression and secretion triggered by this chemical barrier damage.

Our results demonstrated that the AhR agonist was able to counteract the chemical barrier damage on paracellular permeability to ions (TEER), on tight junctions (occludin, tricellin and ZO-1) location (Figure 3 in article 2) and on proinflammatory cytokines secretion and expression (Figure 4 in article 2). This suggests that AhR activation might improve the intestinal inflammation and epithelial damage triggered by EGTA.

As cell-cell junction integrity is depended on processes controlled by kinases and phosphatases pathways, I studied the implication of PKC, p38MAPK and ERK kinases on AhR action. I analyzed the effects of specific inhibitors of these kinases on the EGTA-dependent decrease of TEER in presence of β NF in Caco-2/TC7 cells (Figure 5 in article 2). I observed that the inhibition of PKC and p38MAPK signaling pathway blocked the action of AhR agonist on TEER. These results suggest the potential implication of these two kinases in the protective effect of β NF on intestinal permeability to ions.

Taken together, our data revealed that the AhR tone is decreased in non-diabetic obese subjects presenting with gut inflammation. Besides, our studies in mice and intestinal epithelial cells showed that AhR activation plays an important role in intestinal barrier integrity and inflammation, both epithelial dysfunctions present in obese subjects. Thus, our work suggests that the administration of AhR agonists might protect the intestine damages reported in obesity.

AhR activation defends gut barrier integrity against damage occurring in obesity

Barbara G. Postal^{1,#,‡}, Sara Ghezzal^{1,‡}, Sébastien André², Kevin Garbin³, Laurent Genser², Edith Brot-Laroche¹, Christine Poitou^{2,4}, Armelle Leturque^{1,2}, Karine Clément^{2,4}, Véronique Carrière^{1,#*}

From the ¹Centre de Recherche des Cordeliers, Sorbonne Université, UPD Univ Paris 05, INSERM, CNRS, Paris, F-75006, France; ²Sorbonne Université, INSERM, NutriOmics research Unit Paris, F-75013 France; ³CHIC platform of Centre de Recherche des Cordeliers, Sorbonne Université, UPD Univ Paris 05, INSERM, Paris, F-75006, France; ⁴Assistance Publique-Hôpitaux de Paris, Pitié-Salpêtrière Hospital, Nutrition Department, CRNH Ile de France, Paris, F-75013 France

Running title: *AhR activation and gut barrier*

#Present address: Centre de Recherche de Saint-Antoine, Sorbonne Université, INSERM, Paris F-75012 France

‡These authors contributed equally to this work

*To whom correspondence should be addressed: Véronique Carrière: Centre de Recherche de Saint-Antoine, UMRS 938, Equipe P. Seksik & H. Sokol, 27 rue de Chaligny, Paris F-75012 France; veronique.carriere@sorbonne-universite.fr; Tel +33 1 40 01 13 89

Keywords: aryl hydrocarbon receptor, intestine, cell junction, permeability, signaling

ABSTRACT

Obesity is characterized by systemic and tissue low-grade inflammation, however the mechanisms involved need further understanding. Accumulation of inflammatory cells is also found in the intestine. The obesity-associated environmental changes might be involved in the development of this inflammation process. In this context, we focused on intestine response to external stimuli mediated by the activation of aryl hydrocarbon receptor (AhR). Jejunum samples from subjects with normal weight and severe obesity were phenotyped according to T lymphocytes infiltration in epithelium from lamina propria, and were assayed for mRNA level of AhR target genes. The effect of an AhR agonist was studied in mice and in Caco-2/TC7 cells. AhR target gene expression, permeability to small molecules and ions and location of cell-cell junction proteins were recorded in condition of altered intestinal permeability. We showed that a low AhR tone correlated with a high inflammatory score in intestinal epithelium in human severe obesity. Moreover, AhR activation protected junctional complexes in intestinal epithelium in mice challenged by an oral lipid load. AhR ligand prevented chemically induced damages of barrier integrity and cytokine expression in Caco-2/TC7 cells. PKC and p38MAPK signaling pathways were involved in this AhR action. Taken together, the results of these series of human, *in vivo* and *in vitro* experiments suggest a protective effect of AhR activation in the intestine. We therefore propose that AhR constitutes a valuable target to protect intestinal functions in metabolic diseases that can be achieved in the future via food or drug ligands.

INTRODUCTION

The development of obesity is characterized by a progressive aggravation of systemic low-grade inflammation together with metabolic deterioration. Inflammation also occurs in tissues such as in adipose tissue (Reilly and Saltiel 2017; Stolarczyk 2017), liver (Loomis *et al.*, 2016) and more recently in the small intestine (Monteiro-Sepulveda *et al.*, 2015). During obesity, adipose tissue contributes to inflammation either directly in the systemic vascular system but also through the portal vein via the release of free fatty acids, cytokines and adipokines (Makki *et al.*, 2013). Recent reports highlighted the role of intestine as an early contributor to low-grade inflammation. Studies in mouse models of high-fat diet induced obesity suggested that the passage of bacterial components such as lipopolysaccharides (LPS) from the intestinal lumen towards the circulation promotes the systemic inflammation through mechanisms involving intestinal barrier damage (Cani *et al.*, 2007; Cani *et al.*, 2008; Araujo *et al.*, 2017). Intestinal permeability was increased during the first week of high-fat diet in mouse (Hamilton *et al.*, 2015; Johnson *et al.*, 2015) and, as we recently demonstrated, after a single gavage with palm oil (Ghezzal *et al.*, in press). These last results suggested that intestinal barrier defects may precede the onset of obesity. In human, we showed that the higher T lymphocytes density in jejunal epithelium of obese compared to lean patients was associated with markers of systemic inflammation (Monteiro-Sepulveda *et al.*, 2015). In fasting state, subtle intestinal barrier alterations were evidenced in jejunum samples of subjects with severe obesity that were further enhanced after an *ex-vivo* lipid challenge (Genser *et al.*, 2018). The patient susceptibility to lipid-induced barrier defect was correlated with both intestinal and systemic inflammation. Altogether these studies established a link between intestinal barrier and low-grade inflammation in obesity, where the molecular actors that orchestrate this relationship need to be deciphered.

Several data highlight the role of AhR in metabolic diseases and inflammation. The aryl hydrocarbon receptor (AhR), a transcriptional factor and a sensor of environmental changes, was extensively studied for its role in the metabolism of xenobiotics (Ramadoss *et al.*, 2005). Investigations using AhR knockout mouse unveiled its important role in the development and control of the immune system (Rothhammer and Quintana 2019). In gut, a protective role of AhR in inflammation or barrier injury conditions have been reported (Rothhammer and Quintana 2019). Its role in the intestinal tract seems acting through intraepithelial lymphocytes differentiation and modulation of innate lymphoid cells (Rothhammer and Quintana 2019). In human, a loss of protective function of AhR was proposed to occur in intestinal bowel diseases, which were linked to reduced production of AhR agonists by gut microbiota of the patients (Lamas *et al.*, 2016). A protective effect of AhR agonist on intestinal barrier in mouse models or in intestinal cells submitted to inflammatory stresses has been reported (Yu *et al.*, 2018; Yu *et al.*, 2018). In metabolic diseases contradictory results were obtained concerning the importance of AhR tone. A set of recent reports showed that AhR deficient mice are protected from

diet-induced obesity and associated metabolic disorders such as insulin-resistance, and hepatic steatosis through mechanisms related to lipid and energy metabolisms (Xu *et al.*, 2015; Moyer *et al.*, 2017). Conversely, the activation of AhR using genetic mouse models or specific ligands such as TCDD promoted hepatic steatosis (Lee *et al.*, 2010; He *et al.*, 2013). This deleterious impact of AhR activation is in contrast with other works showing a protective role of AhR towards liver steatosis in mice (Wada *et al.*, 2016; Natividad *et al.*, 2018). Moreover, in human low levels in feces of AhR agonists were associated with metabolic syndrome, type 2 diabetes, increased body mass index and blood pressure (Natividad *et al.*, 2018).

Combining a series of human, *in vivo* and *in vitro* studies, we aim at determining the potential implication of AhR in the intestinal inflammation and barrier dysfunction reported in obesity.

RESULTS

Increase level of CD3⁺ T cell density in intestinal epithelium of obese subjects negatively correlates with AhR gene targets.

The clinical characteristics of non-obese and severely obese patients included in this study are provided in table 1. The obese patients are free of type 2 diabetes (T2D) and T2D treatment according to clinical record and the measure of fasting glucose and insulin parameters.

We evaluated intestinal inflammation in non-obese and subjects with severe obesity by quantifying the density of CD3⁺ lymphocyte T in jejunum mucosa. The epithelial to lamina propria (Epi/LP) ratio allowed quantifying the T lymphocyte recruitment in the epithelium (Figure 1A). We showed a significantly higher CD3 Epi/LP ratio in obese than in non-obese subjects (0.49 ± 0.06 and 0.811 ± 0.077 respectively, $p < 0.0209$). A more heterogeneous distribution of CD3 Epi/LP was observed within the obese cohort. To study the relevance of AhR activity in the heterogeneity of intestinal inflammation within the obese cohort, we determined in jejunum epithelium from the same samples, the expression of AhR and of its well-known target genes (CYP1A1, CYP1B1) as well as the recently identify AhR-target gene, IL-22 (Parks *et al.*, 2015). We observed strong significant negative relationships between AhR, CYP1A1, IL-22 mRNA levels and CD3 Epi/LP in obese subjects (Figure 2B-D). CYP1B1 mRNA levels and CD3 Epi/LP ratio showed also a tendency ($p = 0.0542$) toward a negative correlation (Figure 1E). However, AhR repressor (AhRR) involved in the feedback regulation of AhR signaling (Vogel and Haarmann-Stemmann 2017) was not correlated with intestinal inflammatory cell accumulation (Figure 2F).

Altogether these results show relationships between intestinal inflammation in obesity and AhR, and its target gene favoring the hypothesis of a low AhR tone, however a direct implication of AhR activity in this context needs to be established.

AhR activation protects junctional complexes in murine intestinal epithelium during lipid load

Inflammation and intestinal permeability is tightly linked in mice. In particular, we previously observed that repeated gavages with palm oil caused a disruption of the intestinal barrier integrity in mice and initiate intestinal inflammation (Ghezzal *et al.*, in press). We thus investigated the effect of AhR activation in a mouse model of lipid-induced impairment of intestinal epithelial barrier. Mice were force-fed with palm oil alone or with β -naphthoflavone (β NF), an AhR agonist (Figure 2). As expected, β NF administration markedly increased the expression of AhR target genes CYP1A1 and CYP1A2 in jejunum (Figure 2A). Importantly, we observed that palm oil feeding impaired tight junction integrity by causing the mislocalization of three tight junction proteins ZO-1, occludin and tricellulin at cell membrane (Figure 2B). The activation of AhR activity by β NF partially restored the localization of these proteins at the membrane (Figure 2B). However, the administration of β NF did not prevent the increase of intestinal permeability to macromolecules (determined by the measure of FITC-dextran 4kDa) induced by palm oil (Figure 2C), suggesting that the partial restoration of cell-cell junction is not sufficient to maintain epithelium integrity in our experimental conditions. Nevertheless, these results suggest a relative protective role for AhR on intestinal epithelium through a direct impact on cell-cell junctions.

AhR activation prevents chemically induced damages of barrier integrity in intestinal epithelial cells

We further investigated the role of AhR on cell-cell junctions in intestinal epithelium monolayer and examined in the human intestinal epithelial Caco-2/TC7 cell line, the effect of AhR activation after barrier damage. Chemical disruption of cell-cell junction disruption was induced by EGTA a calcium chelator known to alter barrier permeability (Rothen-Rutishauser *et al.*, 2002). We first wanted to determine whether AhR activation could prevent the loss of barrier integrity induced chemically by EGTA (Figure 3). Caco-2/TC7 cells were treated for 4 days with β NF and EGTA was added during the last 4h of the experiment. As expected, the treatment of Caco-2/TC7 cells with β NF increased the mRNA level of the AhR target gene CYP1A1 (Figure 3A). The chemical damage of barrier integrity did not further modify CYP1A1 expression. While the permeability to macromolecules measured by the passage of FITC-dextran 4kDa was increased upon chemically induced barrier damage, this increase was not modified in presence of β NF (Figure 3B). Unexpectedly, a small increase of FITC-dextran 4kDa flux is observed when the cells are incubated with β NF alone. We then studied the effects of AhR activation on paracellular permeability to ions measured by the determination of transepithelial resistance (TEER). As awaited, chemically induced barrier damage decreased TEER denoting an enhanced permeability to ions (Figure 3C). We showed that β NF prevented the chemically induced barrier damage by maintaining TEER to control value. A reinforcement of TEER

was also noticed when the cells were incubated with β NF alone. We analyzed by immunofluorescence the localization of the tight junction proteins ZO-1, occludin and tricellulin in cells, pre-treated or not with β NF, in chemically induced barrier damage condition (Figure 3D). We observed a decrease in the fluorescence intensity for these junction proteins in chemically induced barrier damage, which is prevented by β NF. Non-obvious effect was observed in presence of β NF alone. An increase in protein level of occludin and tricellulin was observed in presence of β NF alone, and in chemically induced barrier damage cell treated with β NF (Figure 3E). These results then indicate that pretreatment with the AhR agonist β NF prevent the damages to intestinal epithelium monolayer induced chemically through a mechanism involving an increase of occludin and tricellulin expression and localization at cell-cell contact. We then determined whether the activation of AhR may reinforce the recovery to normal permeability after chemically-induced damages. We incubated the cells for 4h with EGTA and then added new culture medium containing or not the AhR agonist (Figure 3F). We showed that the addition of β NF improved the recovery of TEER in chemically induced barrier damage. This amelioration was apparently initiated the first hours after β NF addition and reached the statistical significance after 5 hours.

Altogether, these results from *in vitro* experiments showed that the activation of AhR protects and restores the intestinal epithelial cell monolayer from damages provoked by a disruptor of cell-cell junctions.

AhR activation prevents the increase of cytokine expression associated with epithelium damages

Intestinal epithelial cells can produce many cytokines and chemokines (Stadnyk 2002; Miron and Cristea 2012). The CXCL8 (IL-8), TNFA and IL1B genes are particularly well expressed in the model of Caco-2/TC7 cells. We previously showed in this model that chemical barrier damage provoked an increase of TNF α , IL1- β and IL-8 expression as well as of IL-8 secretion (Ghezzal *et al.*, in press). We determined whether the presence of the AhR agonist counteracts these effects. Despite barrier damage, we showed that the pre-treatment with β NF indeed prevented the increase in the mRNA level of the three investigated cytokines (Figure 4A-C) and of the secretion of IL-8 in the basal medium (Figure 4D). These results showed that *in vitro* activation of AhR precludes the inflammatory response of intestinal epithelial cells during a treatment disrupting epithelial barrier.

AhR activation exerts its preventive effect through PKC and p38MAPK signaling pathways

The assembly and maintenance of cell-cell junction and in particular of tight-junctions are controlled and regulated by phosphorylation/dephosphorylation processes (Garcia *et al.*, 2018) involving several regulatory proteins such as kinases or phosphatases (Stein and Kottra 1997; Mitic and Anderson 1998;

Matter and Balda 2003). We investigated the implication of such signaling pathways in the protective effect of AhR activation on the EGTA-dependent decrease of TEER in Caco-2/TC7 cells. Cells were pre-incubated, daily for 4 consecutive days, with inhibitors of protein kinases C (Ro 31-8220) or p38MAP kinase (SB203580) or ERK1/2 (U0126) one hour before the addition of β NF. Chemical barrier damage was induced for the last 4 hours of treatment. We confirmed that barrier damage is associated with a decrease of TEER that is counteracted in presence of this AhR agonist and further showed that the maintenance of TEER by β NF treatment in barrier-damaged cell monolayer did not occur when the cells were pretreated with Ro-318220 (Figure 5A) or with SB203580 (Figure 5B). However, in the absence of β NF treatment, we found a decrease of TEER in presence of Ro-318220 in chemically damaged epithelial barrier (Figure 5A), indicating that the inhibition of protein kinase C is sufficient to modify TEER. Contrary to the effects of PKC and p38MAPK inhibitors, the inhibition of protein kinase ERK1/2 by U0126 treatment did not block the β NF dependent-maintenance of TEER to control value (Figure 5C).

These results suggest the potential implication of PKC and p38MAPK in the protective effect of β NF on intestinal permeability to ions (TEER).

DISCUSSION

The molecular mechanisms involved in the intestinal and systemic low-grade inflammation in human subjects need deeper understanding. Reports suggest that intestinal barrier defect may represent an early event in the onset of intestinal and systemic inflammation (Laugerette *et al.*, 2011; Vors *et al.*, 2015; Genser *et al.*, 2018; Ghezzal *et al.*, in press). We here showed a negative correlation between the expression of AhR target genes and inflammation in jejunum samples of subjects with obesity albeit without diabetes. This observation suggests that low AhR activity is linked to low-grade inflammation in human obesity. Several mediators recently suggested, including changes in gut microbiota composition in obesity, may be related to this potentially decreased AhR tone in obesity. It was reported that endogenous AhR ligands are produced by intestinal microbiota from the metabolism of nutrients-derived metabolites such as tryptophan (Hubbard *et al.*, 2015) and butyrate, a short chain fatty acid (Marinelli *et al.*, 2019), to activate the AhR tone at least in the intestine. Intestinal inflammation and changes in fecal microbiota composition of subjects suffering from intestinal bowel diseases were associated with low level of fecal AhR ligands (Lamas *et al.*, 2016). The diet supplementation with Lactobacillus strains, which display a high natural capacity to produce AhR ligands, improved the metabolic impairments induced by the high fat diet (Kang and Cai 2018; Natividad *et al.*, 2018) or reduced the severity of chemically induced colitis in mice (Lamas *et al.*, 2016). However, obesity is often associated with an increased fecal abundance of Lactobacillus species (Armougom *et al.*, 2009; Million *et al.*, 2012; Peters *et al.*, 2018), thus one wonders whether

this change in abundance is accompanied by an increase in the function of this bacterial species. Indeed, low levels of AhR ligands in feces were correlated with high corpulence in Human (Natividad *et al.*, 2018). Moreover, the use of probiotics showed beneficial effects, albeit moderate, of *Lactobacillus* strains administration in fat mass reduction in obese subjects (Kang and Cai 2018). The mechanisms involved in these effects need understanding.

We demonstrated here that AhR activation prevented the increase of cytokine expression induced by a chemical treatment disrupting cell-cell junctions. Several mechanisms are involved in the expression of cytokine in immune cells, most of them are linked to the activation of NF- κ B pathways and protein kinases, however little is known on those specifically responsible for the expression of cytokines by intestinal epithelial cells (Andrews *et al.*, 2018). We here provide mechanistic information regarding the link between AhR activation and intestinal barrier properties. We showed in mice after a lipid load or in human intestinal epithelial cell line that AhR activation by the agonist β NF preserved the integrity of intestinal epithelium by maintaining paracellular permeability to ions and by preserving cell-cell junction. Experimental approaches in Caco-2/TC7 cells revealed increased occludin and tricellulin protein levels after β NF treatment and the potential implication of protein kinase C and p38MAPK in the protective effect of AhR action on paracellular permeability. AhR is a transcription factor known to bind specific responsive elements in the promoter of its target genes (Guyot *et al.*, 2013). To date no AhR responsive elements have been described on the promoter of occludin and tricellulin suggesting that the effects of AhR occurs through indirect pathways. Numerous observations indicated that AhR triggers several cellular pathways via the activation of protein kinases or via its E3 ubiquitin protein ligase activity (Larigot *et al.*, 2018; Rothhammer and Quintana 2019). Studies suggest that the Src tyrosine kinase is a member of the multiprotein AhR complex located in the cytosol in absence of AhR ligands. The interaction of AhR with its ligands provoked the dissociation of this complex, the release and the activation of src kinase as well as the translocation of AhR in the nucleus (Rothhammer and Quintana 2019). Most interestingly src and other protein kinases activated by AhR such as PKC and p38MAPK are involved in the maintenance of tight junctions. Paracellular permeability is tightly regulated by mechanisms controlling the localization and the expression of tight junctions proteins through phosphorylation/dephosphorylation or protein stabilization processes (Turner 2009; Van Itallie and Anderson 2018; Schuhmacher *et al.*, 2019). For example it has been observed that PKC and the protein Par-6 are members of a multiprotein complex involved in the maintenance of cell polarity (Schuhmacher *et al.*, 2019) and that Par-6 is also involved in the protective effects of AhR on intestinal epithelial barrier (Yu *et al.*, 2018).

Further studies are required to fully understand the mechanisms involved in the protective effect of AhR on intestinal epithelium.

Altogether, our study shows an important protective role of AhR on intestinal barrier integrity including on cell-cell junction and expression of inflammatory markers and suggest that in obesity this integrity might be altered through an imbalance between AhR tone and low-grade inflammation. The lower AhR activity in intestine of obese subject may thus contribute to local and systemic inflammation through a loss of intestinal barrier integrity. AhR might thus constitute to protect intestinal functions in metabolic diseases via food or drug ligands action.

EXPERIMENTAL PROCEDURES

Human subjects, Clinical and biological characteristics

This study is ancillary to a previously published study (Monteiro-Sepulveda *et al.*, 2015) that included a population of severely obese patients involved in a bariatric surgery program (Roux-en-Y gastric bypass), occurring at the Pitié-Salpêtrière University Hospital, Nutrition and visceral surgery departments, Paris, France. Non-obese subjects underwent pancreaticoduodenectomy or gastrectomy allowing access to proximal jejunal samples. For this study purpose, a subgroup of 36 subjects including 26 severely obese and 10 non-obese subjects, were selected being free of diabetes based on international definition and with no personal or familial history of inflammatory bowel disease. Their levels of white blood count were under 10.109/mm³ and of CRP inferior to 5 mg/l. We excluded non-obese subjects with diabetes, renal- cardiac- or hepatic failure. The study was conducted in accordance with the Declaration of Helsinki, received approval from the local ethics committee (CPP Ile de France I) and was registered on the ClinicalTrials.gov website <https://clinicaltrials.gov/ct2/show/NCT00476658> (Monteiro-Sepulveda *et al.*, 2015). Informed written consent was obtained from all patients prior to study inclusion. Medical history and clinical variables were recorded for non-obese and obese patients before surgery as described in (Monteiro-Sepulveda *et al.*, 2015). Venous blood samples were collected after a 12-h fast for routine assessment of biological metabolic as previously described (Dalmás *et al.*, 2011). Insulin resistance was assessed using the HOMA-IR index [insulinemia (mIU/L) x fasting blood glucose (mmol/L)/22.5].

Human jejunum tissue sampling, epithelium and lamina propria fractions and analyses

Proximal jejunum samples from obese subjects and non-obese subjects were collected during surgery, conditioned and transported as described (Monteiro-Sepulveda *et al.*, 2015). Briefly, proximal jejunal samples (60–70 cm distal to the ligament of Treitz) were collected from surgical waste (4 cm). Tissue was rapidly opened, washed in DMEM (1g Glucose Glutamax + pyruvate + 10% SVF + 1% Penicilline/Streptomycine) and kept at 4°C before cell isolation, fixation and paraffin imbedding, or freezing at -80°C.

Immunohistology of jejunum (5 mm paraffin-embedded tissue sections) was performed using CD3

rabbit polyclonal (A0452, DAKO, Agilent Technologies, Les Ulis, France). Primary antibody or secondary antibody were incubated for 1h at room temperature and revealed with a streptavidin biotin peroxidase kit (GMR4-61, BioSpa Milan, Italy), DAB staining (DAKO, Agilent technologies), and nuclei hematoxylin counterstaining (Vector, Eurobio, les Ulis, France). Images were obtained by conventional microscopy. Lamina propria and epithelial immune cell densities (cell/mm²), excluding lymphoid structures, were analyzed in longitudinal sections of mucosa in two to four fields (ImageJ 1.46c).

Preparation of epithelial and lamina propria fractions were obtained as previously described (Monteiro-Sepulveda *et al.*, 2015). Briefly, the jejunal mucosa was dissected and minced prior incubation in chelating buffer (5mM EDTA, 2mM DDT, PBS; at 37°C for 20 min). Released epithelial cells were filtered through 70µm mesh cell strainers, centrifuged (600g at 4°C for 5min), and recovered in complete DMEM (10% FCS, 1% Penicilline/Streptomycine). Sucrase activity, used as a witness of enterocyte contamination of lamina propria by epithelial cells, was below 2% of total mucosa activity. Epithelial immune cells were enriched, at the interface of a 40%/70% Percoll gradient (GE Healthcare Europe, Velizy, France). Cells of the lamina propria, including lymphoid tissue, were isolated from fibrous matter by collagenase D digestion (1 mg/ml, Roche, Meylan France) at 37°C for 2h. Red blood cells were removed in lysis buffer (154 mM NH₄Cl, 10 mM KHCO₃, and 0.1mM EDTA). Total RNA of lamina propria and epithelial fractions were extracted with the RNAeasy Mini kit (QIAGEN, Thermofischer scientific, Illkirch, France). RNA concentration and quality were assessed (2100 Bioanalyzer, Agilent Technologies) before reverse transcription of total RNA (Applied Biosystems, Thermofischer scientific). qPCR analyses were performed using Taqman Low Density Arrays (Thermofischer scientific) according to the manufacturer's procedures with the following gene assay IDs : *AhR* (Hs00907314_m1), *AhRR* (Hs01005075_m1), *CYP1A1* (Hs01054797_g1), *CYP1B1* (Hs00164383_m1) and *IL22* (Hs01574154_m1). Results were normalized to the geometric mean of ribosomal 18S (Hs99999901_s1) and peptidylprolyl isomerase B (cyclophilin B, Hs00168719_m1) values.

Mouse treatments and *in vivo* intestinal permeability

Three-month-old male C57BL/6JRj mice (Janviers labs, Le Genest-Saint-Isle, France) were fed *ad libidum* a standard chow diet (A03, SAFE, Augy, France) during all the experiments. Mice were kept with the artificial light-dark cycle 12:12h with lights on 07:00h. Mice were force-fed with 0.2ml of water, palm oil (Sigma-Aldrich, Saint Quentin-Fallavier, France) or βNF (40mg/kg, Sigma-Aldrich) dissolve in palm oil for 4 consecutive days at 18:00h just before the feeding period of mice. For *in vivo* intestinal permeability measurement, fed mice were successively force-fed the 5th day of the experiment, with 0.2ml of palm oil or βNF dissolved in palm oil followed by 0.2ml of 4kDa FITC-

dextran solution (Sigma-Aldrich, 0.5mg/g of mice in water) at 9:00h. Mice were anesthetized and then euthanized 1 hour after the last gavage. Blood and jejunum were then collected. FITC-dextran concentrations were determined in the plasma by fluorometry (FLUOstar Omega; BMG Labtech, Champigny-sur-Marne, France). All experiments involving mice were approved by the French Minister of Education and Research and by the Animal Care and Use Committee N°5 (agreement number: APAFIS#2710-201510301447819).

Cell culture and cell treatments

Caco-2/TC7 cell line is a clonal population of the human colon carcinoma-derived Caco-2 cells, which reproduces to a high degree most of the morphological and functional characteristics of enterocytes (Chantret *et al.*, 1994). Cells were checked for absence of mycoplasma contamination. In all experiments, cells were cultured on 6-well Transwell® filters (Thermofischer scientific) for 3 weeks to obtain fully differentiated enterocyte-like cells as previously described (Beaslas *et al.*, 2009; Morel *et al.*, 2018). Cells were treated with 20µM βNF added in both apical and basal compartments. In some experiment cells were treated with 4.5mM EGTA (Sigma-Aldrich, St. Quentin Fallavier, France) added to the upper compartment. The duration of treatments is indicated in the legend of figures. In some experiments cells were pre-incubated with 5µM Ro 31-8220 (Calbiochem, Merck-Chimie, Fontenay-sous-bois, France) or 10µM U0126 (Cell Signaling, Ozyme, Saint-Cyr-l'École, France) or 20µM SB203580 (5633S, Calbiochem, Merck-Chimie), 1-hour prior βNF addition. These treatments were repeated daily for 4 days. These protein kinases inhibitors were added at both upper and lower compartment of the filters.

Permeability and transepithelial electrical resistance (TEER) measurements in Caco-2/TC7 cells

To assess paracellular permeability, 1mg/ml of 4 kDa FITC-dextran (TdB Consultancy AB, Uppsala, Sweden) was added to the apical medium the last day of treatment. Samples of basal medium were collected after 4h, and fluorescence was determined with a microplate fluorometer (FLUOstar Omega; BMG Labtech, Champigny s/Marne, France). Transepithelial electrical resistance (TEER), which is inversely proportional to permeability to small ions, was measured before and after treatments using a Volt-Ohm Meter (Millipore, Guyancourt, France).

Total RNA extraction and RT-PCR analysis

Total RNA from Caco-2/TC7 cells was extracted with TRI Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. Reverse transcription (RT) was performed with 1 µg of RNA using high-capacity cDNA reverse transcriptase kit (Applied Biosystem, Thermofischer scientific). Semi-quantitative real-time polymerase chain reactions were performed

with the Mx3000P Stratagen system using SYBR Green (Agilent, Les Ulis, France) according to the manufacturer's procedures. The human primer sequences are 5'-AGACAGCAGAGCACACAAGC-3' forward and 5'-ATGGTTCCTTCCGGTGGT-3' reverse for CXCL8 (IL-8), 5'-CTGTCCTGCGTGTTGAAAGA-3' forward and 5'-TTGGGTAATTTTTGGGATCTACA-3' reverse for IL1B (IL1- β), 5'-CAGCCTCTTCTCCTTCCTGA-3' forward and 5'-GCCAGAGGGCTGATTAGAGA-3' reverse for TNFA and 5'-TCCAAGAGTCCACCCTTCC-3' forward and 5'-AAGCATGATCAGTGTAGGGATCT-3' reverse for CYP1A1. PPIB (Cyclophilin B) gene is used as reference gene, the primer sequences are 5'-GCCTTAGCTACAGGAGAGAA-3' forward and 5'-TTTCCTCCTGTGCCATCTC-3' reverse.

Cytokine secretion

Cytokine IL-8 protein level was quantified in basal medium (0.2 ml) of Caco-2/TC7 cells by enzyme-linked immunosorbent assay (ELISA) using kit from R&D System (Lille, France).

Analysis of junctional proteins by immunofluorescence

Immunofluorescence analyses were performed as previously described (Petit *et al.*, 2012). Briefly, Caco-2/TC7 cells were fixed and permeabilized by incubation for 5 minutes in methanol at -20°C . For immunostaining on these cells, we used primary antibodies for tricellulin (1:200; MARVELD2 700191; Thermofischer scientific), ZO-1 (1:200; ZO1-1A12; 33-9100 Thermofischer scientific) and for occludin (1:200; 71-1500; Thermofischer scientific). Jejunum cryosections were fixed for 30 minutes with 4% paraformaldehyde at 4°C , and permeabilized for 30 minutes in 0.1% Triton X-100 at 4°C . For immunostaining of mouse jejunum cryosections, we used primary antibodies for tricellulin (1:10; Tric2469; kindly provided by Dr Furuse (Ikenouchi *et al.*, 2005; Ikenouchi *et al.*, 2008)), ZO-1 (1:200; 617300; Thermofischer scientific) and for occludin (1:10; Moc-37; kindly provided by Dr Furuse (Saitou *et al.*, 1997)). Alexa 488 and Alexa 546-conjugated anti-immunoglobulin G were used as secondary antibodies (1/400; Molecular Probes, Life Technologies, Saint-Aubin, France). Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI) to assess the monolayer integrity. Cells were examined by microscopy using an Axio Imager 2 microscope equipped with an apotome.2, allowing optical sectioning (Zeiss, Oberkochen, Germany). Images were acquired by ZEN 2011® software (Zeiss,) and analyzed by Image J 1.46c.

Simple Westerns™

Caco-2/TC7 cells were lysed as previously described (Beaslas *et al.*, 2009). Protein levels were detected in cell lysates using a Wes™ capillary electrophoresis system (ProteinSimple, San José, CA, USA) according to the manufacturer's instructions. Reconstituted images and quantification were

performed using Compass for SW3.1 software (ProteinSimple). Primary antibodies were rabbit anti-tricellulin (1:2000; MARVELD2 700191; Thermofischer scientific), and rabbit anti-occludin (1:25; 71-1500; Thermofischer scientific). Secondary antibodies and reagents used were provided in the separation and detection module kits (ProteinSimple). Junction proteins levels were normalized to Hsc70 (1:100; sc7298 Santa Cruz, Clinisciences Nanterre, France).

Statistical analysis

Values were expressed as mean \pm SEM. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Two group comparisons were performed using Student's T-test (quantitative variables) or chi-square test (categorical variables). Comparisons involving multiple groups were done using one-way analysis of variance (ANOVA). Correlations were determined using Spearman rank correlation coefficient. A level of $p < 0.05$ was considered as significant.

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AUTEUR CONTRIBUTIONS

BGP, SG and VC designed and conducted the experiments and analyzed the data. KG, SA, EBL conducted CD3 labeling and quantification on human jejunum samples. CP and KC coordinated clinical investigation. LG performed the patient surgery. C.P and KC contributed to subjects recruitment, patient phenotyping and sample collection. VC wrote the paper. BGP, CP, KC, and AL revised the manuscript versions. All authors reviewed the results and approved the final version of the manuscript.

ABBREVIATIONS AND NOMENCLATURE

β NF: betanaphthoflavone; DDT: Dithiothréitol; FCS : fetal calf serum ; FITC-dextran: Fluorescein isothiocyanate–dextran;

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FIGURE LEGENDS

Figure 1: Low expression of AhR target genes in obese subjects with intestinal inflammation

(A) CD3⁺ T cell density (cell/mm²) in epithelium/lamina propria ratio (Epi/LP) was determined by immunohistochemistry in jejunum of non-obese (n =10) and obese subjects (n = 26). Results are expressed as mean ± SEM, *p< 0.05. (B-F) Spearman correlations of AhR (B), CYP1A1 (C), IL-22 (D), CYP1B1 (E), AhRR (F) mRNA levels in intestinal epithelium and CD3 Epi/LP ratio in obese

subjects. AhR: aryl hydrocarbon receptor, AhRR: aryl hydrocarbon receptor repressor, a.u.: arbitrary units. Spearman r and p value are indicated.

Figure 2: Protective role of AhR activation on cell-cell junctions in murine intestinal epithelium.

Mice were submitted to 5 gavages (5x) with 200µl water or palm oil or palm oil + βNF for five consecutive days. (A) The expression of CYP1A1 and CYP1A2 were determined by RT-PCR in the jejunum. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level as mean ± SEM, n=5, ***p< 0.001 as compared to water condition. (B) The distribution of tight junction proteins ZO-1, occludin and tricellulin was analyzed by immunofluorescence on jejunum sections. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). White arrowheads indicate the labeling of junction proteins. Bar= 20µm. (C) The intestinal permeability was assessed after the last gavage with water, palm oil or palm oil+βNF by measuring plasma concentration of FITC-dextran 4kDa (FD4) one hour after an oral FD4 load. Results are expressed in µg/ml as mean ± SEM, *p<0.05, **p<0.01 as compared to water.

Figure 3: Protective role of AhR activation on EGTA-induced damages of barrier integrity in Caco-2/TC7 cells.

(A) Caco-2/TC7 cells were pre-incubated without (Ctrl) or with 20µM βNF for 4 consecutive days before the addition of 4.5 mM EGTA for additional 24h. βNF treatment was maintained during all the experiment. The expression of CYP1A1 was quantified by RT-qPCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level as mean ± SEM, n=6-15, ****p < 0.0001 as compared to control. (B) Cells were cultured in the same conditions as in (A). Paracellular permeability across Caco-2/TC7 cell monolayer was evaluated by measuring the accumulation during 4 h of FITC-4kDa dextran (FD4) in the basal compartment. Results are expressed as percentage of FD4 input (amount added in the apical compartment), mean ± SEM, n=6-15, ***p<0.001 and ****p<0.0001 as compared to control. (C) Cells were cultured in the same conditions as in (A). TEER was assessed in control and treated cells. Results are expressed in ohm.cm² as mean ± SEM, n=6-15, **p<0.01 and ***p<0.001 as compared to control. ###p<0.001 as compared to EGTA. (D) Cells were cultured in the same conditions as in (A). Immunofluorescence analysis was performed to study the localization of ZO-1, occludin and tricellulin. Nuclei were stained DAPI. Bar=20µm (E) Cells were cultured as in (A). Occludin and tricellulin protein levels were determined in cell lysates using a capillary-based western blot. Reconstituted images were displayed. Hsc70 protein levels were used as loading control. (F) Caco-2/TC7 cells were first incubated in presence or not of 4.5mM EGTA for 4h. Medium were then removed and replaced with fresh medium in presence or not of 20µM βNF for 20h. TEER was

measured before EGTA treatment (T0), after 4h of EGTA treatment (T4) and at different times after β NF addition. Results are expressed in percentage of TEER measured at T0 for each cell culture condition as mean \pm SEM, n=6. *p<0.05 and ****p<0.0001 as compared to control and ##p<0.01 as compared to EGTA.

Figure 4: AhR activation prevents the increase of cytokine expression induced by EGTA

Caco-2/TC7 cells were cultured in the same conditions as in Figure 3. The mRNA levels of TNF- α (A), IL-1 β (B) and IL-8 (C), were quantified by RT-PCR. Cyclophilin was used as reference gene. Results are expressed in arbitrary units (a.u.) as the ratio of target gene to cyclophilin (cyclo) mRNA level as mean \pm SEM, n=20-30. (D) The concentration of IL-8 protein in the basal compartment was quantified by ELISA 24h after EGTA treatment. Results are expressed in pg/ml as mean \pm SEM, n=6. Fold-increase as compared to control condition is indicated at the top of histograms. **p<0.01, ***p<0.001, ****p<0.0001 as compared to control unless otherwise indicated.

Figure 5: Signaling pathways are involved in the preventive effect of AhR activation on transepithelial resistance

Caco-2/TC7 cells were pre-incubated with (A) 5 μ M Ro 31-8220 (Ro) or (B) 20 μ M SB203580 (SB) or (C) 10 μ M U0126, 1-hour prior β NF addition. These treatments were repeated daily for 4 days. EGTA was added for the last 4 hours of treatment. Transepithelial resistance (TEER) was measured at the end of the experiment. Results are expressed in percentage of TEER measured at T0 for each cell culture condition as mean \pm SEM, n=3. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 as compared to control unless otherwise indicated.

Table 1. Clinical and biological baseline characteristics of non- obese and obese patients enrolled in the study

	non-obese (n= 10)	obese (n= 26)	pvalue obese vs non- obese
Demographic data			
sex ratio M/F, %	100 / 0	4 / 96	< 0.0001
age (years)	58.8 ± 4.9 (42-84)	39.3 ± 2.3 (21-64)	0.0002
Corpulence and adiposity			
weight (kg), mean ± SEM (min-max)	69.5 ± 3.2 (50-87)	124.3 ± 4.7 (83.1-184.4)	< 0.0001
BMI (kg/m ²), mean ± SEM (min-max)	22.6 ± 0.82 (16.9-26.37)	46.5 ± 1.3 (38.1-56.9)	< 0.0001
Comorbidities			
type 2 diabetes, %	0	0	-
hypercholesterolemia, %	20	35	0.6749
hypertriglyceridemia, %	-	30	
hypertension, %	0	25	0.1310
Glucose metabolism			
glycemia (mmol/L), mean ± SEM (min-max)	-	5.3 ± 0.1 (4.3-6.4)	-
insulinemia (mU/L), mean ± SEM (min-max)	-	25.1 ± 3.9 (9.5-67.5)	-
HOMA-IR index, mean ± SEM (min-max)	-	6.3 ± 1.1 (2.4-18.7)	-
Lipid metabolism			
total cholesterol (mmol/L), mean ± SEM (min-max)	-	5.0 ± 0.2 (3.3-7.2)	-
triglycerides (mmol/L), mean ± SEM (min-max)	-	1.3 ± 0.08 (0.47-1.96)	-
HDL (mmol/L), mean ± SEM (min-max)	-	1.1 ± 0.06 (0.7-1.6)	-
LDL (mmol/L), mean ± SEM (min-max)	-	3.3 ± 0.2 (1.9-5.2)	-

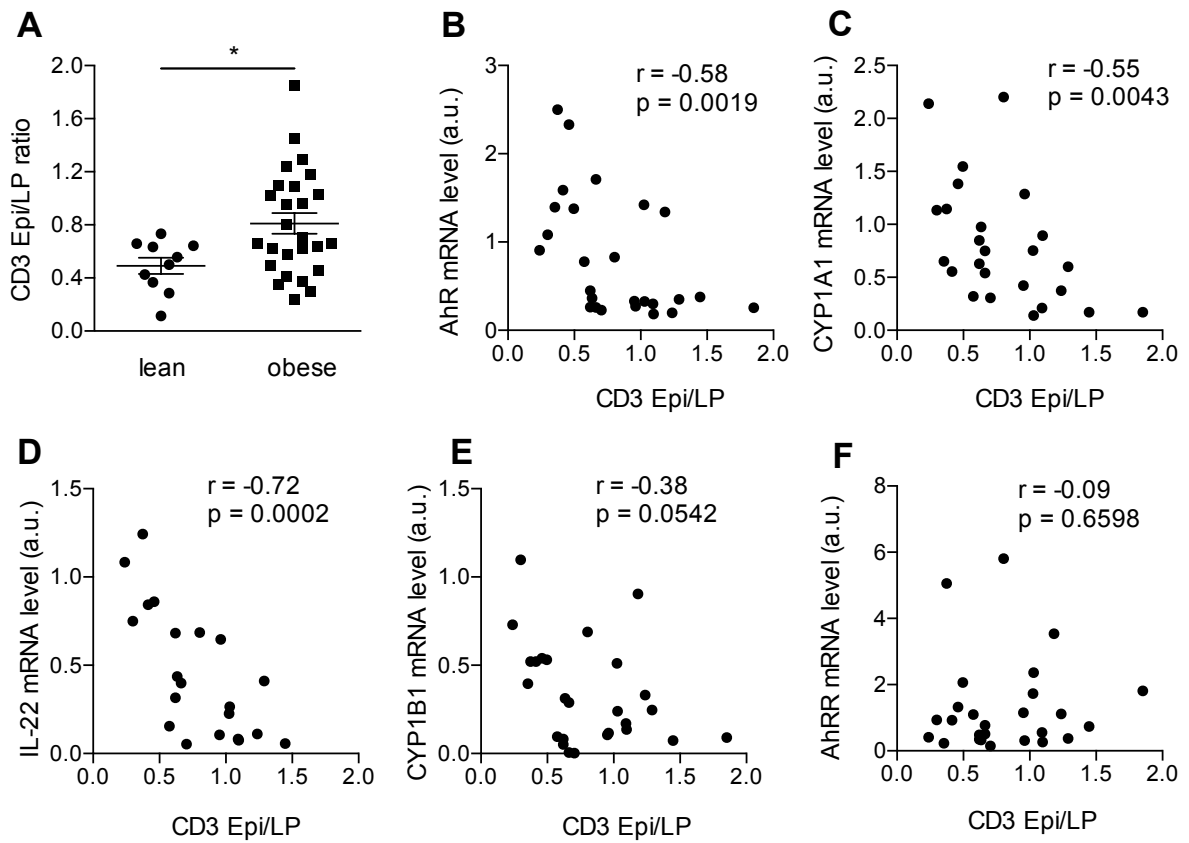


Figure 1

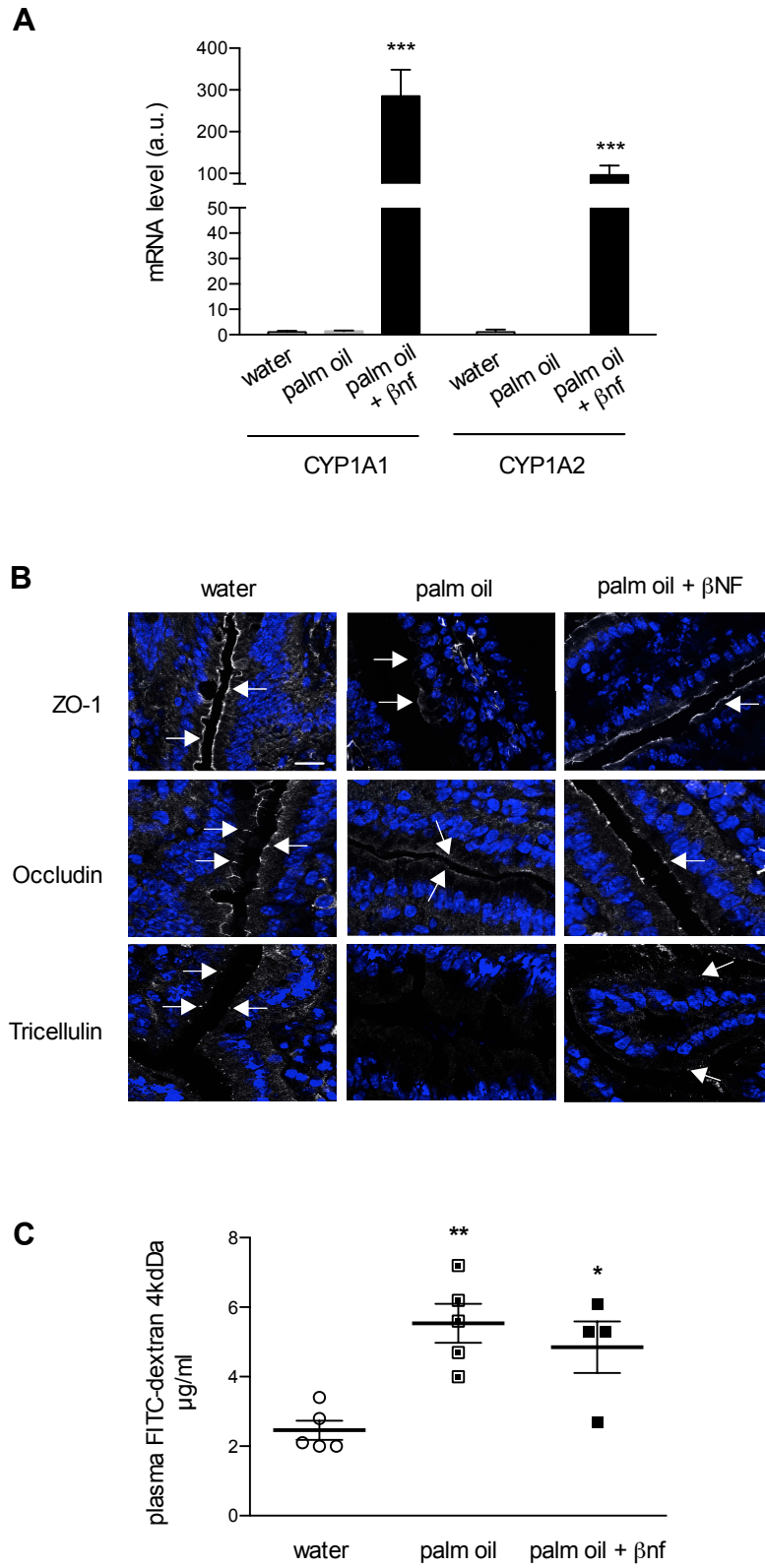


Figure 2

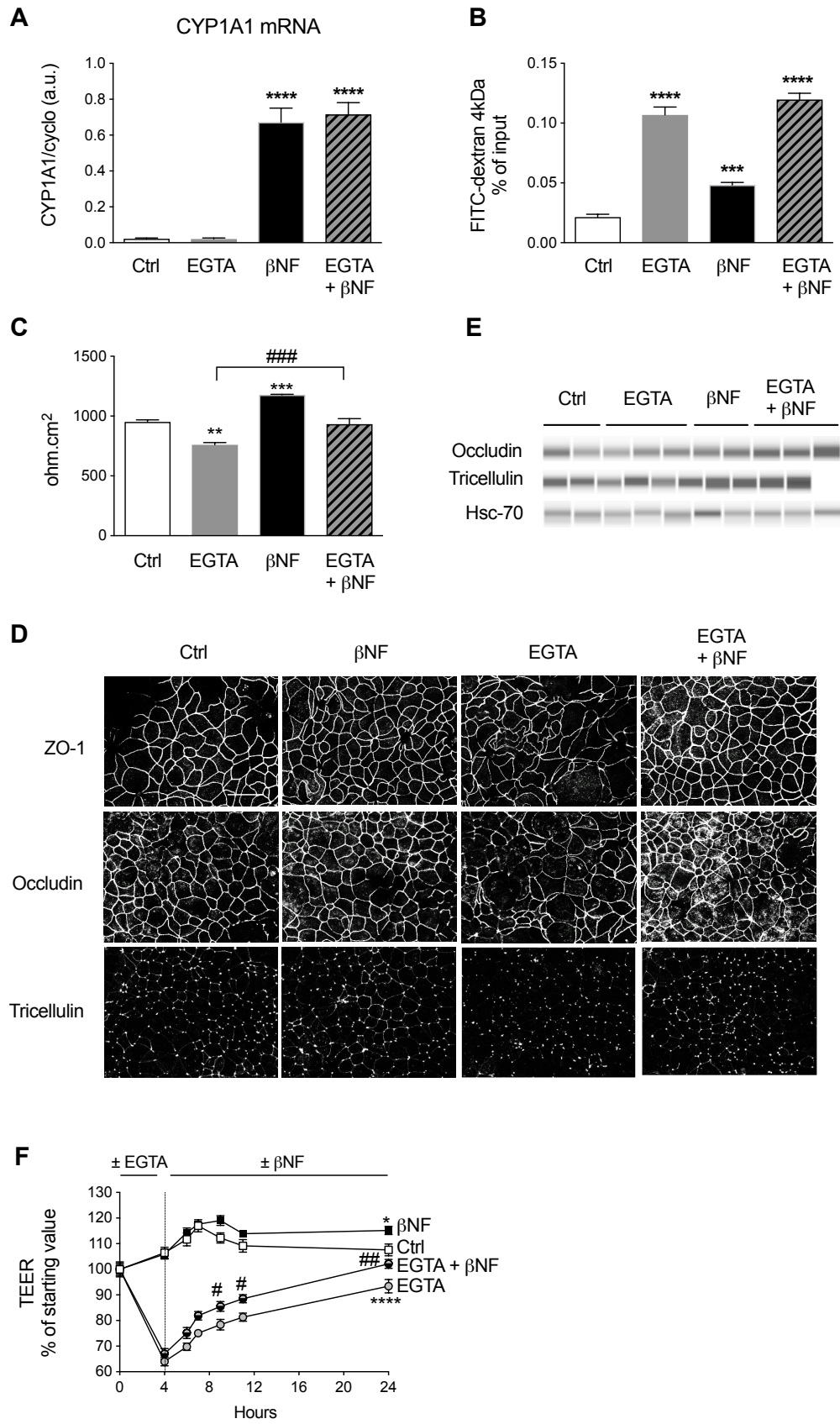


Figure 3

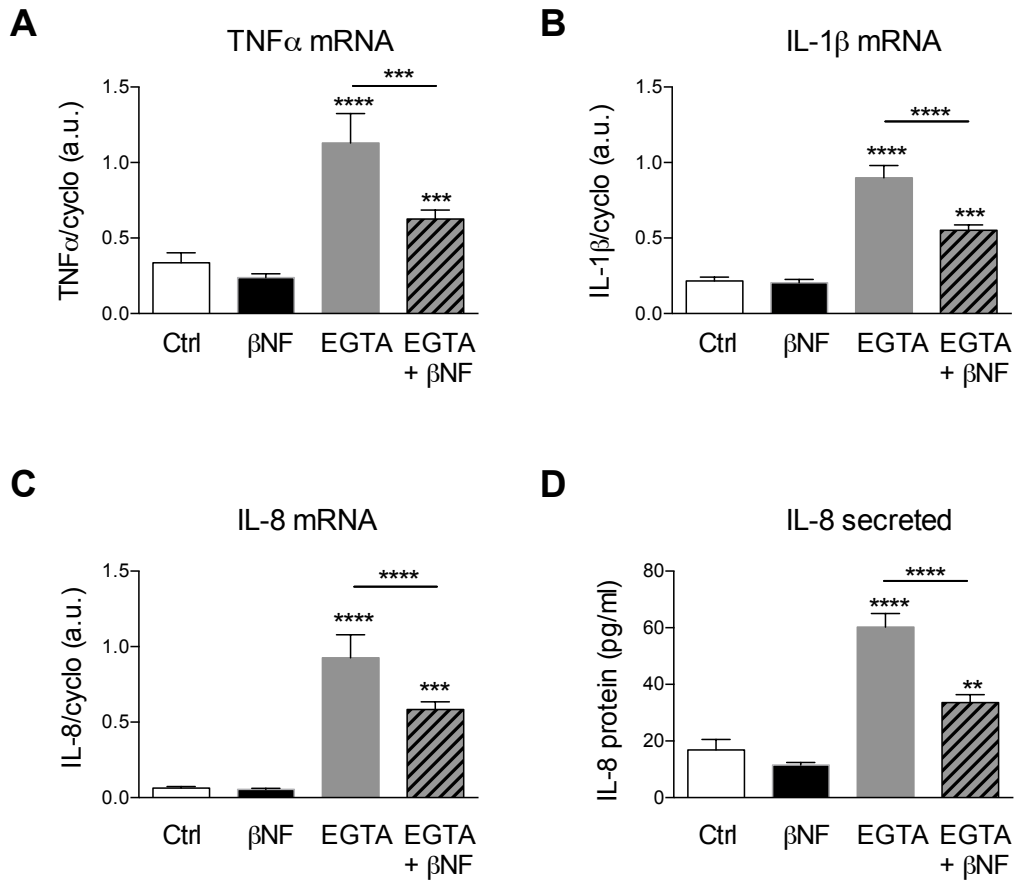


Figure 4

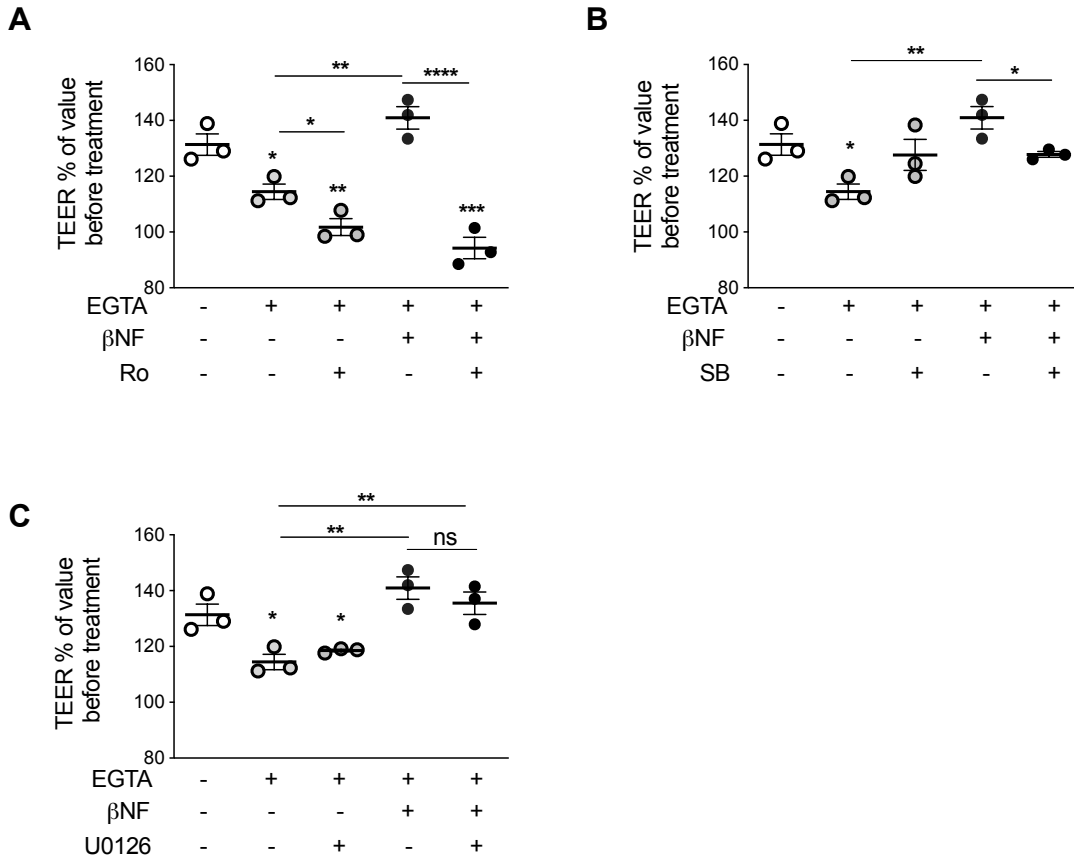


Figure 5

Supplementary results

During my thesis, I focus on the molecular mechanisms by which AhR is implicated in the maintenance of intestinal barrier integrity. However, I also analyzed in the human intestine of obese patients, the activity of another transcription factor the nuclear farnesoid X receptor (FXR) known to trigger an immune response and barrier dysfunction to environmental changes in the intestine (Gadaleta *et al.*, 2011). This transcription factor is especially important in the intestine because it is involved in the entero-hepatic cycling of bile acids, which are important substrates for microbiota metabolism. It is also involved in intestinal homeostasis and displays intestinal-specific target genes as FGF19.

FXR is a nuclear hormone receptor mainly involved in bile acid, lipid and glucose metabolism as well as in inflammation. Bile acids are the major FXR endogenous ligands. They are produced in hepatocytes, stored in the gallbladder, and released into the duodenum upon ingestion of a meal to facilitate absorption of nutrients. In intestinal lumen, bile acids are actively transformed by microbiota controlling their re-absorption in the ileum. The bile acids in the gut lumen influence microbiota composition and reciprocally, changes in microbiota composition modify bile acid transformation and metabolism (Chavez-Talavera *et al.*, 2017). Conflicting results are showing that either an FXR activation or an inhibition can occur in metabolic and gut inflammatory diseases (Stojancevic *et al.*, 2012; Jiang *et al.*, 2015; Verbeke *et al.*, 2015; Gonzalez *et al.*, 2016). Indeed, it was observed that FXR activation improved inflammation in chemically-induced colitis in mice (Vavassori *et al.*, 2009; Gadaleta *et al.*, 2011). In contrast, FXR-deficient mice displayed intestinal barrier defect (Inagaki *et al.*, 2005; Blacher *et al.*, 2017). Focusing on human obesity, a positive correlation between BMI and the expression of FXR or target genes in ileum samples was reported (Jiang *et al.*, 2015). However, the secretion of the intestine-specific FXR target gene FGF19 in the blood is decreased in obese patients (Gallego-Escuredo *et al.*, 2015; Gomez-Ambrosi *et al.*, 2017). In this context, the FXR tone in the jejunum of obese patients was assessed according to their intestinal inflammatory score.

I used the same non-diabetic obese subjects as for the AhR study. I correlated their jejunum inflammation score, (established by the ratio T cells CD3 Epi/LP) with the mRNA levels of FXR and its target genes (SHP, IBABP, ASBT, OST alpha and beta, MRP3) in jejunum epithelium fraction. Spearman correlations showed that the jejunum of obese patients with high recruitment of T lymphocytes display a lower FXR tone defined by lower RNA

levels of FXR itself and of six target genes (Figure 20). This result suggests that when non-diabetic obese patients display an intestinal inflammation it is accompanied by an impaired FXR activation, as shown earlier for AhR tone.

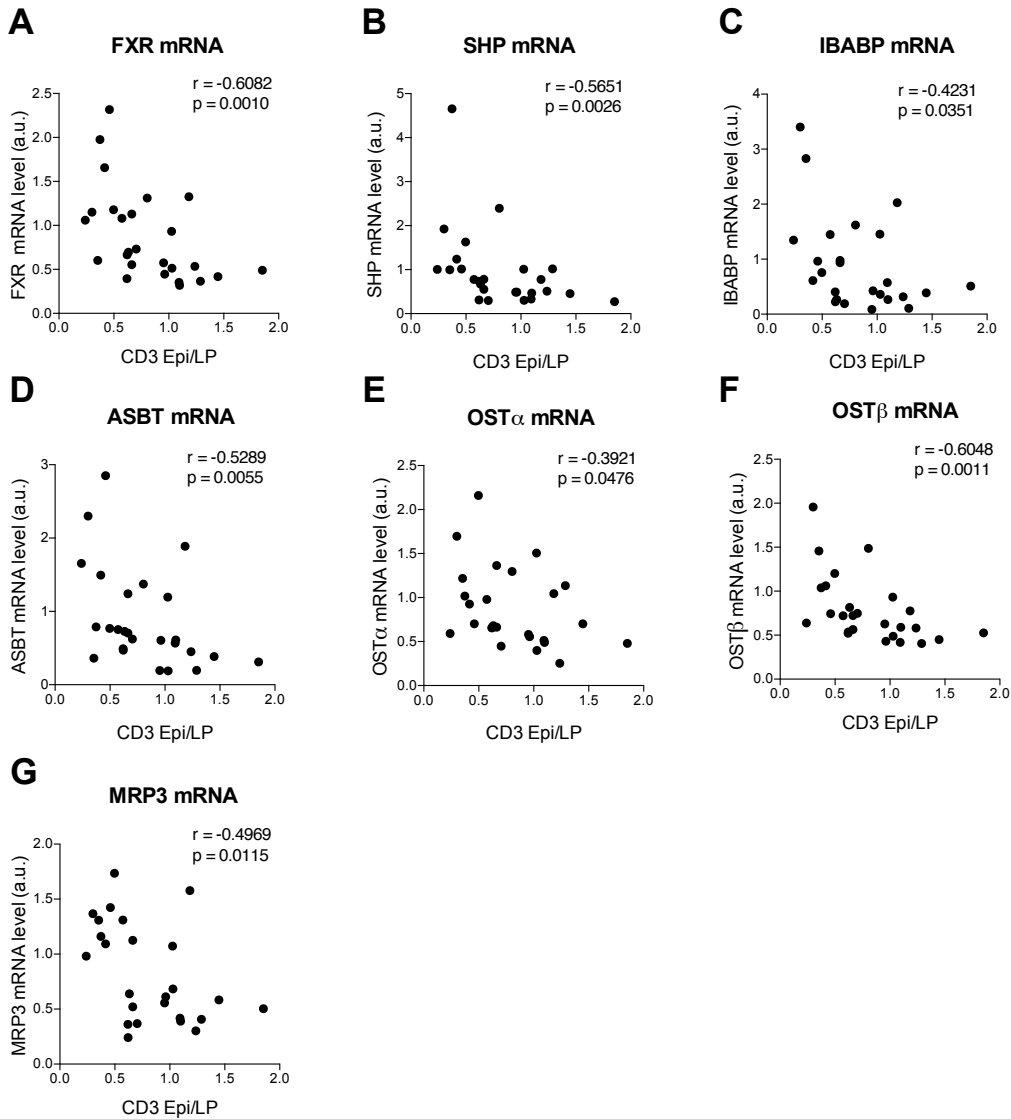


Figure 20: Low FXR tone in jejunal epithelium from obese subjects with intestinal inflammation

(A-G) Spearman correlations of FXR (A), SHP (B), IBABP (C), ASBT (D), OST α (E), OST β (F) and MRP3 (G) mRNA levels in intestinal epithelium and CD3 Epi/LP ratio in obese subjects. FXR: Farnesoid X Receptor, SHP: small heterodimer partner, IBABP: ileal bile acid-binding protein, ASBT: sodium-dependent bile acid transporter, OST α/β : organic solute transporter α/β , MRP3: multidrug resistance protein 3, a.u.: arbitrary units. Spearman r and p value are indicated.

To have a more complete analysis of AhR and FXR tone in human jejunum of obese subjects I include, below, complementary data to those presented as Spearman correlations. First, I arbitrary separated obese subjects in two groups according to the value of CD3 Epi/LP ratio, the cut-off value chosen was 0.8. The CD3^{Low} group of obese displays a similar CD3 ratio as lean group (0.51 ± 0.04 and 0.50 ± 0.06 respectively), while the CD3^{High} obese group displays a significantly higher ratio (1.16 ± 0.08) than obese CD3^{Low} and lean subjects (Figure 21).

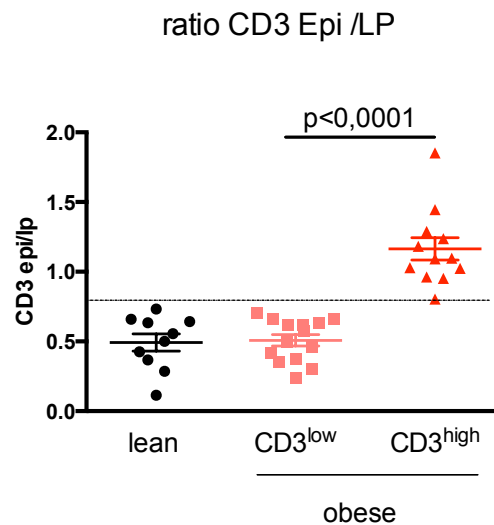


Figure 21: Lymphocyte T density in jejunum of lean and obese subjects.

CD3⁺ T cell density (cell/mm²) in epithelium/lamina propria ratio (Epi/LP) was determined by immunohistochemistry in jejunum of non-obese (n =10) and obese subjects (n = 26). Obese subjects were separated in two groups according to the CD3 ratio value, CD3^{Low} contains subjects with a CD3 ratio < 0.8, and CD3^{High} group contains subjects with a CD3 ratio > 0.8. Dashed line represents the cut-off line at value 0.8. Results are expressed as mean ± SEM.

Besides the quantification of AhR and FXR target genes mRNA levels in the epithelium fraction of jejunum human samples, I also quantified their expression in the lamina propria fraction prepared as previously described (Monteiro-Sepulveda *et al.*, 2015). The Table 2 below reports the statistical analysis of variation of mRNA levels of AhR, FXR and their target genes in obese subjects with gut inflammation (CD3^{High} group) compared to lean and to CD3^{Low} obese group in both epithelium and lamina propria fractions of jejunum. No modification of the expression of AhR and FXR target genes were observed between CD3^{Low} obese group and lean group. Only obese subjects with intestinal inflammation, CD3^{High} group, presented a decreased expression of AhR and FXR tone in human jejunum.

RESULTS

		Epithelium		Lamina Propria	
		Obese CD3 ^{High}		Obese CD3 ^{High}	
		vs. Lean	vs. Obese CD3 ^{Low}	vs. Lean	vs. Obese CD3 ^{Low}
FXR Target Genes	IBABP				*
	OSTa			*	
	OSTb	*	*	*	
	SHP			**	
	ASBT, IBAT		*	*	
	MRP3			p=0.076	
	FXR	*	*		
AhR Target Genes	CYP1B1	*			
	CYP1A1				
	IL22	p=0.079	**		p=0.086
	AHR		*		
	AHRR				

Decrease of the expression (mRNA)		
p < 0.01 **	p < 0.05 *	Tendency

Table 2: Variation of mRNA levels of AhR, FXR and their target genes in epithelium and lamina propria from jejunum of lean, CD3^{low} and CD3^{high} obese groups.

DISCUSSION

General Discussion

In 2005, Chris Wild introduced the innovative “exposome” concept (Wild, 2005). This concept considers all exposures to environmental factors that undergo a human body from its conception to its end of life that complete the effect of the genome. It aims to understand the response of the human body, or any other organism, to combined and cumulative exposures to chemicals and diseases. This concept has been enlarged, mentioning that the assessment of body exposures should not be restricted to exogenous chemicals coming from air, water, food, smoking, etc., but should also include endogenous toxicants produced by the gut flora, inflammation, oxidative stress, lipid peroxidation, infections, and other natural biological processes (Liory and Rappaport, 2011; Wild, 2012). Thus, this concept can easily be applied to the multifactorial processes involved in obesity since the exposure to environmental and endogenous factors could be implicated in obesity etiology.

Obesity is associated with both tissue and systemic inflammation and several intestinal dysfunctions are reported in humans and mice (Cotillard *et al.*, 2013; Everard *et al.*, 2013; Monteiro-Sepulveda *et al.*, 2015; Nehra *et al.*, 2016; Birchenough *et al.*, 2017; Genser *et al.*, 2018; Tseng and Wu 2018). However, the etiology of defects such as local intestinal barrier disruption and tissue inflammation observed in obese subjects remains unclear. Studies conducted in obese individuals or in obese animal models induced by a long-term high fat diet make it difficult to establish a causal relationship and by consequence the identification of the actors involved in the origin of these dysfunctions.

The main hypothesis of my thesis work was to consider that intestinal and systemic inflammation might originate from intestinal barrier defects and to envisage the implication of different environmental factors in the intestinal dysfunction. Based on these findings, I studied the contribution of dietary lipids, in particular, palmitic acid, and of the transcription factors AhR in the onset and the modulation intestinal barrier dysfunction and intestinal inflammation. We hypothesized, that all these events could participate in low-grade inflammation reported in obesity. I also analyze the FXR tone in the jejunum of human obese subjects. We choose to study AhR and FXR in the intestine for their importance in inflammation, their sensitivity to environmental changes and because some studies have shown that FGF21 and FGF19, target genes of AhR and FXR respectively (Cheng *et al.*,

2014), present opposite concentrations in the blood of obese patients. While FGF21 is increased, FGF19 is decreased in these patients (Zhang et al., 2008, Gomez-Ambrosi et al., 2017).

All these points are commented separately to facilitate the reading in the following discussion.

Dietary lipids involvement on the onset of intestinal inflammation and epithelial dysfunctions

As mentioned previously in the results part of my thesis, several studies conducted in healthy human and in rodents showed that one lipid meal is sufficient to provoke a transient endotoxemia and elevation of plasma level of pro-inflammatory cytokines (Cani *et al.*, 2007; Cani *et al.*, 2008); (Erridge *et al.*, 2007) (Laugerette *et al.*, 2011; Vors *et al.*, 2015) (Ghoshal *et al.*, 2009) suggesting that lipid meal-induced alteration of intestinal barrier. Moreover, in mice submitted to a long-term high-fat diet, this metabolic inflammation seems to be more severe when saturated fatty acids were present (Laugerette *et al.*, 2012); (Ravussin *et al.*, 2012). In human, our group has recently shown that defect of the intestinal barrier in intestinal permeability in obese subjects was revealed by a dietary lipid challenged and was linked with systemic and intestinal inflammation (Genser *et al.*, 2018). I thus determined whether a short-term supply of palmitic acid, a main saturated fatty present in the human diet, can trigger intestinal barrier alteration and induce intestinal and systemic inflammation.

In a mouse model, I demonstrated the existence of an intestinal barrier alteration from the first intake of palm oil. It is characterized by an increase in intestinal permeability and an alteration of the localization of some junction proteins. All deleterious effects were exacerbated after 5 palm oil gavages. Associated with intestinal epithelial barrier perturbation, we observed a modification in the expression of inflammatory genes in mouse intestine (IL-1 β , Reg-3 γ , Gata-3). Moreover, in Caco-2/TC7 cell model, a single treatment of palmitic acid also triggers an increase in epithelial paracellular permeability, an alteration on junctional proteins location at the cell membrane and an increase in the expression of the pro-inflammatory cytokine IL-8. These results suggest that alteration of the intestinal barrier integrity and the increase of pro-inflammatory cytokines in response to dietary lipids may represent early events in the onset of intestinal inflammation reported in obesity.

The intestinal inflammation that we observed *in vivo* remains moderate. We reported an increased IL-1 β expression and a decreased Gata-3 transcription factor. The Gata-3 protein

controls the Th-2 anti-inflammatory response by inducing the expression of anti-inflammatory cytokines (IL-4, IL-5 and IL-13) and repressing the expression of interferon- γ (IFN- γ) associated with the Th-1 pro-inflammatory response (Ho *et al.*, 2009; Seidelin *et al.*, 2015). Thus, the modification of IL-1 β and Gata-3 expression suggests a modification of immune profile in the intestine towards a pro-inflammatory profile.

Interestingly, an increase in Reg-3 γ expression, an antimicrobial peptide exhibiting a beneficial role in intestinal immunity and barrier function (Hogan *et al.*, 2006) is observed after one palm oil gavage but not in animals that received palm oil for 5 days. A previous report showed that Reg-3 β and Reg-3 γ mRNA levels were reduced in mice intestine after 10 and 30 days of HFD (Garidou *et al.*, 2015). These findings suggest that after one lipid challenge, the intestine rapidly engages defense mechanisms but that the repetition of palm oil gavage decreased the efficiency of intestinal defense response.

The low intestinal inflammation observed in our experiments is consistent with the observations of Johnson *et al.* (Johnson *et al.*, 2015) where limited alteration of the intestinal immune system is observed after several weeks of a high-fat diet in mice. Intestinal inflammation in diet-induced obesity differs from the marked inflammation observed in other tissues such as the liver and adipose tissue (van der Heijden *et al.*, 2015). Several mechanisms could explain the low gut inflammation, the rapid renewal of the intestinal epithelium or the efficient mechanisms of gut defense.

Four days of palm oil gavage changes the composition of the microbiota as did several weeks of high-fat diet (Clarke *et al.*, 2012; Matsuoka and Kanai 2015). Our data showed that palm oil gavages induced an increase in the abundance of *Bacteroides*, which are known to be increased following an obesogenic diet enriched in saturated fatty acids (Devkota *et al.*, 2012), and a decrease in bacterial species such as *Clostridium leptum*, *Akkermensia muciniphila* and Segmented Filamentous Bacteria (SFB), described to play a protective role in the intestinal host cells (Everard *et al.*, 2013; Marchix *et al.*, 2018). This fast switch of microbiota composition was also demonstrated in healthy humans after a dietary change (David *et al.*, 2014) and in rats fed a diet rich in lipids (Vaughn *et al.*, 2017). Also, obese subjects undergo a by-pass surgery present rapidly changes in their microbiota in a starvation-like situation induced by RYGB (Furet *et al.*, 2010). Since the microbiota have an important role on intestinal barrier integrity, such as shaping the intestinal immune system by contributing to immune system development and maturation (Rolhion and Chassaing 2016),

maintain of microvilli formation patterns and cell renewal (Yu *et al.*, 2012; Takiishi *et al.*, 2017), changes in its composition observed after 4 gavages can also contribute to the alteration of the intestinal barrier and/or intestinal inflammation.

I investigated whether intestinal barrier dysfunctions and intestinal inflammation in response to a short-term palm oil treatment can be associated with systemic inflammation. The plasma IL-6 and IL-1 β levels show no difference between animals gavaged with palm oil or with water. This result suggests that palm oil gavages for 4 days are not enough to trigger systemic inflammation. Meanwhile, our data could also indicate that intestinal epithelial barrier disruption and intestinal inflammation may precede the systemic inflammation induced by fat diet. In literature, studies on the effect of diets on endotoxemia and systemic inflammation are still contradictories. While some studies observed that a single high-fat meal is sufficient to increase endotoxemia and pro-inflammatory cytokines in healthy humans (Laugerette *et al.*, 2011; Vors *et al.*, 2015) and animals (Ghoshal *et al.*, 2009). Other studies performed in mice, showed the activation of signaling pathways involved in cytokine expression after 2 weeks of high-fat diet and increased intestinal expression of TNF- α after the 6th week (Ding and Lund 2011). However, this increased intestinal immune activation did not trigger a systemic inflammation (Ding and Lund 2011).

Altogether our experiments in mice showed that short-term supply of palm oil alters intestinal barrier, intestinal immune response and microbiota composition. As for Human studies, causal relationships are difficult to establish. We thus used a cellular model of human enterocytes, the Caco-2/TC7 cell line to disconnect further the effects of short-term palmitic acid on intestinal barrier and cytokine expression without the interference of other factors or cell types such as microbiota and immune cells. Studies in a cell culture model render possible the analyses of molecular mechanisms involved.

In Caco-2/TC7 cells, we observed that 24h treatment with palmitic is sufficient to alter intestinal permeability, to perturb the localization of junction proteins and to modify the expression of IL-8 and TGFB1 cytokines. Repeated palmitic acid supply exacerbated these effects. These events are not observed in the presence of a monounsaturated fatty acid, oleic acid, suggesting that they are fatty acid nature dependent. IL-8 is a proinflammatory chemokine involved in the recruitment and activation of immune cells, in particular, neutrophils (David *et al.*, 2016). This cytokine participates in the host cells defense against

pathogenic bacteria invasion (Wera *et al.*, 2016). Interestingly, IL-8 is elevated in the serum of obese patients compared to non-obese patients and is positively correlated with BMI and fat mass (Strackowski *et al.*, 2002; Kim *et al.*, 2006).

I then analyzed by which mechanisms palmitic acid could alter the intestinal barrier.

My first hypothesis is to consider that palmitic acid induces specific cellular events leading to alteration of the intestinal barrier. A recent study published by our team, in which I participated, showed that a single intake of palmitic acid, but not oleic acid, triggers the *de novo* synthesis of ceramides (Tran *et al.*, 2016). The ceramides are known for their deleterious effects in the cell and our work demonstrated that ceramides produced in response to this acute intake of palmitic acid, disrupt the insulin signaling in Caco-2/TC7 cells and in the intestine of mice after a single palm oil gavage (Tran *et al.*, 2016). Moreover, a recent study has highlighted the importance of ceramide levels, particularly C-16 ceramide in intestinal barrier defects (Kim *et al.*, 2017). Determining and assaying the species of ceramides produced by palmitic acid and comparing them with those produced by the addition of C2-ceramide in the cells would make it possible to identify the species that is mainly involved in the deleterious effects induced by palmitic acid.

Based on this study and others, we wondered whether the ceramides could be involved in the disturbances of intestinal barrier integrity caused by palmitic acid. We observed that inhibition of *de novo* synthesis of ceramides prevented the increase of IL-8 expression but not paracellular permeability in cells incubated with palmitic acid. This discrepancy could be explained by the fact that the inhibition of *de novo* synthesis of ceramides was not totally effective. A residual production of ceramides could be sufficient to alter paracellular permeability through their properties to form channels on membranes (Siskind *et al.*, 2002; Siskind 2005) and thus maintaining elevated paracellular flux through these artificial pores. To test these hypotheses, we could compare the amount of ceramides produced by palmitic acid with and without the inhibitor and to performed immunofluorescence staining of ceramides in the membrane to detect the presence of channels in treated Caco-2/TC7 cells. Moreover, ceramides are known to be located in rafts domains of the membrane, where tight junctions proteins are localized (Eum *et al.*, 2015; Head *et al.*, 2014). Several works showed that ceramides may affect the stability of rafts membrane domains and their composition of protein (Yu *et al.*, 2005; Megha *et al.*, 2007). Thus, it is possible that ceramides by destabilizing rafts domains, alter junctional complexes and increase paracellular permeability.

Another hypothesis is to consider that the cytokines secreted by intestinal epithelial cells could alter directly cell-cell junctions. Indeed several studies have elucidated the role of pro-inflammatory cytokines on the intestinal epithelial barrier dysfunctions (Ma *et al.*, 2004; Al-Sadi *et al.*, 2010; Al-Sadi *et al.*, 2012; Lee 2015). Beyond the described effects of cytokines such as TNF- α , INF- γ or IL-1 β (Al-Sadi *et al.*, 2008; Al-Sadi *et al.*, 2012; Kominsky *et al.*, 2014; Al-Sadi *et al.*, 2016), deleterious and dose-dependent effects of IL-8 on tight junction proteins, have been also reported on endothelial permeability (Yu *et al.*, 2013). However, the underlying mechanisms driving this IL-8 effect remains unclear. Since IL-8 expression and secretion is increased here, after short-term palmitic acid treatment, we presume that this cytokine could participate in epithelial barrier homeostasis. Other studies have shown that TGF- β 1 is implicated on tight junctions' homeostasis. In response to TGF- β 1, proteins of the SMAD (Small Mothers Against Decapentaplegic) family are able to reduce the expression of genes encoding junction proteins such as E-cadherin and occludin (Lamouille *et al.*, 2014). In Caco-2 cells, one of the TGF- β 1 receptors, TGF- β RI, co-localizes with occludin (Yakovich *et al.*, 2010), suggesting a possible interaction between TGF- β 1 and occludin. Thus, IL-8 and TGF- β 1 cytokines can be involved in epithelial barrier alteration. It would be interesting to analyze the signaling pathways controlled by these cytokines by blocking their action, by invalidating their expression or by blocking the binding on their receptor (via blocking antibodies).

Another interesting point is the specificity of the effects of palmitic acid versus the absence of effect of oleic acid after 24 hours of treatment. However, our team showed increased cell permeability to macromolecules and decreased the tricellulin intensity in Caco-2/TC7 cells 4 hours after oleic acid (Genser *et al.*, 2018). This indicates that oleic acid, as probably other fatty acids, may exert transient effects on the intestinal barrier. This may be explained by the differences in the metabolism of these 2 fatty acids, oleic acid being rapidly used for triglycerides secretion and secreted as lipoproteins in a more efficient manner than palmitic acid. Moreover, oleic acid did not increase intracellular ceramides levels either the induction of pro-inflammatory cytokines, which could explain that the long-term treatment with oleic acid does not cause alteration of the epithelial barrier. It could suggest also that intestinal epithelial cells have the capacity to cope with a massive supply of lipids by engaging host defense but a lipid overload according to the nature of the fatty acid may exceed this host defense.

AhR and FXR in intestinal inflammation in human obesity

According to the objectives of my thesis, I explored the relationship between intestinal inflammation and AhR and FXR activities in obese subjects. I observed negative correlations between the expression of AhR and FXR target genes in one hand and jejunum inflammation score (T lymphocyte recruitment) in the other hand. Thus, intestinal inflammation in obese subjects is correlated with a low activity of these 2 transcription factors. The main challenge is now to determine whether the low activity of AhR and FXR in the intestine is a consequence of obesity, linked to associated modifications of host metabolism or microbiota composition or whether AhR and FXR activity may trigger gut inflammation by direct control of inflammatory processes or intestinal barrier integrity.

My results concerning the low AhR tone in human jejunum of obese subjects with intestinal inflammation can be put in regard with the recent observation of Natividad et al showing that obese subjects with metabolic disease present low levels of endogenous AhR ligands in feces (Natividad *et al.*, 2018). Although in Natividad et al, the presence of gut inflammation was not studied, the low intestinal AhR activity was correlated with high BMI, type-2 diabetes and high blood pressure. The same authors observed also low fecal levels of AhR ligands in the high-fat-induced model of obesity in mice and established a link with changes in gut microbiota composition. Interestingly, the supplementation of mice with *Lactobacillus* strains, which display a high natural capacity to produce AhR ligands, improved the metabolic impairments induced by the high-fat diet through mechanisms related to intestinal barrier integrity and production of the incretin hormone GLP-1 (Natividad *et al.*, 2018). Besides, some studies have highlighted the important role of AhR in lipid homeostasis (La Merrill *et al.*, 2013). AhR seems to be activated by LDL, and AhR-knockout mice have higher levels of serum LDL (McMillan and Bradfield 2007). Altogether these data reinforce the potential important role of AhR in obesity and its metabolic complications.

Several reports addressed the importance of FXR in obesity however as for AhR, conflicted results were obtained (De Magalhaes Filho *et al.*, 2017). It has been observed that a FXR deficiency protected mice against diet-induced obesity (Prawitt *et al.*, 2011). Tissue-specific invalidation of FXR in mice revealed that intestinal FXR is required for the development of diet-induced obesity and its metabolic complications (insulin resistance, non-alcoholic fatty liver disease). However, fexaramine a potent FXR agonist acting specifically

in the intestine, exerts an anti-obesity effect in high-fat-fed mice (Fang *et al.*, 2015). These controversial findings of the role of FXR on obesity prove the complexity of this transcription factor and the mechanisms triggered by their activation and also how complex is this disease, which displays a lot of metabolic disturbances in different organs including the intestine. FXR may be also involved in intestinal inflammation during obesity through its role in the modulation of immune response and barrier function (Vavassori *et al.*, 2009). Indeed, bile acids, the natural FXR ligands have been described to regulate many different aspects of intestinal physiology, such as cell survival and death, fluid secretion and production of mucus and epithelial barrier function, tight junction integrity, and proinflammatory cytokines production (Barcelo *et al.*, 2001; Dossa *et al.*, 2015) (Maran *et al.*, 2009). Moreover, a study has associated the increase of luminal bile acids to a deterioration of intestinal permeability *in vivo* (Stenman *et al.*, 2012). On the other hand, the immune cell modulation by the FXR signaling pathway could improve intestinal inflammation (Gadaleta *et al.*, 2011). FXR itself and its natural ligands bile acids appear to play an important role in the modulation of inflammatory responses and barrier function (Mroz *et al.*, 2018).

These findings evidence the difficulty to understand the causal relationship between transcription factor activities and the onset or the modulation of intestinal inflammation and low-grade inflammation reported in obesity.

AhR involvement in the intestinal inflammation and epithelial dysfunctions

Recently, studies emphasized the key role of AhR in the maintenance of intestinal homeostasis, by acting on the proliferation of stem cells in the colon crypt, the formation of lymphoid tissue and the prevention of bacterial overload (Stockinger *et al.*, 2014). This role was essentially described in colitis animal models or in human suffering of intestinal bowel disease (Takamura *et al.*, 2010; Furumatsu *et al.*, 2011; Monteleone *et al.*, 2011; Kiesler *et al.*, 2015; Goettel *et al.*, 2016; Lamas *et al.*, 2016; Lanis *et al.*, 2017; Lamas *et al.*, 2018). In the intestinal immune system, AhR regulates the function and development of innate lymphoid cells and also the differentiation and function of the intestinal lymphocytes (Cervantes-Barragan and Colonna 2018), especially those secreting IL-22, a cytokine responsible for maintaining the intestinal barrier function and expression of REG3, an antimicrobial gene (Kiss *et al.*, 2011; Lee *et al.*, 2011; Gulhane *et al.*, 2016; Wang *et al.*,

2017). AhR knock-out mice displayed a decreased expression of IL-22 and REG3 in the intestine. When these animals were exposed to a bacterial infection, they developed more serious colitis than wild-type animals (Stockinger *et al.*, 2014). Although IL-22 is characterized by several studies' relevance in IBD, about its ability to induce mucosal healing, further works still need to fully understand the mechanism that determines its beneficial effect on colitis. In this context, AhR activation as a stimulator of IL-22 expression would be considered a promising therapeutic target for IBD.

In our experimental conditions, we did not detect IL-22 in mouse intestine submitted to repeated gavage with palm oil, however, in obese subjects presenting an intestinal inflammation, we observed that the mRNA level of IL-22 in jejunum epithelium is decreased and negatively correlated to the recruitment of T lymphocytes into the epithelium. It is known that IL-22 exerts diverse metabolic benefits, as it improves insulin sensitivity, preserves gut mucosal barrier and endocrine functions, decreases endotoxemia and chronic inflammation, and regulates lipid metabolism in liver and adipose tissue (Dalmas and Donath 2014; Wang *et al.*, 2014; Sabat and Wolk 2015). Besides, IL22 is an important cytokine in wound healing through stimulating survival of intestinal stem cells and enhancing anti-bacterial peptide production from epithelial cells (Rubino *et al.*, 2012; Aparicio-Domingo *et al.*, 2015). IL-22 expression can be stimulated by AhR activation (Alam *et al.*, 2010; Lamas *et al.*, 2016; Lamas *et al.*, 2016), suggesting that IL-22 is an AhR stimulated gene. Thus, in my study in obese subjects, the decreased expression of AhR itself or, by consequence, the decreased expression of IL-22 could be involved in the intestinal inflammation reported in obesity.

In vivo in mice, I observed that the short-term treatment with the AhR agonist was not sufficient to prevent the increased intestinal permeability caused by palm oil and did not allow paracellular permeability to reach normal value even though a clear beneficial effect on junctional complexes can be observed. The protective role of AhR on cell-cell junction integrity is supported by other studies, especially in mouse models of colitis, where the expression of tight junction proteins and paracellular permeability were significantly decreased but restored after the activation of AhR by its agonist, FICZ (Park *et al.*, 2015; Park *et al.*, 2015; Yu *et al.*, 2018). The same protective role of AhR on junctional proteins and paracellular permeability was observed in Caco-2 cells treated with the pro-inflammatory cytokines TNF- α and IFN- γ (Natividad *et al.*, 2018; Yu *et al.*, 2018). Moreover, in my study

in mice, short term AhR activation was not able to mitigate the inflammatory profile disturbance on mice treated with palm oil. Thus, our data *in vivo* suggest that AhR activation could act directly on junctional complexes but a more sustained activation might be necessary to improve the intestinal barrier dysfunction. A specific-dependent effect of the different AhR agonists used can also be responsible for some discrepancy between my results and the results described above. FICZ and β NF are distinct AhR ligands and their triggering intracellular signaling pathways remains to be explored. Moreover, emerging data suggest the importance of concentration of AhR agonist on AhR function. It is suggested that depending of AhR ligands/agonists dose, no beneficial or deleterious effects could occur (Esser *et al.*, 2018).

The mechanisms involved in epithelial barrier improvement modulated by AhR

I used the human intestinal epithelial cell line, Caco-2/TC7 cells to study the role of the AhR on the intestinal epithelium and the molecular mechanisms involved without the interference of microbiota, immune cell regulation, etc.

I showed that AhR activation prevented chemical barrier damage induced by both exposure of Caco-2/TC7 cells to lipid micelles containing palmitic acid, and upon chemical disruption of junctional protein complexes, the calcium chelator EGTA. Indeed, I demonstrated that AhR activation by its agonist β NF prevented the barrier disruption triggered by palmitic acid and EGTA. While β NF was not able to counteract the inflammatory response to palmitic acid, it diminished the expression and the secretion of cytokines induced by EGTA treatment. My results are showing a protective role of AhR on intestinal barrier dysfunction via an action on junctional complexes.

Recent studies emerged on the protective effect of the AhR agonist FICZ on barrier function, especially on colitis models. However, the mechanisms driving its beneficial effect remain not well understood. Some papers showed that AhR protective effect acts through tight junctions regulation, mainly occludin and ZO-1 (Han *et al.*, 2016; Liu *et al.*, 2018; Yu *et al.*, 2018) and implicates mainly the myosin light chain (MLC) kinase (MLCK) pathway (Han *et al.*, 2016; Yu *et al.*, 2018). The MLCK-dependent MLC phosphorylation is an important regulatory point involved in the maintenance of tight junction (Cunningham and Turner 2012). In our experiments, MLCK pathways do not appear to be implicated in the protective effects of β NF, since the expression of MLCK and the phosphorylation of MLC were not

modified under our experimental conditions (data not shown), so the beneficial effect of AhR activation on chemically-induced tight junctions disassembly involves other pathways.

Other protein kinases and phosphatases activities have been involved in junctional proteins disruption. In particular, it is known that occludin dephosphorylation on threonine residues without affecting the serine-phosphorylation accompanied a rapid disassembly of tight junctions in Caco-2 cell monolayers caused by EGTA-induced calcium depletion (Seth *et al.*, 2007). Thus, I investigated the links between AhR activation and protein kinases pathways involved in the control of junction proteins. We observed that the beneficial effect of AhR activation on paracellular permeability to ions was abolished when we blocked the PKC and p38MAPK phosphorylation pathway but not the MEK dependent pathway. It has been observed that PKC is a member of a multiprotein complex involved in the maintenance of cell polarity acting on the tight junction proteins ZO-1 and occludin (Schuhmacher *et al.*, 2019). Interestingly, another member of this complex is the protein Par-6, which has been recently involved in the protective effects of AhR activation on the intestinal epithelial barrier in response to lipopolysaccharide (Yu *et al.*, 2018). It is now important to measure *in situ* interaction of junction proteins with their partners inside junctional complexes using *in situ* proximity ligation assay experiments. Indeed it has been shown that threonine phosphorylation on T770/T772 residues in the GuK (guanylate kinase) domain of ZO-1 by PKC ϵ disrupts its interaction with occludin (Chattopadhyay *et al.*, 2014). Moreover, the determination of phosphorylation status of tight junction proteins using antibodies against serine, threonine or tyrosine phosphorylated residues could also support our hypothesis.

The expression of the total and phosphorylated form of Src and JNK kinases was analyzed here (data not shown) and no difference in protein expression was observed neither upon AhR activation or chemical barrier damage. However, changes in the phosphorylation status of a protein are highly dynamic and reversible and might be difficult to catch experimentally.

Interestingly, we observed that chemically induced barrier damage (EGTA treated cells) is accompanied by an increased pro-inflammatory cytokines expression and secretion that was counteracted by AhR activation. NF- κ B pathway is an important regulator involved in the expression and secretion of cytokines but also in the effect of cytokines on intestinal epithelial barrier (Ma *et al.*, 2004; Al-Sadi *et al.*, 2008; Al-Sadi *et al.*, 2010; Al-Sadi *et al.*, 2012; Kominsky *et al.*, 2014; Lee 2015; Al-Sadi *et al.*, 2016). I observed that barrier damage

by EGTA did not increase the NF- κ B activation (results not shown), suggesting that the NF- κ B pathway was not involved in this process. Others kinases such as ERK and p38MAPK regulates IL-8 expression (Jijon *et al.*, 2002) or autophagy regulates Il-1 β and TNF α expression (Liu *et al.*, 2017). Cytokine expression can also be controlled by posttranscriptional mechanisms *via* many cis-acting sequence elements and transcription factors (Kovarik *et al.*, 2017). Further experiments are required to fully decipher the mechanisms involved in the protective effects of AhR on intestinal barrier.

Our findings reinforce the link between obesity and low-grade intestinal inflammation to environmental factors such as diet, microbiota and the activation of transcription factors expressed in the intestine. Furthermore, our data suggest the direct implication of all these factors, especially the palmitic acid and AhR activation, in the intestinal barrier integrity. Henceforth, it needed to go further to investigate the molecular mechanisms involved in the activation of AhR and its reduced activity in obese subjects, jointly with FXR, to better understand their specific role on gut inflammation and intestinal epithelium damage in obesity.

CONCLUSION

Conclusion

In my thesis work, I studied the participation of two actors (palmitic acid and AhR) related to environmental changes in the development of intestinal inflammation reported in obesity. My starting hypothesis was to consider that intestinal inflammation is linked to intestinal barrier perturbation and can imply mechanisms occurring before the onset of obesity. My studies conducted in enterocytic cell line help me to identify the mechanisms that occur in the intestinal epithelium independently of microbiota or immune cells influence.

Firstly, my work shows that obese subjects presenting with gut inflammation in their jejunal mucosa, display a decreased epithelial AhR and FXR tone, suggesting that these 2 transcription factors exert a potential role in epithelial intestinal dysfunction, as reported in intestinal bowel diseases in human (Lamas *et al.*, 2016).

Moreover, I showed that the short-term palm oil intake in mice is capable to trigger an early disruption on the intestinal barrier integrity, initiate an inflammatory response and modify the intestinal microbiota composition. Iterative short-term supply of palmitic acid is sufficient to induce persistent alterations in the enterocytic cell line, supporting the idea that the irreversibility of these effects deleterious, contribute to a vicious circle.

Further, my findings showed that the AhR activation by β NF improves the intestinal barrier dysfunction both *in vivo* and in intestinal epithelial cells. The mechanisms driving this protective effect of AhR involve here the tight junction proteins assembly. The AhR activation maintains the tight junction proteins at the membrane, preventing their loss of location triggered by nutrient or chemically induced barrier damage. The AhR protective action appears to be regulated by the phosphorylation of junctional proteins.

Take our findings together and regarding the mechanism involved, our results show that palmitic acid may initiate a vicious cycle starting at the gut barrier, leading to gut inflammation, that may contribute to systemic inflammation. I showed also AhR activation might improve the intestinal inflammation and epithelial damage, both epithelial dysfunctions present in obese subjects (Figure 22). Hence, the administration of AhR agonists might protect the intestine damage in obesity.

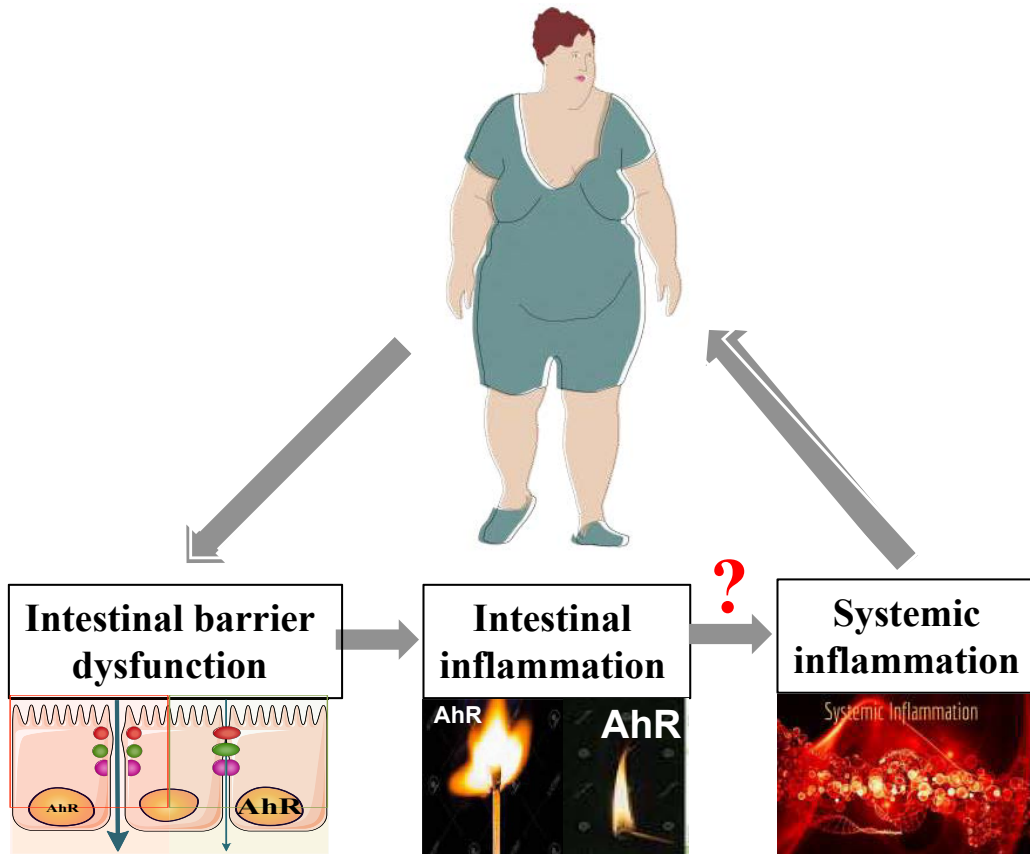


Figure 22: Gut - A nexus between inflammation and obesity?

PERSPECTIVES

Perspectives

The actors studied here can induce cross-talk between host cells, thus it would be now useful to deepen the sequence of events occurring *in vivo* taking into account the role of microbiota and immune cells on the gut barrier in physiological and pathological situations. The jejunum samples of well-phenotyped obese patients might help to further understand the tight junction protein status in the damaged gut.

To better characterize the AhR tone in obese subjects, it would be interesting to quantify the amount of AhR ligands/agonists in feces at different time after by-pass surgery to determine whether changes in AhR ligands amount (total or specific ligand) is correlated with improvement of metabolic parameters and if possible, with intestinal permeability.

To better characterize the AhR action on intestinal functions, it would be interesting to quantify: 1) the recruitment of immune cells of the intestine and the infiltration of immune cells from lamina propria to the epithelium using *in situ* markers such as T lymphocytes (CD3 T cell marker) (Monteiro-Sepulveda *et al.*, 2015), Macrophages (F4/80 marker) (Wagner *et al.*, 2018), and neutrophil (MPO marker) (Helke *et al.*, 2018); 2) the injury of the epithelium by the quantification in feces of lipocalin-2 (neutrophil marker) (Moschen *et al.*, 2017); 3) the protection by the thickness of mucus layer *via* histological staining with alcian blue, which stains the mucins and mucopolysaccharides (Rohe *et al.*, 2018); 4) the systemic consequences by using markers of neutrophil infiltration in plasma (MCP-1 marker) (Osaka *et al.*, 2016) or of acute inflammation (SAA-1, -2,-3) (Tannock *et al.*, 2018); 5) the apparition and maintenance of AhR protective effect by time course of intestinal barrier readouts and 6) better explore the role of AhR in the kinases pathways and tight junctions phosphorylation through the tight junctions immunoprecipitation followed by western blot analysis and the kinases activity.

Additionally FXR activation is supposed to participate to the improvement of intestinal barrier function (Gadaleta *et al.*, 2011; Stojancevic *et al.*, 2012; Verbeke *et al.*, 2015; Liu *et al.*, 2017). Obese subjects showed a decreased FXR target gene expression correlated with intestinal inflammation. Henceforth, to better clarify the role of FXR activation on intestinal barrier function, it is necessary to carry out *in vivo* and *in vitro* experiments. Since FXR is highly expressed in the intestine and activated by bile acids,

intestinal barrier function would be addressed in the basal state and after a lipid load. Constitutive activation of FXR via genetic models might be useful to complement the drug-induced FXR activation. The enterocytic cellular model will help to establish the relative importance of AhR and FXR on intestinal barrier.

A comprehensive view of the reduction of AhR and FXR tone in the intestine of obese subjects is not reached yet. Indeed, according to our findings, a reduction of AhR activity in inflammatory conditions would lead to intestinal epithelium damages and seems to present importance in metabolic diseases.

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ANNEXES

Annexes

1) Article as second author: Tran *et al.*, 2016

During my thesis, I participated in a study investigating in mice and in Caco-2/TC7 cells the mechanism involved in the insulin resistance of intestinal epithelial cells induced by palmitic acid (Tran *et al.*, 2016). In this study of which I am the second author, we showed that:

- Insulin was not able to exert its physiological action on intestinal lipid metabolism when mice have received one bolus of saturated fatty acid-rich palm oil rather than of mono-unsaturated fatty acid-rich olive oil.

- Insulin-signaling pathway, assessed by the phosphorylation of protein kinase B (AKT), is impaired under palmitic acid supply in mouse intestine and Caco-2/TC7 cells.

- Palmitic acid supply, contrary to oleic acid supply, triggered intracellular ceramide production

- The inhibition of palmitic-acid-dependent production of ceramides blocked part of the palmitic acid effects on AKT signaling pathways.

Altogether, this work demonstrates that a palmitic acid-ceramide pathway accounts for the impairment of intestinal insulin sensitivity occurring as soon as the first lipid supply.

Short Term Palmitate Supply Impairs Intestinal Insulin Signaling via Ceramide Production*

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Thi Thu Trang Tran^{†1}, Bárbara Graziela Postal^{‡2}, Sylvie Demignot[‡], Agnès Ribeiro[‡], Céline Osinski[‡], Jean-Paul Pais de Barros[§], Agnieszka Blachnio-Zabielska[¶], Armelle Leturque[‡], Monique Rousset[‡], Pascal Ferré^{||}, Eric Hajduch^{||3}, and Véronique Carrière^{‡3,4}

From the [†]Centre de Recherche des Cordeliers, INSERM, UPMC Univ Paris 06, Sorbonne Universités, Université Paris Descartes, Sorbonne Paris Cité, Ecole Pratique des Hautes Etudes (EPHE), Université Paris Sciences et Lettres, Université Paris Diderot, CNRS, Institute of Cardiometabolism and Nutrition, F-75006 Paris, France, [§]INSERM UMR866, Université de Bourgogne, F-21070 Dijon, France, [¶]Medical University of Białystok, P-15-089 Białystok, Poland, and ^{||}INSERM UMRS 1138, Sorbonne Universités, UPMC Univ Paris 06, Sorbonne Paris Cité, Université Paris Descartes, Université Paris Diderot, Institute of Cardiometabolism and Nutrition (ICAN), Centre de Recherche des Cordeliers, F-75006 Paris, France

The worldwide prevalence of metabolic diseases is increasing, and there are global recommendations to limit consumption of certain nutrients, especially saturated lipids. Insulin resistance, a common trait occurring in obesity and type 2 diabetes, is associated with intestinal lipoprotein overproduction. However, the mechanisms by which the intestine develops insulin resistance in response to lipid overload remain unknown. Here, we show that insulin inhibits triglyceride secretion and intestinal microsomal triglyceride transfer protein expression *in vivo* in healthy mice force-fed monounsaturated fatty acid-rich olive oil but not in mice force-fed saturated fatty acid-rich palm oil. Moreover, when mouse intestine and human Caco-2/TC7 enterocytes were treated with the saturated fatty acid, palmitic acid, the insulin-signaling pathway was impaired. We show that palmitic acid or palm oil increases ceramide production in intestinal cells and that treatment with a ceramide analogue partially reproduces the effects of palmitic acid on insulin signaling. In Caco-2/TC7 enterocytes, ceramide effects on insulin-dependent AKT phosphorylation are mediated by protein kinase C but not by protein phosphatase 2A. Finally, inhibiting *de novo* ceramide synthesis improves the response of palmitic acid-treated Caco-2/TC7 enterocytes to insulin. These results demonstrate that a palmitic acid-ceramide pathway accounts for impaired intestinal insulin sensitivity, which occurs within several hours following initial lipid exposure.

The worldwide obesity epidemic has stimulated numerous research efforts to identify factors that affect energy balance. As compared with the liver, pancreas, muscle, or adipose tissues,

the intestine has received little attention with regard to its potential role in the onset of metabolic disorders. Nevertheless, the intestine could also contribute to the development of metabolic disease, especially through its role in postprandial lipemia.

The increased amplitude and duration of the postprandial peak of circulating triglyceride-rich lipoproteins (TRL)⁵ are known risk factors for atherosclerosis and cardiovascular diseases (1, 2). Postprandial hypertriglyceridemia can be due to impaired TRL catabolism, and/or lipoprotein remnant uptake, and may also result from intestinal TRL overproduction (3). Thus, investigating intestinal lipoprotein secretion might help to understand aberrant postprandial lipemia (4).

The intestine ensures the transport of alimentary fat, which is the most calorie-dense nutrient. Enterocytes produce intestinal TRLs (chylomicrons) along a multistep pathway, which includes long chain fatty acid uptake, triglyceride synthesis in the endoplasmic reticulum, and assembly with apolipoproteins (apo) such as apoB48. Chylomicrons are then secreted into the lymph and ultimately into the blood. Overproduction of intestinal TRL may be an important contributor of both fasting and postprandial dyslipidemia (3, 5). Microsomal triglyceride transfer protein (MTP) transports neutral lipids (6) and plays a central role in the efficiency of lipid absorption by modulating chylomicron size. Alterations in MTP protein expression can impact intestinal fat transport and lipoprotein metabolism (7). However, in metabolic syndrome, which encompasses dyslipidemia, hypertension, and insulin resistance, the mechanisms underlying perturbed chylomicron assembly and secretion remain poorly explored.

Insulin also plays a central role in regulating energy metabolism. In the liver, the other TRL-secreting organ, insulin inhibits the production of very low density lipoproteins via the inhibition of MTP expression and apoB secretion (8–10). In the human intestine, insulin inhibits the production of apoB48-containing lipoproteins by both direct and indirect mecha-

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³ Both authors contributed equally to this work.

⁴ To whom correspondence should be addressed: UMRS U1138, Centre de Recherche des Cordeliers, Équipe Armelle Leturque, 15 Rue de l'École de Médecine, 75006 Paris, France. Tel.: 33-144272407; Fax: 33-143251615; E-mail: veronique.carriere@crc.jussieu.fr.

⁵ The abbreviations used are: TRL, triglyceride-rich lipoprotein; apo, apolipoprotein; DHCer, dihydroceramide; IRS, insulin receptor substrate; MTP, microsomal triglyceride transfer protein; OA, oleic acid; OKA, okadaic acid; PA, palmitic acid; PP2A, protein phosphatase 2A; SPT, serine palmitoyltransferase subunit.

nisms (11). In healthy chow-fed hamsters, a decrease in circulating levels of triglyceride-rich apoB48-containing lipoproteins was observed 60 min after insulin administration (12). The mechanisms involved in the inhibitory effect of insulin on chylomicron production remain unclear.

In hamsters, intestinal insulin resistance can be induced by a high fructose diet (12, 13), a high fat diet (15), or by TNF- α infusion (14). In these conditions, an overproduction of intestinal lipoproteins and/or an induction of MTP expression are observed. Moreover, several groups have reported that the intestine plays a role in the onset of insulin resistance in humans. Indeed, hyperinsulinemic insulin-resistant human subjects display increased production rates of intestinal apoB48-containing lipoproteins (16), and in individuals with type 2 diabetes, intestinal chylomicron production is resistant to insulin's acute suppressive effects (17).

Dyslipidemia is often reported during the progression from obesity to type 2 diabetes, thus exposing tissues to a variety of elevated lipids. Among these lipid species, ceramides have recently gained attention as important in the development of insulin resistance and impaired glycemic control (18). With excess saturated fat intake, ceramides accumulate in insulin-sensitive tissues, either as a consequence of *de novo* synthesis or through mobilization from complex sphingolipids (18). Insulin-resistant rodents and humans often display elevated ceramide concentrations in the liver, muscle, or serum, as compared with lean or untreated control subjects (19). At the cellular level, ceramide accumulation impairs insulin signaling and intracellular handling of glucose and lipids, resulting in deleterious effects on cell metabolism in liver, muscle, and adipose tissue (20, 21). However, in these studies the intestine was not investigated.

The cellular mechanisms that link lipid overload, the intestine, and insulin resistance remain to be clarified. Moreover, most of the above reported results were obtained under established states of insulin resistance or after long term high fat or fructose diets. Considering the rapid renewal of intestinal epithelium, local insulin resistance may occur rather quickly. In this work, we aimed to determine whether an acute supply of the saturated fatty acid palmitic acid would be sufficient to interfere with insulin action in intestinal cells and, if so, by which mechanisms.

Results

In Mouse Intestine, a Single Oral Gavage with Palm Oil Impairs Insulin Effects on Lipid Absorption—Following a single administration of those oils that are enriched in monounsaturated fatty acids (olive oil) or saturated fatty acids (palm oil) (Table 1), the effects of insulin on lipid absorption were analyzed in mice. In the absence of insulin treatment and 1 h after an olive or palm oil bolus, the postprandial plasma triglyceride content was increased as compared with control (water), as expected (Fig. 1A). Insulin injection reduced the plasma triglyceride level rise in mice that received an olive oil bolus, although no effect was observed in mice that received a palm oil bolus (Fig. 1A). Modifications of MTP activity (Fig. 1B) and expression (Fig. 1C) were analyzed in intestinal cells of mice force-fed palm oil, olive oil, or water and treated with or without insulin.

TABLE 1

Fatty acid composition of olive and palm oils, according to the manufacturer

Component	Olive oil (100 g)	Palm oil (100 g)
Palmitic acid	11.3	43.5
Oleic acid	71.3	36.6
Linoleic acid	12.5	9.1
Others	4.9	10.8
Total saturated fatty acids	13.8	49.3
Total monounsaturated fatty acids	72.9	37
Total polyunsaturated fatty acids	10.5	9.3

Gavage with olive or palm oil both increased MTP activity when compared with water. However, insulin exerted an inhibitory effect on MTP activity in mice force-fed water or olive oil but not in mice force-fed palm oil (Fig. 1B). Concerning changes of *Mtp* gene expression in intestinal epithelial cells, in the absence of insulin treatment, MTP mRNA levels were similar in mice that received either water or palm oil boluses (Fig. 1C), although they increased after an olive oil bolus. Insulin treatment reduced the rise of MTP mRNA in intestinal epithelial cells of mice force-fed olive oil (Fig. 1C) but led to increased MTP expression in mice force-fed palm oil (Fig. 1C). Differences between MTP mRNA levels and MTP activity following treatment may be explained by the complexity of the mechanisms responsible for MTP regulation, which has been shown to occur at transcriptional, translational, and/or post-translational levels (6).

These results indicate that palm oil prevents insulin exerting its inhibitory action on triglyceride secretion, *Mtp* gene expression, and MTP activity, suggesting that palm oil may interfere with the insulin-signaling pathway.

Palmitic Acid Impairs Insulin Signaling in Mouse Intestinal Epithelial Cells and in Human Enterocytes—To further understand the mechanisms by which saturated fatty acids alter intestinal insulin actions, we analyzed the effects of olive and palm oils on insulin signaling in mouse intestinal epithelial cells (Fig. 2). We observed that the increased AKT serine 473 phosphorylation in mouse intestinal epithelial cells, after insulin injection, was significantly impaired in mice that were force-fed palm oil but not olive oil. This suggests that a single bolus of palm oil is sufficient to alter insulin signaling *in vivo*.

Because palmitic acid is the major fatty acid component of palm oil (Table 1), we tested the specific effect of this saturated fatty acid on insulin signaling in intestinal cells as compared with oleic acid (the main monounsaturated fatty acid in olive oil). For this experiment, we used cultured human Caco-2/TC7 enterocytes incubated for 24 h with lipid micelles containing either palmitic (PA) or oleic acid (OA) before an insulin challenge. As shown in Fig. 3, in control and oleic acid-treated cells, insulin treatment induced a significant increase in insulin receptor substrate (IRS) phosphorylation at tyrosine 612 (Fig. 3A) and in AKT phosphorylation at both serine 473 and threonine 308 (Fig. 3B). In contrast, no increase in IRS or AKT phosphorylation occurred in Caco-2/TC7 cells treated with palmitic acid-containing lipid micelles, indicating that this saturated fatty acid prevents insulin signaling in enterocytes. This inhibitory effect of PA-containing lipid micelles in Caco-2/TC7 cells was observed after 24 h of treatment but

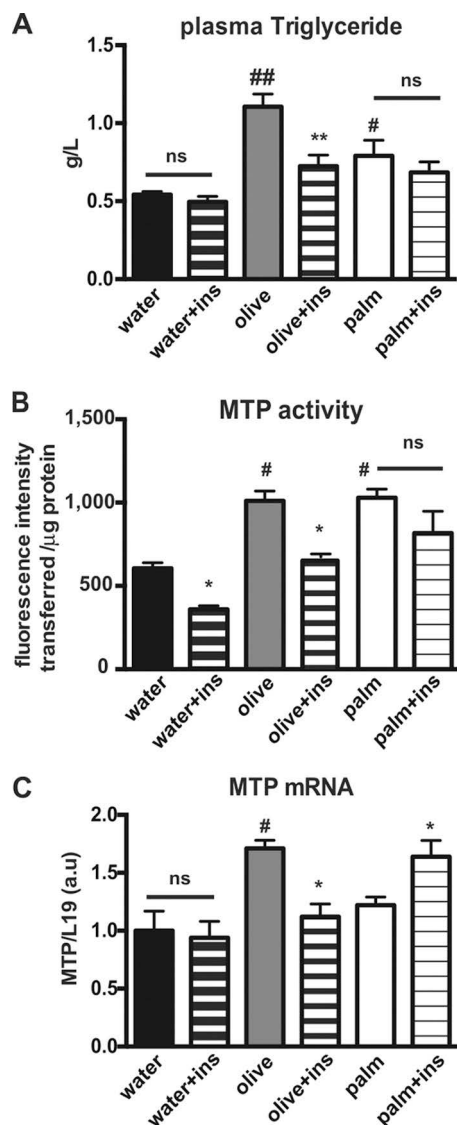


FIGURE 1. Effects of palm and olive oils on insulin-dependent intestinal lipid absorption in mice. *A*, fasted mice were force-fed water, olive oil, or palm oil and either subjected or not to an intraperitoneal injection of insulin (*ins*) 30 min later. Blood was collected 1 h after the bolus. Plasma triglyceride levels were quantified. #, $p < 0.05$, and ##, $p < 0.01$, as compared with water; **, $p < 0.01$ as compared with the same condition without insulin; *ns*, not significant, mean \pm S.E., $n = 8$. *B*, fasted mice were force-fed either water, olive oil, or palm oil and were either subjected or not to an intraperitoneal insulin (*ins*) injection 4 h later. Mice were euthanized 30 min after insulin injection. MTP activity was assayed in cellular homogenates of intestinal epithelial cells. Results display mean \pm S.E., $n = 4$, for each condition. #, $p < 0.05$ as compared with water; *, $p < 0.05$, as compared with the same condition without insulin; *ns*, not significant. *C*, fasted mice were force-fed water, olive oil, or palm oil and were either subjected or not to an intraperitoneal insulin (*ins*) injection at the same time as lipid bolus. Four hours later, mice were euthanized, and the jejunum was collected. MTP mRNA levels were quantified by RT-PCR in mouse intestinal epithelial cells, and by using L19 mRNA as a reference. Results display mean \pm S.E., $n = 4$, for each condition. #, $p < 0.05$, as compared with water; *, $p < 0.05$, as compared with the same condition without insulin; *ns*, not significant.

not after 6 h of treatment (Fig. 3C). This suggests that metabolic processing of palmitic acid is required for its effect on insulin signaling.

Palmitic Acid Increases Ceramide Production in Mouse Intestinal Cells—Palmitic acid has been described as a precursor of *de novo* ceramide synthesis, which contributes to insulin resis-

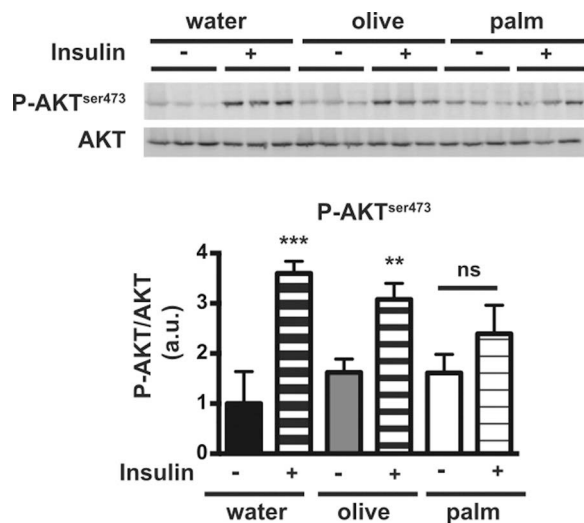


FIGURE 2. Effects of palm and olive oils on insulin signaling in the mouse intestine. Fasted mice were force-fed water, olive oil, or palm oil, and 4 h later were either subjected or not to an intraperitoneal insulin injection. Mice were euthanized 30 min after insulin injection, and cell lysates from intestinal epithelial cells were analyzed by Western blotting for AKT phosphorylation (P-AKT) at serine 473 (*top panel*). AKT phosphorylation was reported as the ratio of P-AKT to total AKT content (arbitrary units, *a.u.*) (*bottom panel*). The P-AKT/AKT ratio obtained in the water condition without insulin was set at 1. Results display mean \pm S.E., $n = 4$, for each condition. **, $p < 0.01$, and ***, $p < 0.001$, compared with the same treatment without insulin; *ns*, not significant.

tance (20, 27). We thus analyzed whether this saturated fatty acid increases ceramide production in intestinal cells.

We first compared the amount of ceramide in mouse plasma 4 h after gavage with water, olive oil, or palm oil. An increase in plasma ceramide was observed following palm oil bolus and to a lesser extent after olive oil bolus (Fig. 4A). The effect of palm oil on ceramide production was further analyzed in mouse plasma and intestinal samples. Mice were treated or not with myriocin, an inhibitor of serine palmitoyltransferase (SPT) that is the first enzyme in *de novo* ceramide synthesis (28). Mice were then force-fed palm oil. Both treatments were administered daily for 4 consecutive days. Plasma ceramide levels were quantified in all mice before and 2 h after the last palm oil bolus. As shown in Fig. 4B, an increase (+21%) of total ceramide content was observed in mouse plasma 2 h after the palm oil bolus. This increase in plasma ceramide level was not observed in animals treated with myriocin, suggesting the occurrence of *de novo* ceramide synthesis in the small intestine during the 2-h period immediately following the palm oil bolus. The total ceramide content was also measured in the intestine of mice treated with palm oil in the presence or absence of myriocin (Fig. 4C). In epithelial intestinal cells, total ceramide remained unchanged after 2 h of palm oil treatment; however, an inhibitory myriocin effect was observed. According to the increase in total plasma ceramide after a palm oil bolus (Fig. 4B), we hypothesized that ceramides were rapidly secreted after synthesis. Thus, we assayed the intestinal content of dihydroceramides (DHCer), ceramide precursors that are specifically produced via *de novo* synthesis (20). We observed a +48% increase in total DHCer levels 2 h after palm oil bolus, which was prevented with myriocin treatment (Fig. 4D). DHCer species analysis revealed that palm oil mainly increased C20 and C22 DHCer in this tissue (8.3- and 2.1-fold, respectively) (Fig. 4E).

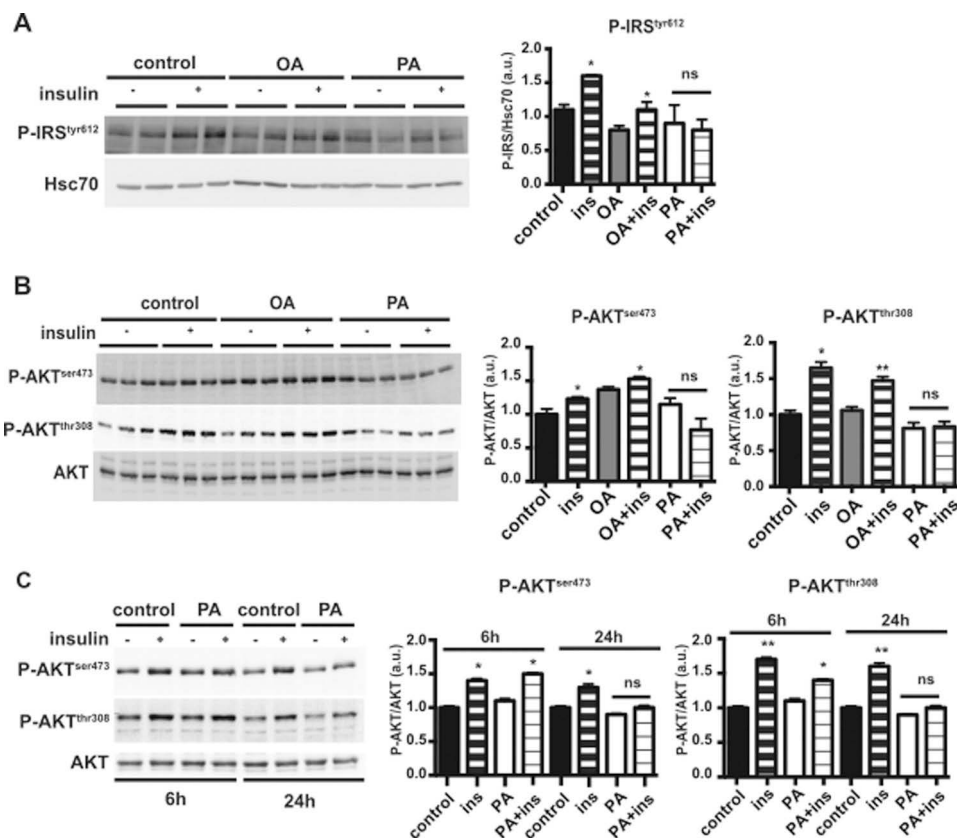


FIGURE 3. Effects of palmitic or oleic acid on AKT phosphorylation in cultured human Caco-2/TC7 enterocytes. *A*, Caco-2/TC7 cells were incubated with oleic acid- or palmitic acid-containing lipid micelles (OA and PA, respectively) for 24 h. As indicated, insulin (*ins*) was added to the culture 10 min prior to harvesting. Cell lysates were used for Western blotting analysis of IRS phosphorylation at tyrosine 612, with Hsc70 as loading control (*left panel*). Ratios of phosphorylated IRS to Hsc70 protein, expressed as arbitrary units (a.u.), are reported in the *right panel*. Results display mean \pm S.E., $n = 4$. *, $p < 0.05$, as compared with the same condition without insulin; ns, not significant. *B*, Caco-2/TC7 cells were cultured in the presence or absence of OA- or PA-containing lipid micelles for 24 h. Cells were incubated with or without insulin (*ins*) for 10 min prior to harvest. AKT phosphorylation at serine 473 ($P\text{-AKT}^{\text{Ser473}}$) or threonine 308 ($P\text{-AKT}^{\text{Thr308}}$) was analyzed by Western blotting (*left panel*) using specific antibodies against the phosphorylated residues. Total AKT was used as control, and the ratios of the respective P-AKT to total AKT content are reported (*right panels*). The P-AKT/AKT ratio obtained under control conditions (without lipid micelles and insulin) was set at 1. *, $p < 0.05$; **, $p < 0.01$, as compared with the same conditions without insulin. ns, not significant, mean \pm S.E., $n = 4$. *C*, Caco-2/TC7 cells were incubated in the presence or absence of palmitic acid-containing lipid micelles (PA) for 6 or 24 h and treated or not with insulin for 10 min prior to harvest. AKT phosphorylation in cell lysates was analyzed by Western blotting (*left panel*). Quantification of AKT phosphorylation was expressed as the ratio of P-AKT to total AKT (*right panels*) in arbitrary units (a.u.). Results display mean \pm S.E., $n = 4$. *, $p < 0.05$, and **, $p < 0.01$, as compared with the same conditions without insulin. ns, not significant.

Because palm oil and olive oil contain a mixture of fatty acids (Table 1), we analyzed the specific effect of palmitic acid on ceramide production by comparing the intracellular content of ceramide in Caco-2/TC7 cells incubated with palmitic acid or oleic acid (Fig. 5). As compared with control cells, the intracellular content of C_{14} -, C_{18} -, C_{20} -, C_{22} -, and C_{24} -ceramide species was increased in PA-treated cells but not in OA-treated cells (Fig. 5A). Interestingly, we observed a 46% increase in serine palmitoyltransferase 2 subunit (SPT2) expression in PA-treated cells as compared with control cells (Fig. 5B). SPT2 expression remained unchanged in OA-treated cells as compared with control cells.

Altogether, these results demonstrate that palmitic acid induces *de novo* ceramide synthesis in intestinal cells. They indicate that the effects of palmitic acid on insulin signaling could occur through increased ceramide synthesis in enterocytes.

Alteration of Insulin Signaling in Intestinal Cells by Palmitic Acid Is Dependent on *de Novo* Ceramide Synthesis—We then determined the direct impact of ceramide on insulin signal-

ing in intestinal cells. Caco-2/TC7 cells were incubated with C_2 -ceramide, a short chain cell-permeable biologically active analogue of ceramide (Fig. 6A) (29, 30). Compared with control cells, the addition of C_2 -ceramide reduced the basal phosphorylation level of the AKT threonine 308 residue, and it significantly impaired the response to insulin, as early as 6 h after the beginning of the treatment. C_2 -ceramide addition also tended to decrease both basal and insulin-stimulated AKT phosphorylation at serine 473 (Fig. 6A), but it did not reach statistical significance when compared with untreated cells. These results demonstrate that ceramide addition in enterocytes partially reproduces the effects of palmitic acid on insulin signaling.

We then analyzed whether inhibiting *de novo* ceramide synthesis reversed the effect of palmitic acid on insulin signaling (Fig. 6B). As myriocin treatment is toxic to Caco-2/TC7 cells (data not shown), we used L-cycloserine, another potent inhibitor of *de novo* ceramide synthesis (31). L-Cycloserine treatment partially restored serine 473 and threonine 308 AKT phosphorylation upon insulin treatment of PA-treated cells (Fig. 6B).

Palmitic Acid, Ceramides, and Intestinal Insulin Resistance

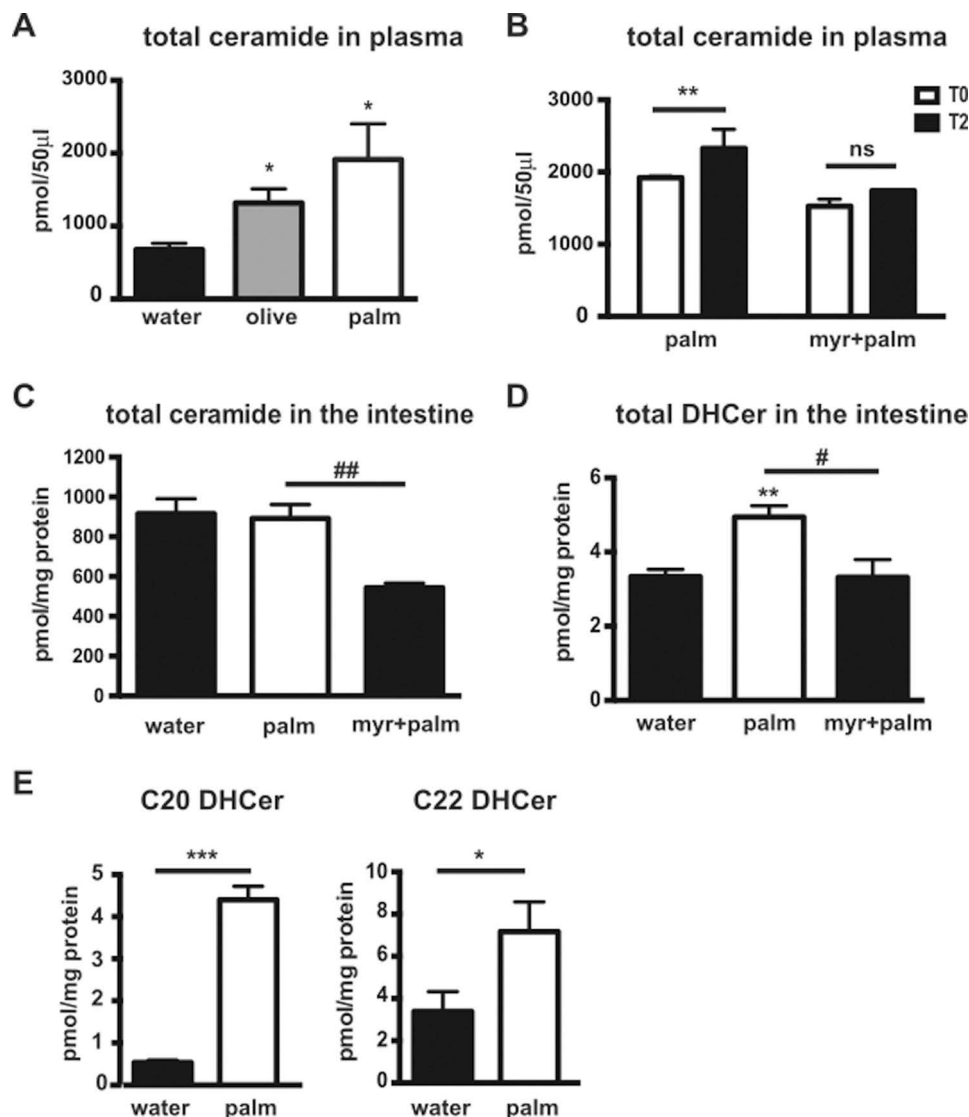


FIGURE 4. Effects of palm oil on ceramide production in mouse intestinal epithelial cells. *A*, total ceramide was quantified in mouse plasma 4 h after a water, olive oil, or palm oil bolus. Results (mean \pm S.E.) are expressed as pmol/50 μ l plasma. *, $p < 0.05$ versus water, $n = 4$, for each condition. *B–E*, mice received or not myriocin (*myr*) by gavage and a bolus of water or palm oil 30 min later. These treatments were repeated for four consecutive days ($n = 4$, for each condition). On the 4th day of the experiment, ceramides and dihydroceramides were quantified in plasma and intestinal epithelial cells by mass spectrometry. *B*, total plasma ceramides were quantified before (*T0*, white boxes) and 2 h after the gavage (*T2*, black boxes). Results (mean \pm S.E.) are expressed as pmol/50 μ l plasma. **, $p < 0.01$, as compared with palm oil at *T0*. *C*, total ceramide content was quantified in intestinal epithelial cells 2 h after the final water or palm oil gavage, with or without myriocin treatment. Results (mean \pm S.E.) are expressed as pmol/mg of protein. ##, $p < 0.01$, as compared with palm oil. *D*, total DHCers were quantified in mouse intestinal epithelial cells 2 h after the final gavage. Results (mean \pm S.E.) are expressed as pmol/mg of protein. **, $p < 0.01$, as compared with control; #, $p < 0.05$, as compared with palm oil. *E*, quantification of C_{20} - and C_{22} -dihydroceramides (*C20 DHCer* and *C22 DHCer*, respectively) was carried out in mouse intestinal epithelial cells 2 h after the bolus. Results (mean \pm S.E.) are expressed as pmol/mg of protein. *, $p < 0.05$, and ***, $p < 0.001$, as compared with water.

Altogether, these results show that the effects of palmitic acid on insulin signaling are directly linked to *de novo* ceramide synthesis in enterocytes.

Inhibitory Effect of Ceramide on Insulin-stimulated AKT Phosphorylation Is Mediated by PKC Activity but Not by PP2A Activity—In 3T3-L1 adipocytes and L6 muscle cells, it has been reported that protein kinase C (PKC) and protein phosphatase 2A (PP2A) are involved in the inhibitory effect of ceramide on insulin signaling (31, 32). To determine whether PKC and/or PP2A pathways are also involved in enterocytes, we pre-treated Caco-2/TC7 cells with either a broad PKC inhibitor (Ro 31.8220) or a PP2A inhibitor (okadaic acid, OKA), before the addition of C_2 -ceramide and insulin (Fig. 7). When Caco-2/

TC7 cells were incubated in the presence of ceramide and insulin, we observed that the ceramide-dependent low level of AKT phosphorylation was prevented by pre-treatment with Ro 31.8220 (Fig. 7A) but not with OKA (Fig. 7B). These results indicate that a ceramide-activated PKC pathway is likely to be involved in the inhibitory effect of ceramides on insulin signaling in Caco-2/TC7 cells.

Inhibiting *de Novo* Ceramide Synthesis Partially Restores Insulin Effects on ApoB48 Secretion and MTP Activity in PA-treated Caco-2/TC7 Cells—We next determined the impact of palmitic acid supply and inhibiting *de novo* ceramide synthesis on apoB48 secretion and MTP activity in Caco-2/TC7 cells. As observed previously (33), palmitic acid-containing micelles

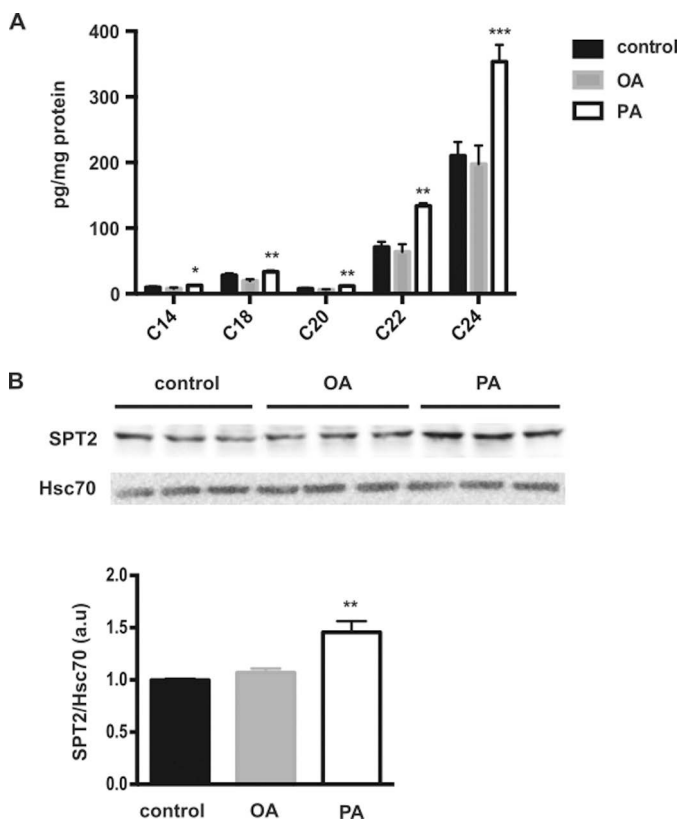


FIGURE 5. Effects of palmitic acid on ceramide production in Caco-2/TC7 enterocytes. A, cellular ceramide species content was quantified in control cells and in cells treated for 24 h with OA- or PA-containing lipid micelles. Results (mean \pm S.E.) are expressed as pg/mg protein. *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$ as compared with control cells, $n = 4$. B, representative Western blot of SPT2 (upper panel) in cell lysates from Caco-2/TC7 cells cultured under the same conditions as in A. Hsc70 protein was used as loading control. The lower panel represents the quantification of SPT2 protein levels expressed as the SPT2/Hsc70 ratio in arbitrary units (a.u.), the control value being set to 1. Results display mean \pm S.E., $n = 6$; **, $p < 0.01$, as compared with control.

were less potent than oleic acid-containing micelles in inducing the secretion of apoB48 (Fig. 8A). Insulin decreased apoB48 secretion in OA-treated cells but not in PA-treated cells. Upon insulin stimulation, apoB48 secretion tended to be lower in PA-treated cells that were pre-treated with L-cycloserine as compared with the observed apoB48 level in the absence of the *de novo* ceramide synthesis inhibitor.

Furthermore, MTP activity was determined in Caco-2/TC7 cells (Fig. 8B). As observed previously in these cells (23, 34), MTP activity was not modified by the supply of oleic acid-containing micelles. However, we observed an increase of MTP activity in PA-treated cells as compared with control cells. Insulin decreased MTP activity in OA-treated cells but not in PA-treated cells. Upon insulin stimulation, the pre-treatment of PA-treated cells with L-cycloserine decreased MTP activity as compared with the same conditions but without L-cycloserine pre-treatment.

Altogether, these results indicate that the inhibitory effects of insulin on apoB48 secretion and MTP activity observed in OA-treated cells do not occur in PA-treated cells and that an inhibition of the *de novo* ceramide synthesis pathway can restore insulin action.

Discussion

It has been established that enterocytes express all of the receptors and mediators involved in the insulin-signaling pathway (35). However, few data exist on the intestinal role of insulin during fat absorption under healthy conditions. The inhibitory effect of insulin on intestinal lipoprotein secretion has previously been observed (11, 36, 37), but the mechanisms implicated remain unknown. Here, we report for the first time that in enterocytes (*in vivo* as well as in Caco-2/TC7 cells) insulin modulates the expression and/or activity of MTP, which has a crucial role in the assembly of intestinal apoB-containing lipoproteins (6).

Most of the knowledge concerning the effects of insulin on intestinal lipoprotein secretion has been obtained in pathological situations with an overt insulin-resistant state, such as in obesity or type 2 diabetes. In such pathologies, the increased production of chylomicrons and their reduced clearance contribute to the postprandial dyslipidemia observed in insulin-resistant individuals (38). The physiological suppressive effect of insulin on chylomicron production is reportedly absent in type 2 diabetes subjects (17). Duodenal explants from insulin-resistant obese subjects undergoing bariatric surgery were shown to express higher mRNA levels of free fatty acid-binding proteins and MTP and to secrete more intestinal triglyceride-rich lipoproteins, *i.e.* chylomicrons (39). Interestingly, it has also been recently shown that bariatric surgery improves triglyceride-rich lipoprotein metabolism and, in particular, decreases the levels of intestinal lipoproteins in obese subjects (40). These changes were associated with decreases in HOMA-IR (homeostasis model assessment-insulin resistance) index after surgery, indicating an improvement in insulin sensitivity (40). Specific intestinal perturbations in insulin signaling have been observed in enterocytes from the fructose-fed hamster model of insulin resistance (41). In this model, reduced levels of IRS-1 phosphorylation were observed (12). Although chronic administration of fructose led to increased intestinal lipoprotein secretion, such an effect was not observed after short term treatment (2 days) (12), suggesting that the perturbation of intestinal lipoprotein secretion in this model could be a consequence of intestinal adaptation to an overt insulin-resistant state. In this study, we demonstrate that an acute supply of palm oil in mice, or of palmitic acid in Caco-2/TC7 enterocytes, is sufficient to impair insulin signaling as well as insulin effects on lipid-induced MTP expression and/or activity. This indicates that intestinal insulin resistance rapidly occurs within a time frame compatible with an enterocyte's short life span in the intestinal epithelium (42). It is thus possible that repeated consumption of saturated fat containing large amounts of palmitic acid might maintain this intestinal insulin-resistant state, leading to altered intestinal function, which in turn could affect the function of peripheral tissues.

Our results demonstrate that the effects of palmitic acid on insulin signaling in enterocytes depend on the production of ceramides. Ceramides are generated through three different pathways as follows: 1) *de novo* synthesis, in which the condensation of serine and palmitoyl-CoA by SPT constitutes the rate-limiting step; 2) from sphingomyelin through the activation of sphingomyelinases; and 3) the salvage pathway, through the

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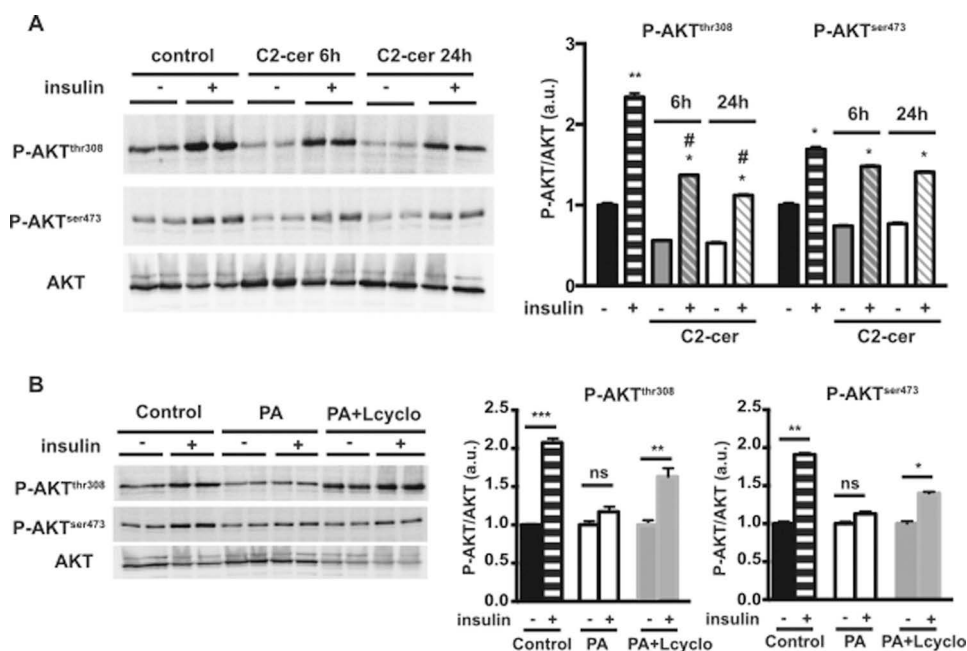


FIGURE 6. Effects of C₂-ceramide addition and *de novo* ceramide synthesis inhibition on insulin-dependent AKT phosphorylation. *A*, Caco-2/TC7 cells were incubated for 6 or 24 h with or without C₂-ceramide. For some conditions, insulin was added 10 min prior to harvest. AKT phosphorylation in cell lysates was analyzed by Western blotting (left panel). Specific antibodies against phosphorylated serine 473 (P-AKT^{Ser473}) and threonine 308 (P-AKT^{Thr308}) AKT residues were used. Total AKT was used as control. The quantification of AKT phosphorylation is displayed in the right panel. Results are expressed as the P-AKT/total AKT ratio in arbitrary units (a.u.). Results represent mean \pm S.E., $n = 4$. *, $p < 0.05$, and **, $p < 0.01$, as compared with the same condition without insulin. #, $p < 0.05$, as compared with control cells in the presence of insulin. *B*, Caco-2/TC7 cells were incubated 24 h with or without palmitic acid-containing lipid micelles (PA). For some conditions, cells were pre-treated with L-cycloserine for 1 h before incubation with PA-containing lipid micelles (PA+Lcyclo). When appropriate, insulin was added 10 min prior to harvest. AKT phosphorylation in cell lysates was analyzed by Western blotting (left panels). Specific antibodies against phosphorylated serine 473 (P-AKT^{Ser473}) and threonine 308 (P-AKT^{Thr308}) AKT residues were used. Total AKT was used as control. Quantification of AKT phosphorylation (right panels) was expressed as the P-AKT/total AKT ratio in arbitrary units (a.u.); the ratio value obtained without insulin for each condition was set at 1. *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$, as compared with the same condition without insulin, ns, not significant, $n = 4$.

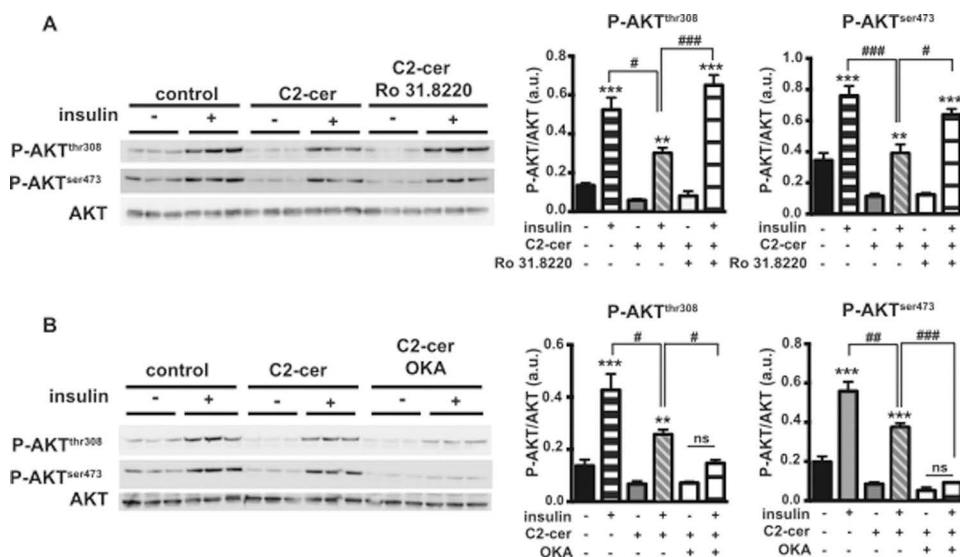


FIGURE 7. Effects of PKC and PP2A inhibitors on insulin-dependent AKT phosphorylation. Caco-2/TC7 cells were incubated with or without 100 μ M C₂-ceramide (C2-cer) for 6 h. For some conditions, insulin was added 10 min prior to harvest. When indicated, cells were treated for 24 h with 5 μ M Ro 31.8220 (A) or 100 nM OKA (B). AKT phosphorylation in cell lysates was analyzed by Western blotting (left panels). Specific antibodies against phosphorylated serine 473 (P-AKT^{Ser473}) and threonine 308 (P-AKT^{Thr308}) AKT residues were used. Total AKT was used as control. Quantifications of AKT phosphorylation are displayed in the right panels. Results are expressed as the P-AKT/total AKT ratio in arbitrary units (a.u.). Results represent mean \pm S.E., $n = 3$. *, $p < 0.01$, and ***, $p < 0.001$, as compared with the same condition without insulin; #, $p < 0.05$, ##, $p < 0.01$, and ###, $p < 0.001$, as compared with the indicated condition.

breakdown of complex sphingolipids (43). In palm oil-treated mice and in palmitic acid-treated Caco-2/TC7 cells, our results show that the observed increase in ceramide production mainly occurs via the *de novo* synthesis pathway. Indeed, in intestinal cells, we observed an increase in dihydroceramides (Figs. 4 and

5), which are intermediate metabolites specifically generated during the *de novo* ceramide synthesis pathway (43). Moreover, an increase of SPT2 expression, one of the SPT subunits, is observed in palmitic acid-treated Caco-2/TC7 cells (Fig. 5). Finally, the inhibition of the *de novo* ceramide synthesis path-

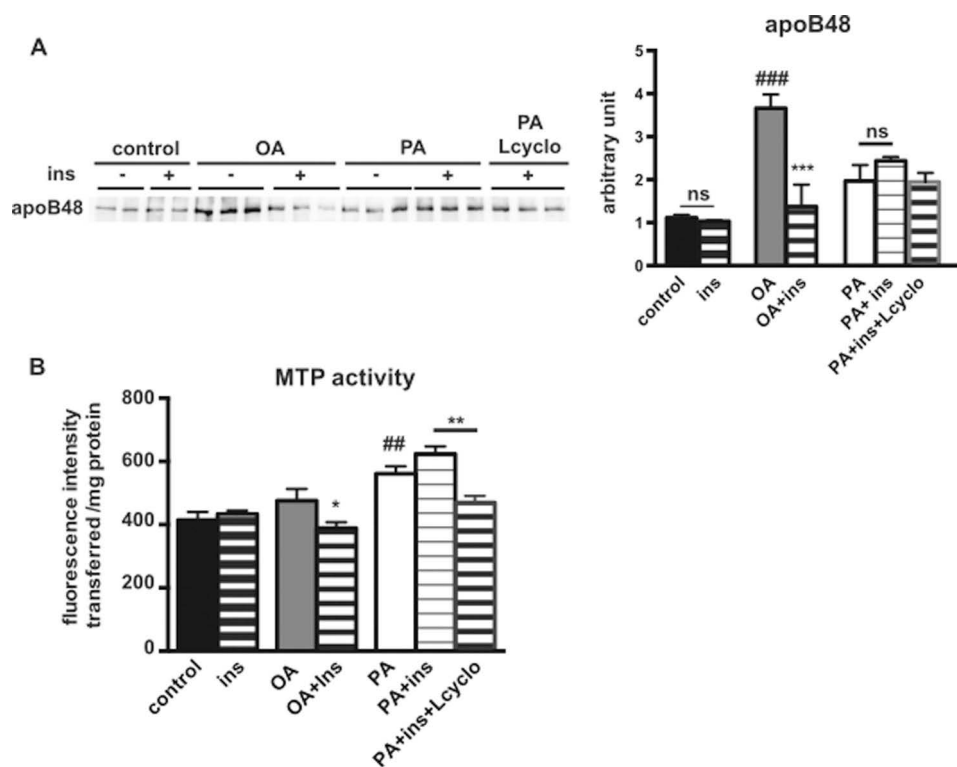


FIGURE 8. Effects of *de novo* ceramide synthesis inhibition on MTP activity and apoB48 secretion in Caco-2/TC7 cells. Caco-2/TC7 cells were incubated with or without OA- or PA-containing lipid micelles (PA) for 24 h. When indicated, L-cycloserine was added 1 h before incubation with PA-containing lipid micelles (PA+Lcyclo). Insulin (ins) was added 30 min prior to harvest. *A*, apoB48 secretion in basal culture medium was determined by Western blotting (left panel). Quantification of the Western blotting is displayed in the right panel. Results are expressed as arbitrary units (mean \pm S.E., $n = 6$). ***, $p < 0.001$, as compared with the same condition without insulin; ###, $p < 0.001$, as compared with control; ns, not significant. *B*, MTP activity was assayed in cell homogenates. Results display mean \pm S.E., $n = 6$, for each condition. *, $p < 0.05$, as compared with the same condition without insulin; **, $p < 0.01$, as compared with the indicated condition; ##, $p < 0.01$, as compared with control.

way partially reversed the effects of palmitic acid in these cells (Figs. 6B and 8). We delved deeper into the mechanism and showed that in enterocytes the effects of C_2 -ceramide on AKT phosphorylation involved the activation of the PKC pathway. This ceramide-activated pathway has previously been observed to mediate deleterious ceramide effects on insulin signaling in different insulin-sensitive cell types (31, 32).

Interestingly, we show that both C_2 -ceramide addition and inhibition of the *de novo* ceramide synthesis pathway mainly exert their effects on the AKT threonine 308 residue and to a lesser extent on its serine 473 residue (Fig. 6). These AKT serine and threonine residues are also known to be phosphorylated upon insulin stimulation by distinct protein kinases, PDK-1 and mTORC2, respectively (44). Further studies will be needed to determine whether palmitic acid and ceramides could also act negatively on these kinases in intestinal cells.

Our present results must be taken into account in the context of the augmented saturated fat consumption and the concomitant increased incidence of obesity and type 2 diabetes in humans. Indeed, elevated ceramide levels associated with metabolic disease have been observed in the plasma of subjects (45–47). Interestingly, gastric bypass surgery, which improves insulin sensitivity in severely obese patients, also reduces levels of plasma ceramide subspecies (48). Moreover, inhibiting *de novo* ceramide synthesis by myriocin treatment, which decreased ceramide levels in tissues and/or plasma (49–51), also diminishes atherosclerotic lesions in rodents (51) and

reverses diet-induced insulin resistance (49, 50, 52). Altogether, these data suggest that elevated plasma ceramide levels could contribute to the onset of metabolic diseases. In our study, we observed that a single oral administration of palmitic acid is sufficient to augment plasma ceramide levels in mice. Increased plasma ceramide levels resulting from intestinal metabolism after consumption of saturated fatty acid-rich diet, may thus contribute to the altered insulin sensitivity in peripheral tissues.

In summary, our study has demonstrated for the first time that the palmitic acid-ceramide-AKT pathway modifies intestinal insulin sensitivity. We show that the small intestine is able to rapidly and locally produce ceramides from palmitic acid, which then alter intestinal insulin responses. An elevation of intestine-derived plasma ceramides due to a saturated fat-containing diet may thus contribute to the onset of metabolic diseases.

Experimental Procedures

Animals—Male 10–12-week-old C57BL/6 mice were obtained from Janvier (St Berthevin, France), housed in standard cages, and supplied with food and drinking water *ad libitum*. Animals were kept in a 12-h light/dark cycle and at a controlled temperature (22 °C). All animal care and experimental procedures were conducted in accordance with the French Law of April 6, 2010, and they were approved by the local ethics committee (Charles Darwin, Ce5/2012/052).

Cell Culture—Cell culture media and supplements were obtained from Invitrogen (Cergy-Pontoise, France) and FCS (fetal

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calf serum) from AbCys Biowest (Paris, France). Microporous polyethylene terephthalate membrane inserts were from Corning (Avon, France) (23.1 mm diameter, 3- μ m pore size high density). C_2 -ceramide was purchased from Cayman Chemical Co. (Montigny-le-Bretonneux, France), and insulin, myriocin, or L-cycloserine was from Sigma. Ro 31.8820 and OKA inhibitors were purchased from Merck Millipore (Molsheim, France).

Caco-2/TC7 cells were plated at a density of 0.25×10^6 cells on each insert in 6-well plates and grown and differentiated as described previously (22) in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX I containing 25 mM glucose, supplemented with penicillin (100 IU/ml) and streptomycin (100 μ g/ml), 1% non-essential amino acids, and 20% (v/v) heat-inactivated (56 °C, 30 min) fetal calf serum. Cells were cultured for 1 week in media containing FCS in the upper and lower compartments and then for 1 week with FCS in the lower compartment only. Media were changed daily. The day before and during treatment with insulin, cells were cultured in media devoid of FCS. After incubation, media and cells were immediately collected and processed for Western blotting as described below. In some experiments, cells were treated with insulin (100 nM), L-cycloserine (20 mM), C_2 -ceramide (100 μ M), OKA (100 nM), or Ro 31.8220 (5 μ M), and the duration of treatment is indicated in figure legends.

Preparation of Lipid Micelles—Lipid micelles were prepared as described (23). Briefly, stock solutions (100 mM) of PA, OA, L- α -lysophosphatidylcholine, 2-mono-oleoylglycerol, and 25 mM cholesterol (all from Sigma) were prepared in chloroform/methanol (2:1, v/v). To prepare 1 ml of lipid micelles, 6 μ l PA or OA was mixed with 2 μ l of other lipids in a sterile glass tube. The mixture was dried under a stream of nitrogen gas; the residue was dissolved in 83 μ l of a sterile solution of 24 mM sodium taurocholate in serum-free medium, and the volume was brought to 1 ml with serum-free medium. The final lipid micelle preparation therefore consisted of serum-free medium containing sodium taurocholate (2 mM), PA or OA (0.6 mM), L- α -lysophosphatidylcholine (0.2 mM), and cholesterol (0.05 mM) with 2-mono-oleoylglycerol (0.2 mM).

Mouse Treatments—Mice were fasted overnight and forced either 0.2 ml of olive or palm oil. The lipid composition of olive and palm oils is described in Table 1. Insulin (0.5 units/25 g of mouse weight) was injected intraperitoneally. Treatment duration and biological sample collection time points are indicated in the figure legends. For myriocin treatment, mice were subjected to a daily oral gavage for 4 days with vehicle (100 μ l of 5% w/v carboxymethylcellulose) or myriocin (0.3 mg/kg/day) 30 min before a palm oil bolus (0.2 ml). On day 5, mice received the same gavage, 2 h before euthanasia. Five minutes before treatment or euthanasia, blood samples were collected into EDTA tubes from the tail vein of conscious animals by gentle massage following tail snip and kept chilled on ice. Following centrifugation of blood samples, plasma was used for triglyceride measurements using a kit from DiaSys (DiaSys, Condom, France) according to the manufacturer's instructions. Intestinal epithelial cells were prepared as described previously (24). Briefly, the jejunum was cut into small pieces and incubated at 4 °C for 2 h in 3 ml of cell recovery solution (BD Biosciences, Le Pont de Claix, France) containing protease and phosphatase inhibitor mixtures (Roche Diagnostics, Meylan, France). Epi-

thelial cell homogenates were filtered, washed with PBS, centrifuged to obtain villus epithelial cells, and then homogenized for protein extraction.

Protein Extraction and Measurement—Proteins were solubilized from isolated intestinal epithelial cells or Caco-2/TC7 cells in high sucrose buffer containing protease and phosphatase inhibitors (25). Protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad, Marnes-La-Coquette, France) with BSA standards.

Western Blotting—Cell lysates (30 μ g of protein) were fractionated by SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. After an overnight incubation at 4 °C in TBS-T (20 mM Tris/HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) supplemented with 5% (w/v) BSA, blots were probed with primary antibodies, *i.e.* anti-AKT, anti-P-AKT^{Thr-308}, anti-P-AKT^{Ser-473} (Cell Signaling Technology, Ozyme, Saint Quentin en Yvelines, France), anti-P-IRS1^{Tyr-612} (Millipore, Guyancourt, France), anti-HSC70 (Santa Cruz Biotechnology, CliniSciences, Nanterre, France), anti-apoB (Chemicon, Millipore, Guyancourt, France), and anti-SPT2 (a gift from Hornemann Thorsten, Institute of Clinical Chemistry, Zurich, Switzerland), followed by peroxidase-conjugated secondary antibodies. Blots were developed with enhanced chemiluminescence (ECL[®]) reagents according to the manufacturer's instructions (Amersham Biosciences, Orsay, France).

Quantitative Real Time PCR—Total RNA was extracted from intestinal epithelial cells using Tri-Reagent (Invitrogen) according to the manufacturer's recommendations. Quantitative real time PCR was performed with the SYBR Green PCR kit (Life Technologies, Inc.) using a Stratagene system according to instructions. Relative quantification was determined using the $2^{-\Delta\Delta Ct}$ method. Oligonucleotide sequences were as follows: forward, 5'-GGCAGTGCCTTTTCTCTGCT-3', and reverse, 5'-TGAGAGGCCAGTTGTGTGTGAC-3' for murine *Mtp* mRNA and forward, 5'-ATGTATCACAGCCTGTACCTG-3', and reverse, 5'-CGTGCTTCCTTGGTCTTAGAC-3' for murine *L19* mRNA (reference gene).

Quantification of Ceramides and Dihydroceramides by LC-MS/MS—Ceramide standards (d18:1-14:0 Cer, d18:1-16:0 Cer, d18:1-17:0 Cer, d18:1-20:0 Cer, d18:1-22:0 Cer, d18:1-24:0 Cer, and d18:1-24:1 Cer) were obtained from Avanti Polar Lipids (Coger SAS, Paris, France). Chemicals of the highest available grade were purchased from Sigma. LC-MS/MS quality grade solvents were purchased from Fisher (Illkirch, France).

Ceramides and dihydroceramides were extracted according to the Bligh and Dyer method as described by Reis *et al.* (26). Briefly, plasma aliquots (50 μ l of plasma + 150 μ l of saline) or cell lysates (200 μ l) were mixed with d18:1-17:0 Cer, used as an internal standard, and extracted with 750 μ l of 2:1 chloroform/methanol for 10 min. Chloroform (250 μ l) was then added and extraction followed for 10 min. Distilled water (250 μ l) was added and extraction continued for 10 more min. After centrifugation (10,000 \times g, 10 min, 4 °C), the organic phase was collected. The aqueous phase was acidified with hydrochloric acid (8 μ l, 3 mol/liter) and further extracted with 600 μ l of chloroform for 10 min. After centrifugation (10,000 \times g, 10 min, 4 °C) the organic phase was collected and combined with the previous sample. Pooled organic phases were washed with 800 μ l of

the upper phase from a chloroform/methanol/water (96.7:93.3:90) mixture. The organic phase was evaporated under vacuum. Extracts were finally dissolved with 200 μ l of 60:30:4.5 chloroform/methanol/distilled water, and 3 μ l were injected on a 1200 6460-QqQ LC-MS/MS system equipped with an ESI source (Agilent Technologies, Les Ulis, France). Separation was achieved on a Poroshell C8 2.1 \times 100 mm, 2.7- μ m column (Agilent Technologies) at a flow rate of 0.3 ml/min, 30 $^{\circ}$ C, with a linear gradient (solvent A) of formic acid/ammonium formate (0.2%/1 mM final concentration) and (solvent B) methanol containing formic acid/1 mM ammonium formate as follows: 70% B for 1 min, up to 100% B in 4 min, and maintained at 100% for 5 min. Acquisition was performed in positive multiple reaction monitoring mode (source temperature, 300 $^{\circ}$ C, nebulizer gas flow rate 10 liters/min, sheath gas flow 11 liters/min, temperature 325 $^{\circ}$ C, capillary 3500 V, nozzle 1000 V, fragmentor 180 V, collision energy 27 V). Transitions, $[M - 18]^+ \rightarrow 262.2$, $[M - 18]^+ \rightarrow 264.2$, and $[M - 18]^+ \rightarrow 266.2$, were used for quantitation of d18:2-ceramide, d18:1-ceramide, and d18:0-ceramide, respectively.

Calibration curves were obtained for each molecule using authentic standards extracted by identical methods as used for plasma samples and cell lysates. Quadratic regression and linear regression were applied to calculate plasma and cell lysate ceramide concentrations, respectively.

MTP Activity—MTP activity was assayed using 100–200 μ g of cell homogenate according to the manufacturer's instructions (MAK110 kit, Sigma, Saint-Quentin Fallavier, France).

Statistics—Comparisons between the two groups were performed with Student's *t* tests. Comparisons involving multiple groups were done using one-way analysis of variance. A level of $p < 0.05$ was considered as significant.

Author Contributions—T. T. T. designed and conducted the experiments and analyzed the results. B. G. P. carried out *in vitro* experiments. V. C. designed experiments and performed *in vivo* and some *in vitro* experiments. E. H. designed experiments on ceramides. S. D. contributed to the design of cell culture treatments and experiments. C. O. performed some *in vitro* experiments. J.-P. B. and A. B.-Z. performed quantification of ceramides and analyzed the results. A. R. conducted experiments on insulin signaling in the intestine with T. T. T., P. F., M. R., and E. H. conceived the project and obtained financial support from Institute of Cardiometabolism and Nutrition and Fondation pour la Recherche Médicale. V. C. and T. T. T. wrote the paper. P. F., E. H., A. L., S. D., and M. R. revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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2) Article at *Médecine/Science*, 2017

During my thesis, I participated in the organization of the event: "Journées de l'Ecole Doctorale Physiologie Physiopathologie et Thérapeutique " which resulted in the publication of a report in the French journal *Médecine/Science*.

> Les doctorants organisateurs de ces journées proposent un résumé des principaux événements scientifiques qui se sont déroulés les 25 et 26 mai 2016 au centre de recherche des Cordeliers (CRC) lors des journées de l'École « physiologie, physiopathologie et thérapeutique » (ED 394). Outre la présentation de leurs travaux scientifiques lors des sessions « communications orales » et « posters », les participants ont entendu une conférence plénière du Professeur Pierre Corvol sur l'intégrité scientifique et participé à une table ronde consacrée à la médiation scientifique. Ces deux conférences ont permis d'échanger les points de vue sur ces problématiques actuelles. <

Partenariat médecine/sciences - Écoles doctorales (5)

**Nos jeunes pousses
ont du talent !**



Ce travail de rédaction a été encadré par Isabelle Cremer et Catherine Monnot, directrice et directrice adjointe de l'ED394.

isabelle.cremer@crc.jussieu.fr

catherine.monnot@college-de-france.fr

Série coordonnée par Laure Coulombel.

COMPTE RENDU DE CONGRÈS

Les journées 2016 de l'école doctorale « physiologie, physiopathologie et thérapeutique » de l'Université Pierre et Marie Curie*

Sami Ayari¹, Cécile Bandet², Marion Dajon³, Ray El Boustany⁴, Sara Ghezzal¹, Annaelle Jarossay⁵, Hélène Kaplon³, Barbara Postal¹, Élodie Pramil⁶, Jules Russick⁵, pour le comité d'organisation des journées

Des résultats scientifiques à l'honneur !

Les doctorants ont récompensé les présentations de plusieurs de leurs collègues dans les domaines de la physiologie cardio-vasculaire, de l'inflammation, des pathologies virales et génétiques ou de la fonction intestinale, sélectionnées sur l'intérêt scientifique et la qualité de la présentation (orale ou poster).

* « L'école doctorale physiologie, physiopathologie et thérapeutique (P2T)/ED394 est une école doctorale accréditée par l'Université Pierre et Marie Curie (UPMC) et associée à l'Institut Pasteur, l'Institut Curie, l'INRA, l'IRSN et le Collège de France. Cette école doctorale est pluridisciplinaire, regroupant environ 250 doctorants dans le domaine des sciences de la vie avec une forte composante en biologie, médecine et santé ». (présentation tirée du site http://www.ed394.upmc.fr/fr/l_ecole_doctorale.html).

• *Un modèle murin d'hypertension d'origine sympathique.* L'intervention d'Émilie Simonnet¹ a été très remarquée. Dans le cadre de son projet de thèse, Émilie a mis en évidence le rôle crucial de l'innervation sympathique des artérioles. Celle-ci participe à la régulation de leur diamètre, et donc du flux sanguin dans les tissus périphériques. Émilie a en effet démontré *in vitro* et *in vivo* que lorsque l'innervation

¹Équipe « physiopathologies intestinales : nutrition et fonction de barrière », UMRS 1138 (Armelle Leturque), centre de recherche des Cordeliers, Paris, France ; ²équipe « pathogenèse cellulaire et clinique du diabète », UMRS 1138 (Fabienne Foufelle), Centre de recherche des Cordeliers ; ³équipe « Cancer et immunité anti-tumorale », UMRS 1138, (Isabelle Cremer et Jean-Luc Teillaud), Centre de recherche des Cordeliers ; ⁴équipe « Physiopathologie et thérapeutiques des maladies vasculaires et rénales liées au diabète et à la nutrition » (Ronan Roussel), Centre de recherche des Cordeliers ; ⁵équipe « Immunopathologie et immuno-intervention thérapeutique » UMRS 1138 (Sébastien Lacroix-Desmazes), Centre de recherche des Cordeliers ; ⁶équipe « Mort cellulaire et résistance aux traitements dans les hémopathies malignes » UMRS 1138 (Santos A. Susin), Centre de recherche des Cordeliers, Paris, France.

artérielle se met en place, les artères expriment une molécule de guidage axonale, l'éphrine-A4, qui possède une action répulsive sur les neurones sympathiques. Les souris génétiquement inactivées pour le récepteur Ephrine-A4 présentent donc une innervation artérielle accrue, associée à une hypertension. Ces souris pourraient ainsi constituer le premier modèle murin d'hypertension d'origine sympathique, permettant de tester de nouvelles voies thérapeutiques dans le traitement de l'hypertension artérielle.

¹ Équipe « développement et physiologie des interactions neurovasculaires », Inserm U1050 - UMR7241 CNRS, Centre interdisciplinaire de recherche en biologie, Collège de France.



• *Récepteur des minéralocorticoïdes et inflammation*. Mathieu Buonafine² a démontré que dans les cellules dendritiques, l'activation du récepteur aux minéralocorticoïdes par l'aldostérone jouait un rôle fondamental au cours de l'inflammation observée lors d'une hypertension induite par l'aldostérone chez la souris. Il a pu mettre en évidence, grâce à des modèles de souris transgéniques, que cet effet implique la protéine *neutrophil gelatinase-associated lipocalin*, dont le rôle est encore mal connu. Cette dernière pourrait jouer un rôle majeur dans la médiation des effets pathologiques de l'activation du récepteur aux minéralocorticoïdes, notamment lorsqu'elle est exprimée par certains types de cellules immunitaires.

• *Réponses immunitaires différentielles et statut des patients VIH*. Concernant les pathologies virales, deux travaux portant sur le VIH (virus de l'immunodéficience humaine) se sont particulièrement démarqués. Tout d'abord, Angeline Rouers³ a montré que la fonctionnalité des lymphocytes T folliculaires *helper* (Tfh) était altérée lors de l'infection par le VIH. Elle a également démontré que dans le sang des patients *elite controllers*⁴, la réponse des lymphocytes B mémoires anti-VIH était maintenue, contrairement à celle des patients non *controllers* traités. Reste à savoir si chez ces patients *elite controllers*, les fonctionnalités des Tfh sont également intactes. Ainsi, une meilleure compréhension des mécanismes cellulaires lors de l'infection par le VIH pourrait contribuer à l'élaboration de nouvelles stratégies vaccinales anti-VIH.

• *Macrophages et contrôle du réservoir du VIH*. Ester Gea-Mallorqui⁵ a mon-

tré que les macrophages font partie des cellules réservoirs qui empêchent l'éradication complète du VIH. Le VIH-2, très semblable au VIH-1, est mieux contrôlé par le système immunitaire. L'objectif du travail d'Esther est d'analyser cette différence, et notamment de comparer des macrophages infectés *in vitro* par l'un ou l'autre de ces deux virus. Ce travail a permis d'identifier des différences majeures de la distribution dans les macrophages d'une protéine virale, la production virale et la transmission aux lymphocytes T du virus présent dans les macrophages. La connaissance de ces différences pourrait contribuer à un meilleur contrôle de l'infection par le VIH-2.

• *Une nouvelle stratégie thérapeutique dans la mucoviscidose ?* Dans le domaine de la génétique, le travail de Florence Sonnevill⁶ porte sur la mucoviscidose : il démontre que le canal chlorure ANO1 (*anoctamin-1*) pourrait être un canal alternatif pour compenser la protéine déficiente (le canal chlorure CFTR, *cystic fibrosis transmembrane conductance regulator*). Florence a montré que le microARN miR-9 inhibe le gène *ANO1*. Pour lever cette inhibition, une molécule spécifique a été synthétisée : le TSB ANO1. Dans le contexte de la mucoviscidose (modèles de lignées cellulaires épithéliales bronchiques, ou de cultures de cellules primaires issues de patients et de souris), cette molécule restaure les efflux de chlorure ainsi que de nombreux paramètres dérégulés *in vitro* et *in vivo*. Lever l'inhibition de l'expression de cette protéine pourrait donc être considéré comme une nouvelle stratégie thérapeutique.

• *Absorption des sucres intestinaux*. Enfin, dans la thématique métabolisme et nutrition, le travail de Charlotte Schmitt⁷ a été récompensé. Il porte sur les mécanismes de contrôle des fonc-

tions intestinales dans l'obésité et le diabète. Charlotte s'intéresse en particulier à l'absorption intestinale des sucres alimentaires, fonction vitale pour l'organisme. Elle a montré que le transporteur GLUT2 est impliqué dans l'absorption des sucres et dans la plasticité des cellules endocrines de l'intestin.

• *Cellules immunitaires intestinales et obésité*. Sothea Touch⁸ présentait un *poster* sur le rôle des cellules immunitaires intestinales dans la physiopathologie de l'obésité. Ses résultats ont révélé l'importance du recrutement des cellules immunitaires contribuant à l'inflammation dans cette pathologie. Elle a entrepris une étude des cellules immunitaires sanguines et intestinales de sujets obèses, qui démontre l'altération globale de l'immunité dans ces deux compartiments. Ces données sont actuellement confrontées aux paramètres cliniques et à la composition du microbiote intestinal à la recherche d'un lien entre les modifications du microbiote, l'état inflammatoire et les dysfonctions métaboliques liés à l'obésité humaine.

Le respect de l'intégrité scientifique : une conférence de Pierre Corvol

Docteur en médecine et chercheur, Pierre Corvol, professeur émérite (chaire de médecine expérimentale) et ancien administrateur du Collège de France, vice-président de l'Académie des Sciences, a été chargé par le ministre de l'Enseignement supérieur et de la Recherche d'établir un rapport sur l'intégrité scientifique et de faire des recommandations pour la promouvoir. Il est intervenu lors d'une séance plénière des Journées de notre École doctorale « physiologie, physiopathologie et thérapeutique » sur ce sujet de grande actualité, la formation des doctorants à l'intégrité scientifique étant désormais stipulée dans l'arrêté du 25 mai 2016 fixant le cadre national de la formation

² Équipe « récepteur minéralocorticoïde : physiologie et innovations thérapeutiques », UMR51138 Inserm, centre de recherche des Cordeliers.

³ Équipe « immunobiologie des infections virales et présentation des antigènes », Inserm U1135, CR7 UMRS UPMC, centre de recherche immunologie et maladies infectieuses (CIMI).

⁴ Sur cette dénomination, voir la Nouvelle de V. Martinez et B. Autran, *Med Sci (Paris)* 2008 ; 24 : 7-9.

⁵ Équipe « transport intracellulaire et immunité », Inserm U932, Institut Curie.

⁶ Équipe « mucoviscidose : physiopathologie et phéno-génomique », UMR5938 Inserm, Centre de recherche Saint Antoine.

⁷ Équipe « physiopathologies intestinales : nutrition et fonction de barrière », UMR51138 Inserm, centre de recherche des Cordeliers.

⁸ Équipe « nutriOMICs », Inserm U1166, ICAN, hôpital la Pitié-Salpêtrière.



conduisant à la délivrance du diplôme national de doctorat.

Lors de son intervention, Pierre Corvol a défini l'intégrité scientifique puis en a exposé les enjeux. Rapidement, il a lancé un dialogue constructif avec les doctorants sur l'importance de l'intégrité scientifique dans notre travail au quotidien. En effet, la recherche scientifique se doit de contribuer à faire évoluer les connaissances de la Science de façon honnête et intègre. La communauté scientifique, et plus largement la société, doivent avoir confiance dans les différents résultats publiés. Le débat sur l'intégrité scientifique a permis de pointer du doigt des méconduites scientifiques, voire des fraudes. Nous avons pu échanger nos points de vue concernant l'arrangement ou la falsification des données, telle que l'augmentation de l'échantillonnage de façon factice pour rendre un résultat statistiquement significatif. Nous avons conclu que ce genre de pratiques est considéré comme une fraude. De plus, ces pratiques peuvent avoir un impact néfaste et important dans les domaines de la santé et de l'environnement. Le coût financier qu'elles peuvent engendrer est loin d'être négligeable pour la société. Nous avons également discuté d'une autre problématique concernant l'intégrité scientifique, le plagiat. N'oublions pas que le plagiat est une forme de méconduite assez répandue et qui fait l'objet de sanctions. Ce comportement peut conduire à des poursuites pénales. Les causes des dérives évoquées ci-dessus sont multiples. Entre 2010 et 2015, dans 27 universités et 8 établissements de recherche en France et à l'international, il a été relevé 22 falsifications de données ; 46 cas de plagiat ; 6 conflits d'intérêt. Par ailleurs, la surinterprétation de données ou de résultats publiés peut conduire à émettre des conclusions scientifiques erronées. Toutes ces formes de méconduites peuvent être motivées par des enjeux de carrière, notamment l'obtention d'un poste stable chez le jeune chercheur, mais

aussi par la recherche de financement ou encore par un besoin de reconnaissance. Afin de contrôler et de pouvoir repérer ce type de comportement, la communauté scientifique internationale a élaboré des codes ou des chartes. De ce fait en 2011, *The European Code of Conduct for Research Integrity*⁹ a été mis en place. En 2015, les universités et les principaux organismes de recherche en France ont signé une charte nationale de déontologie des métiers de la recherche¹⁰. D'autres moyens ont également été développés pour lutter contre tous ces manquements à l'intégrité, notamment sur les réseaux sociaux (<https://www.pubpeer.com> et <https://www.retractionwatch.com>). Ainsi, cela permet de repérer et de diffuser les diverses falsifications de données dans un article. En effet, certains auteurs prétendent avoir réalisé leur expérience en « triplicate », or la figure correspondant à la dite expérience ne présente ni moyenne ni barre d'erreur.

L'accès aux données brutes de la recherche et le projet de Loi « Pour une République numérique¹¹ » devraient contribuer à une recherche intègre et responsable. Enfin, rappelons que c'est à chaque scientifique qu'incombe la responsabilité d'avoir une conduite exemplaire et de produire une science pleinement intègre. Cette conférence a permis de souligner l'importance de la génération et de la divulgation de données scientifiques originales fiables pour la société d'aujourd'hui et de demain.

Transmettre la science : un enjeu de société

Nous avons également choisi d'organiser une table ronde sur le thème de

la médiation scientifique. Nous avons souhaité ouvrir le débat sur les enjeux de la communication scientifique avec les doctorants et nos invités qui en ont fait leur métier. Cette table ronde a été animée par Ange Ansur (coordinatrice éducative « Les Savanturiers¹² »), Mathieu Buonafine (doctorant et grand vainqueur national du concours « Ma thèse en 180 secondes¹³ »), Anne Nassif (directrice de publication de « Nutrimedia¹⁴ ») et Noémie Naguet (chargée de communication au CNRS et à « L'esprit sorcier¹⁵ »).

Le profil varié des invités a rapidement soulevé la question suivante : qui est le mieux placé aujourd'hui pour informer le public de la science ?

En effet, de nombreuses formations permettent d'accéder au métier de médiateur scientifique et les scientifiques ne sont plus les seuls acteurs de la communication scientifique. Noémie Naguet explique avoir suivi une formation en communication pendant un an avec le Cnam (centre national des arts et métiers) car elle souhaitait obtenir la légitimité de pouvoir faire de la communication scientifique après l'obtention de son doctorat en biologie. De même, Ange Ansur dévoile un parcours de traductrice avant de devenir professeure des écoles et de se mettre au service des Savanturiers. Les moyens de communication (vidéos publiées sur Internet, réseaux sociaux, blogs, milieux associatifs, etc.) sont de plus en plus nombreux et permettent de s'adresser à un public plus large (sans qu'une formation scientifique soit indispensable). Leur multiplication témoigne du besoin de diversité des stratégies de communication afin de combler le fossé qui s'est installé entre les scientifiques et le public. Les enjeux sont multiples et loin d'être négligeables. La médiation scientifique intervient non seulement

⁹ Document téléchargeable à l'adresse : http://ec.europa.eu/research/participants/data/ref/h2020/other/hi/h2020-ethics_code-of-conduct_en.pdf

¹⁰ Document téléchargeable à l'adresse : http://www.cnrs.fr/comets/IMG/pdf/charte_nationale_deontologie_signe_e_janvier2015.pdf

¹¹ LOI n° 2016-1321 du 7 octobre 2016 pour une République numérique. Voir le site légifrance <https://www.legifrance.gouv.fr/affichLoiPubliee.do?idDocument=JORFDOLE000031589829&type=general&legislature=14>

¹² <http://les-savanturiers.cri-paris.org/>

¹³ <http://mt180.fr/>

¹⁴ <http://www.nutrimedia.fr/>

¹⁵ <http://www.lespritsorcier.org/>

pour transmettre le savoir mais également pour répondre à des questions d'ordre sociétal. La situation est parfois délicate comme le montre une enquête de l'Institut d'études d'opinion Ipsos, qui a révélé que certains Français ne font plus confiance aux scientifiques quand il s'agit de dire la vérité sur leurs résultats et leurs travaux. Un étudiant présent dans la salle a tenu à rappeler une polémique récente illustrant la défiance des Français vis-à-vis de l'innocuité des vaccins, notamment ceux qui contiennent des sels d'aluminium utilisés comme adjuvant et suspectés de déclencher une myopathie inflammatoire : la myofasciite à macrophages. D'aucuns se sont interrogés sur la rigueur de la vulgarisation scientifique. À force de trop vouloir simplifier le contenu scientifique, le risque est réel de déformer les propos initiaux quel que soit le public auquel on s'adresse. Ces questions ont ensuite laissé place à de nouvelles interrogations sur les contenus scientifiques du Palais de la découverte ou bien de la Cité des sciences : ceux-ci sont très souvent destinés aux enfants étant donné que de moins en moins d'adultes se rendent dans des musées ayant pour thématique les sciences. En effet, une sorte de cercle vicieux s'est installé : plus les enfants sont nombreux, plus les contenus scientifiques seront adaptés aux enfants, ce qui aura pour conséquence d'attirer encore plus d'enfants et ainsi de suite. Cette tendance génère une base solide d'accès aux sciences pour les enfants, et donc la société à venir, mais ne permet pas de remédier à la méfiance vis-à-vis des sciences des adultes de la société actuelle.

Une réflexion a également été engagée sur les moyens qui pourraient être mis en place pour inciter davantage les gens et les chercheurs à s'investir dans des actions de médiation scientifique. Comment les activités de médiation scientifique d'un chercheur peuvent-elles être évaluées par les instances d'évaluation des organismes de recherche et comment peut-on juger ces activités ? Certains scientifiques estiment que la publication des résultats de leurs travaux fait office de médiation scientifique, d'autres leur font remarquer que ces publications ne sont compréhensibles que par leurs pairs et restent incompréhensibles pour le grand public. Par ailleurs, les doctorants sont désireux de s'investir davantage dans la médiation scientifique malgré les éventuelles difficultés auxquelles ils sont souvent confrontés (manque de temps, manque d'outils et de formation et faible valorisation de cette activité). Néanmoins, les activités de médiation scientifique se diversifient au sein des écoles doctorales. « Ma thèse en 180 secondes » est un exemple d'un nouveau moyen de médiation mis en place pour les doctorants. Le défi pour les doctorants est de présenter leur projet de recherche de manière concise en trois minutes avec l'appui d'une seule diapositive. « *Le concours MT180 est un vrai défi de communication et de vulgarisation scientifique* » témoigne Mathieu Buona-fine, vainqueur de la finale nationale 2016 de Ma thèse en 180 secondes, organisée à Bordeaux. Il a remporté le premier prix du jury pour sa présentation sur l'étude du rôle de la *neutrophil gelatinase associated lipocalin* dans les effets cardiovasculaires de l'activation du récepteur des

minéralocorticoïdes. « *Il [ce concours] m'a permis de faire découvrir mon travail de thèse à un public très large et de lui ouvrir une petite porte sur le monde complexe de la biologie et de la démarche scientifique [...]. C'est un challenge très formateur qui m'a permis de m'aguerrir à la prise de parole en public et d'aborder mon travail de thèse sous un angle complètement différent* », explique-t-il.

En conclusion

Les journées de l'École doctorale 394 ont été une bonne occasion pour les doctorants venant de domaines très différents d'échanger et d'interagir scientifiquement et techniquement, soulignant l'intérêt de la pluridisciplinarité de notre École doctorale. De plus, de nombreuses idées discutées au cours de sessions spéciales (table ronde et conférence plénière), ont permis l'ouverture sur des questions d'avenir, de médiation et d'intégrité. L'ensemble du comité organisateur tient à remercier les doctorants, les sponsors ainsi que les directrices et la gestionnaire de l'École doctorale, acteurs qui ont permis la concrétisation de ces deux journées scientifiques très enrichissantes. ♦


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LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

TIRÉS À PART

I. Cremer et C. Monnot



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Obesity is associated with chronic low-grade inflammation, which contributes to metabolic disorders. However, the origin of systemic inflammation observed at subclinical level in obese patients is still unclear. Recent studies highlighted a central role of the intestine in metabolic diseases. Our team reported a low-grade inflammation in the small intestine of obese subjects, but the underlying molecular mechanisms are still to be revealed. The causes of intestinal inflammation may be due to nutrients and environmental factors. Indeed, studies in healthy subjects and wild-type rodents exposed to a lipid diet reveal an increase in endotoxemia, which is characterized by bacteria wall fragments translocation through the intestinal mucosa into blood. These findings allowed us to hypothesize a role of the intestine and environmental changes such as dietary lipids in the initiation of this inflammation. My specific aims were to investigate the role of palmitic acid and of the transcription factor Aryl Hydrocarbon receptor (AhR), which is sensitive to environmental changes and known to be involved in inflammation, in the onset or modulation of gut inflammation.

In a first study, I analyzed the impact of palm oil on the set up of gut inflammation in mice. My results showed that palm oil gavages during 5 days increased the intestinal permeability, altered the subcellular localization of several junctional proteins and modulated the intestinal expression of pro-inflammatory genes (IL-1 β , Gata-3, Reg-3 γ). Repeated supply of palm oil also modified the composition of intestinal microbiota. I then studied the effect of palmitic acid on a polarized monolayer of the human intestinal epithelial Caco-2/TC7 cell line. The results indicated that palmitic acid treatment disrupted the epithelial barrier and increased IL-8 cytokine mRNA level and secretion. I showed that *de novo* ceramide synthesis pathway induced by palmitic acid in Caco-2/TC7 cells controlled the IL-8 cytokine expression. Together, our results show that dietary lipids can rapidly induce barrier defects and an intestinal inflammatory response.

Afterwards, I evaluated the role of AhR on the intestinal inflammation in obese subjects, and on gut barrier integrity both in mice and Caco-2/TC7 cells. Severely obese patients were phenotyped for multiple bioclinical parameters. Jejunal samples were gathered from obese and lean patients. We measured the expression levels of AhR target genes in jejunal samples of patients according to their score of intestinal inflammation using an index of T cells infiltration in epithelium. We observed that gut inflammation in obese patients was higher than in lean patients. Moreover, obese patients with a high gut inflammation score showed a lower expression of AhR target genes. We hypothesized that the activation of AhR induced by an agonist may thus decrease gut inflammation and improve gut barrier function in mice and in human enterocytic cells. We set up inflammatory environment or epithelial barrier impairment by damaging intestinal epithelium integrity *in vivo* with palm oil and *in vitro* with a calcium chelator. My results showed that AhR activation in mice prevented alteration of cell-cell junctions triggered by palm oil. In Caco-2/TC7 cells, AhR activation counteracted the damages on the epithelium and decreased cytokine expression. These beneficial effects of AhR activation might involve PKC and p38MAP kinase pathways. Thus, we show that a reduced intestinal AhR tone is associated with elevated intestinal inflammation in obese patients and the AhR activation can exert a protective effect on the intestinal barrier integrity and reduce the gut inflammation.

Altogether, these studies demonstrated that alteration of the intestinal epithelial barrier integrity is an important contributor of pro-inflammatory response in gut. Intestinal AhR activation protected the gut epithelium from inflammatory responses and barrier function damage. Although the mechanisms by which AhR activation exert a protective effect on gut need further investigations, AhR might be a possible therapeutic target to limit gut damage in metabolic diseases.

Keywords: intestine, dietary lipids, AhR, intestinal barrier, tight junctions, inflammation, metabolic diseases

L'obésité est associée à un état inflammatoire systémique chronique évoluant à bas bruit, qui contribue aux complications métaboliques observées dans cette pathologie. L'origine de cette inflammation est mal connue, cependant des études récentes ont mis en évidence un rôle central de l'intestin dans les maladies métaboliques. Notre équipe a montré une inflammation de l'intestin grêle chez des patients obèses, dont l'origine pourrait être due à des facteurs nutritionnels ou environnementaux. La présence d'une endotoxémie, signant le passage de fragments bactériens à travers la muqueuse intestinale, chez des sujets sains ou des rongeurs soumis à un régime riche en lipides, a permis d'émettre l'hypothèse d'un rôle de la barrière intestinale et des changements environnementaux comme les lipides alimentaires dans l'initiation de l'inflammation intestinale voir systémique. Pour étayer cette hypothèse, j'ai étudié le rôle d'un lipide alimentaire, l'acide palmitique, et du facteur de transcription Aryl Hydrocarbon (AhR), sensible aux changements environnementaux et connu pour son rôle dans l'inflammation.

Dans une première étude, nous avons analysé l'impact de l'huile de palme sur la mise en place de l'inflammation intestinale chez la souris. Mes résultats ont montré que l'apport répété pendant 5 jours d'huile de palme augmente la perméabilité intestinale, modifie la localisation subcellulaire de plusieurs protéines de jonction et l'expression des gènes impliqués dans les réponses pro-inflammatoires (IL-1 β , Gata-3, Reg-3 γ). Cet apport répété d'huile de palme modifie également la composition du microbiote intestinal. J'ai ensuite étudié l'impact de l'acide palmitique sur les cellules intestinales épithéliales humaines Caco-2/TC7. Les résultats indiquent que l'acide palmitique perturbe la barrière épithéliale et augmente l'expression et la sécrétion de la cytokine pro-inflammatoire IL-8. L'étude des mécanismes moléculaires induits par l'acide palmitique dans les cellules Caco-2/TC7 a révélé l'implication de la voie de synthèse *de novo* des céramides dans l'expression d'IL-8. Nos résultats montrent donc que les lipides alimentaires, en particulier l'acide palmitique, peuvent induire rapidement des défauts de la barrière et une réponse inflammatoire intestinale.

J'ai par la suite évalué le rôle d'AhR sur l'inflammation intestinale chez des patients obèses. Le phénotype bioclinique des sujets obèses a été caractérisé et des échantillons de jéjunum ont été recueillis après chirurgie bariatrique. Nous avons déterminé le score de l'inflammation de l'intestin grêle par l'index d'infiltration épithéliale des lymphocytes T et les taux d'expression des gènes cibles d'AhR ont été mesurés et comparés à des jéjunum de patients minces. Nous avons observé que les sujets obèses présentent une densité de lymphocytes T plus élevée que les sujets minces. De plus, l'inflammation intestinale chez les sujets obèses est négativement corrélée au taux d'expression des gènes cibles d'AhR. Nous avons fait l'hypothèse que l'activation d'AhR par un agoniste pourrait diminuer l'inflammation intestinale et améliorer la fonction de barrière chez la souris et dans les cellules Caco-2/TC7. Nous avons induit un environnement inflammatoire ou une altération de la barrière épithéliale intestinale *in vivo* avec de l'huile de palme et *in vitro* avec un agent chélateur du calcium. Mes résultats ont montré que l'activation d'AhR chez la souris empêchait l'altération des jonctions cellule-cellule déclenchée par l'huile de palme. De plus, dans le modèle cellulaire Caco-2/TC7, l'activation d'AhR par un agoniste a limité les dommages induits chimiquement sur l'intégrité de la barrière épithéliale et sur la sécrétion de cytokines pro-inflammatoires. Les effets bénéfiques de l'activation d'AhR interviendraient via des voies de signalisation des protéines kinases PKC et p38MAPK. Ainsi, nos résultats montrent qu'un niveau réduit d'activation intestinale d'AhR est associé à une inflammation intestinale élevée chez les patients obèses et que l'activation d'AhR peut exercer un effet protecteur sur l'intégrité de la barrière intestinale et réduire l'inflammation dans ce tissu.

L'ensemble de cette étude démontre que la détérioration de l'intégrité de la barrière intestinale contribue à l'inflammation dans l'épithélium intestinal. Un effet protecteur contre les atteintes intestinales pourrait être obtenus par l'activation du facteur de transcription AhR dont une partie des cibles intestinales reste encore à découvrir. AhR pourrait ainsi constituer une option thérapeutique dans les maladies métaboliques pour limiter les dommages de la barrière et l'inflammation tissulaire.

Mots-clés : intestin, lipides, AhR, barrière intestinale, jonctions serrées, inflammation, maladies métaboliques