Development of the gastrointestinal tract in young mammals

Effects of enteral provocation with protease or phytohaemagglutinin in neonatal rats

Arevalo Sureda, Ester

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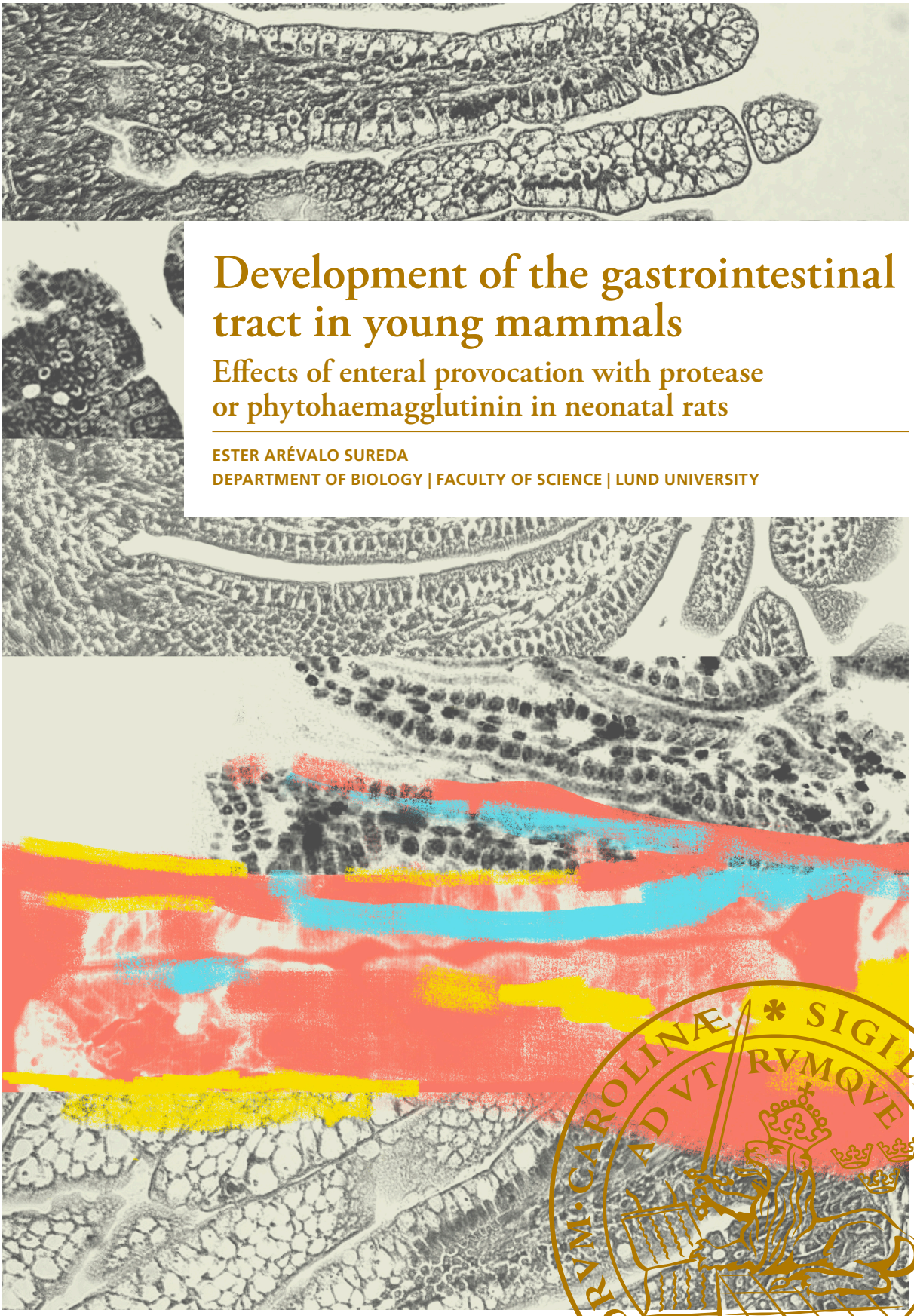

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DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY

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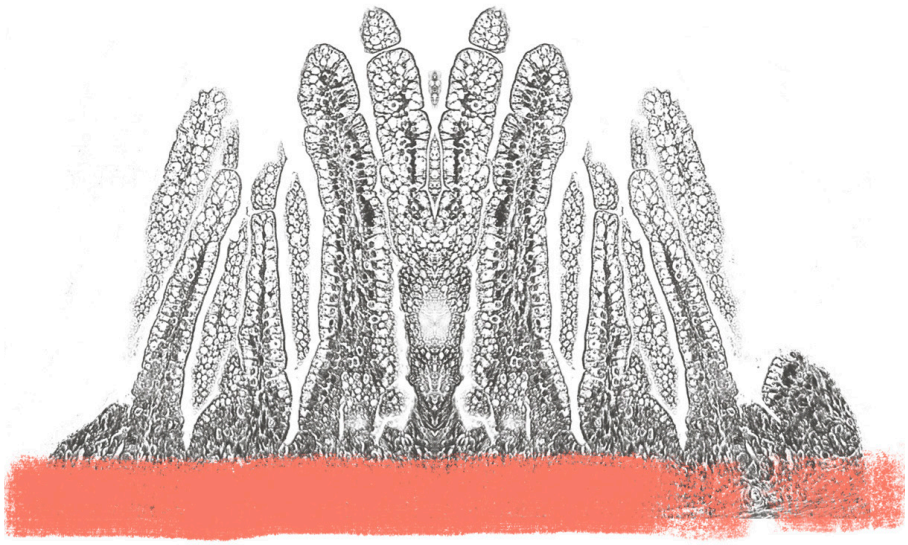

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Ester Arévalo Sureda

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Abstract

The rat, as an altritial species, is born with an immature gastrointestinal tract and intestinal barrier function, which is highly absorptive to milk-borne bioactive molecules that can pass undigested and reach the general circulation of the suckling newborn. This passage occurs by the neonatal-Fc-receptor (FcRn) binding and trancytosis of immunoglobulin G in the proximal small intestine (SI) and by the highly endocytic vacuolated enterocytes non-selectively in the distal SI. Postnatal gut maturation accelerates at weaning, around postnatal day 21, coincident with the dietary transition from milk to solid food. Maturation of the gut can also be precociously induced by provocation with a lectin, phytohaemagglutinin (PHA), mimicking the naturally occurring changes in gut structure and function. The changes occurring during natural or induced gut maturation include stimulation of pancreatic function and cessation of the SI absorptive capacity to macromolecules (gut closure). Intestinal epithelial maturation has been related to the gut immune system and is suggested to depend on T-lymphocytes activation. Recently, the transcription factor B-lymphocyte-induced maturation-protein-1 (Blimp-1) has been proposed to be a key regulator of intestinal maturation in mice. Hence, the present study investigated the events occurring during gut development and the cues initiating the process. The study especially focused on changes in the barrier function and macromolecular permeability, pancreatic function, and the relation to gut immune factors.

A novel animal model of pancreatic and pancreatic-like protease-induced precocious gut maturation was established in neonatal rats, and was used in comparison to the existing PHA-induced model, as well as natural gut development. The gut maturational changes observed during natural or induced maturation, by both protease or PHA, included the transition of foetal- to adult- type SI epithelium, with reduced FcRn expression in the proximal part and disappearance of vacuolated enterocytes in the distal part, associated with a similar change in intestinal epithelial Blimp1 expression. The early effects after exposure to the provocative agents, PHA and protease, revealed that both agents hampered macromolecular permeability and only protease also caused an increase in epithelial leakiness of the distal SI. These results indicated that protease and PHA affected the intestinal barrier function differently. Furthermore, the provocative agents were also tested in neonatal athymic nude rats, T-cell immunodeficient, and they appeared to be susceptible to induced precocious gut maturation. These results suggested that gut maturation is independent of thymus-derived T-celsl, but the involvement of other immune cells types, possibly innate immune cells, should be further investigated.

Thus, the findings of the present thesis will contribute to an increased understanding of initiating cues and the mechanisms of maturation of the intestinal barrier in young mammals. The knowledge obtained could be applied to improve strategies for the treatment of gut-related complications, often affecting premature infants.

Key words: gut, intestine, pancreas, development, precocious, protease, PHA, enterocytes, permeability, endocytosis, IgG, FcRn, Blimp1, T-lymphocyte, passive immunity, altricial, neonatal, suckling, athymic, rat, immunohistochemistry

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To you (not only, but also)

Qui fa tot el que pot, no esta obligat a més
El refranyer popular català i la Mama

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Paper I

Prykhodko, O., Pierzynowski, S. G., Nikpey, E., Arévalo Sureda, E., Fedkiv, O., Weström, B. R. (2015).

Pancreatic and pancreatic-like microbial proteases accelerate gut maturation in neonatal rats.

PLoS ONE, 10(2), e0116947. doi: 10.1371/journal.pone.0116947.

Paper II

Arévalo Sureda E, Weström B, Pierzynowski S, Prykhodko O. (2016).

Maturation of the Intestinal Epithelial Barrier in Neonatal Rats Coincides with Decreased FcRn Expression, Replacement of Vacuolated Enterocytes and Changed Blimp1 Expression.

PLoS ONE, 11 (10), e0164775. doi: 10.1371/journal.pone.0169724.

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Paper III

Arévalo Sureda E, Gidlund C, Weström B, Prykhodko O.

Intestinal precocious maturation can be induced in athymic (nude) neonatal rats despite their T-cell deficiency.

Manuscript submitted to the American Journal of Physiology – Regulatory, Integrative and Comparative Physiology (2017).

Paper IV

Arévalo Sureda E, Prykhodko O., Weström B.

Early effects on the gut and the intestinal permeability after enteral provocation with protease or phytohaemagglutinin in neonatal rats.

Manuscript.

Author Contributions

Paper I

Created the hypothesis: OP SGP
Conceived and designed of the work: OP SGP BRW
Data collection: OP EN
Analysed the data: OP SGP EN EAS OF
Data interpretation: OP SGP EN EAS OF BW
Writing – original draft: OP
Wrote the paper: OP SGP EN EAS OF BW

Paper II

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Writing – original draft: EAS
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Paper IV (Manuscript)

Conception and design of the work: EAS BW
Data collection and analysis: EAS BW
Data interpretation: EAS OP BW
Writing – original draft: EAS
Critical revision of the article: OP BW

Conference abstracts

During the course of this PhD project preliminary results, some of them included in this thesis as additional unpublished data have been presented at international scientific conferences in poster format by the author, or as specified.

Role and expression of neonatal-Fc-receptor in the small intestine during normal and precociously induced maturation in the neonatal rat.

Arévalo Sureda E, Prykhodko O, Pierzynowski SG and Weström B

In: Abstracts from the 26th Meeting of the European Intestinal Transport Group (EITG), 2–5 October 2014. Acta Physiologica. 2015;214 (Supplement S701):1-16. doi: 10.1111/apha.12498. Poster.

***In vivo* macromolecule absorption in wild-type and immunodeficient athymic neonatal rats during lectin-induced maturation.**

Prykhodko O, Arévalo Sureda E, Zhou J, Chopek A, Fedkiv O, Pierzynowski SG and Weström B

In: Abstracts from the 26th Meeting of the European Intestinal Transport Group (EITG), 2–5 October 2014. Acta Physiologica. 2015;214 (Supplement S701):1-16. doi: 10.1111/apha.12498. Oral presentation by Olena Prykhodko.

Expression of the transcription repressor Blimp1 correlates to the disappearance of FcRn expression in proximal and vacuolated cells in distal small intestine during development in neonatal rats.

Arévalo Sureda E, Prykhodko O, Pierzynowski SG and Weström B

In: 48th Annual meeting of the European Society for Pediatric Gatroenterology, Hepatology and Nutrition (ESPGHAN): 2015; Amsterdam, The Netherlands. Poster. PO-G-0009

Increased pancreatic protease activity in relation to PAR2 receptor expression during intestinal postnatal development in rats.

Arévalo Sureda E, Weström B, Pierzynowski SG and Prykhodko O

In: 48th Annual meeting of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN): 2015; Amsterdam, The Netherlands. Poster. PO-G-0014.

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Precocious gastrointestinal maturation can be induced in T-cell deficient athymic (nude) suckling rats

Arévalo Sureda E, Prykhodko O, Zhou J, Weström B

In: 49th Annual meeting of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN): 2016; Athens, Greece. Poster. G-P-002.

Publications not included in the thesis

Lozinska, L., Prykhodko, O., Sureda, E. A., Szwiec, K., Podgurniak, P., Pierzynowski, S., & Weström, B. (2015). **Monitoring changes in plasma levels of pancreatic and intestinal enzymes in a model of pancreatic exocrine insufficiency - induced by pancreatic duct-ligation - in young pigs**. Advances Medical Science, 60(1), 112-117. doi: 10.1016/j.advms.2015.01.003.

Abbreviations

BIgG	Bovine Immunoglobulin G
Blimp1	B-Lymphocyte Induced Maturation Protein – 1
BSA	Bovine Serum Albumin
CCK	Cholecystokinin
CD	Cluster of Differentiation
DAO	Diamine Oxidase
DCs	Dendritic Cells
FcRn	Neonatal-Fc Receptor
FD4	FITC-dextran 4kDa
FD70	FITC-dextran 70kDa
FITC	Fluorescein isothyanate
GI	Gastrointestinal
g bwt	grams body weight
HRP	Horse Radish Peroxidase
HSA	Human Serum Albumin
IECs	Intestinal Epithelial Cells
IELs	Intraepithelial Lymphocytes
Ig	Immunoglobulin
IL	Interleukin
ILCs	Innate Lymphocytes
kDa	kilo Dalton

Lac	Lactulose
MHC	Major Histocompatibility Complex
Man	Mannitol
Nude	Athymic T-Cell Deficient Nude Rats
PARs	Protease Activated Receptors
PBS	Phosphate Buffered Saline
PHA	Phytohaemagglutinin
PPs	Peyer's Patches
Prot	Protease
RIgG	Rat Immunoglobulin G
RT	Room Temperature
SD	Sprague Dawley
SI	Small Intestine
SPF	Specific Pathogen Free
TGF	Transforming Growth Factor
Th	T-helper lymphocyte
TLR	Toll-Like Receptors
TNF	Tumour Necrosis Factor
Treg	Regulatory T-lymphocyte
Å	Ångstrom

Abstract

The rat, as an altritial species, is born with an immature gastrointestinal tract and intestinal barrier function, which is highly absorptive to milk-borne bioactive molecules that can pass undigested and reach the general circulation of the suckling newborn. This passage occurs by the neonatal-Fc-receptor (FcRn) binding and trancytosis of immunoglobulin G in the proximal small intestine (SI) and by the highly endocytic vacuolated enterocytes non-selectively in the distal SI. Postnatal gut maturation accelerates at weaning, around postnatal day 21, coincident with the dietary transition from milk to solid food. Maturation of the gut can also be precociously induced by provocation with a lectin, phytohaemagglutinin (PHA), mimicking the naturally occurring changes in gut structure and function. The changes occurring during natural or induced gut maturation include stimulation of pancreatic function and cessation of the SI absorptive capacity to macromolecules (gut closure). Intestinal epithelial maturation has been related to the gut immune system and is suggested to depend on T-lymphocytes activation. Recently, the transcription factor B-lymphocyte-induced maturation-protein-1 (Blimp-1) has been proposed to be a key regulator of intestinal maturation in mice. Hence, the present study investigated the events occurring during gut development and the cues initiating the process. The study especially focused on changes in the barrier function and macromolecular permeability, pancreatic function, and the relation to gut immune factors.

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Introduction

The digestive system

The digestive system consists of the gastrointestinal (GI) tract and the accessory organs; including the liver and the pancreas. All the organs within the digestive system form a complex system which functions in the digestion and absorption of nutrients, the elimination of food components that are not absorbed or excreted and the protection of the organism. The GI tract is a tube extending from the mouth to the anus, and its inner surface comes into continuous contact with exogenous substances that are consumed via eating. Due to the variety and complexity of the functions of the digestive system, it has become clear that the enteric nervous system (ENS) and the immune system in the gut are of utmost importance. Along the GI tract the following can be found: the oral cavity and the oesophagus, the stomach, the small intestine (or the gut), the caecum, the colon, the rectum and the anus. To facilitate the functions of digestion and absorption it comprises an enormous surface area, which is about 200 m^2 in adult humans [1]. However, at the same time it is a tightly sealed barrier that limits the internal milieu from the external environment.

The small intestine

The small intestine (SI) can be divided into three sections: the duodenum, which begins just after the stomach; the jejunum, and the ileum, which is connected to the colon via the cecum. In the duodenum, decomposition of ingested food by pancreatic and bile secretions takes place, whereas the jejunum and ileum are the main sites of absorption [1].

The architecture of the SI consists of concentric layers, in order from the innermost to outermost (or luminal) layers: serosa, layer of mesothelium; *muscularis*, with two smooth muscle layers (longitudinal and circular); submucosa, connective tissue with blood and lymph vessels, nerve plexus and submucosal glands; and the mucosa. The mucosal layer is organized into the *lamina muscularis*, a smooth muscle layer and the *lamina propria*, a loose connective tissue layer with lymph tissue and blood and lymph vessels, and

finally, the epithelium. The SI epithelium is formed by a monolayer of cells organized in finger-like structures towards the lumen (villi) and invaginations into the submucosa (crypt) (Figure 1). Hence, the functional unit of the SI epithelia is the crypt-villus complex. The cellular types that can be found at the intestinal mucosa are enterocytes (absorptive cells), goblet cells (mucous secreting cells), and endocrine cells (responsible for the secretion of regulatory hormones) distributed in the villi region, and Paneth's cells (responsible for the secretion of antibacterial factors into the lumen of crypts cells) and intestinal stem cells in the crypt region.

All cells in the mucosal epithelium originate from the crypt region, from intestinal stem cells. Two kinds of stem cells have been described in the intestine: crypt base columnar cells (CBCs) [2], which are distributed between Paneth's cells in the bottom of the crypts and can be identified by the marker Lgr5 (leucine group repeated 5) [3]. The second type of stem cells are the transit-amplifying (TA) cells or "+4 cells" [4], which give origin to the other cells of the epithelia and undergo rapid cell proliferation. The intestinal epithelium is in a continuous state of cell turnover, which is completed within 3-5 days [5,6]. Renewal of cells occurs by proliferation, migration and differentiation into the different epithelial cell types from crypts up to the villus and apoptosis and shedding of "old" cells at the tip of villi. Each villus is built on several proliferating crypts.

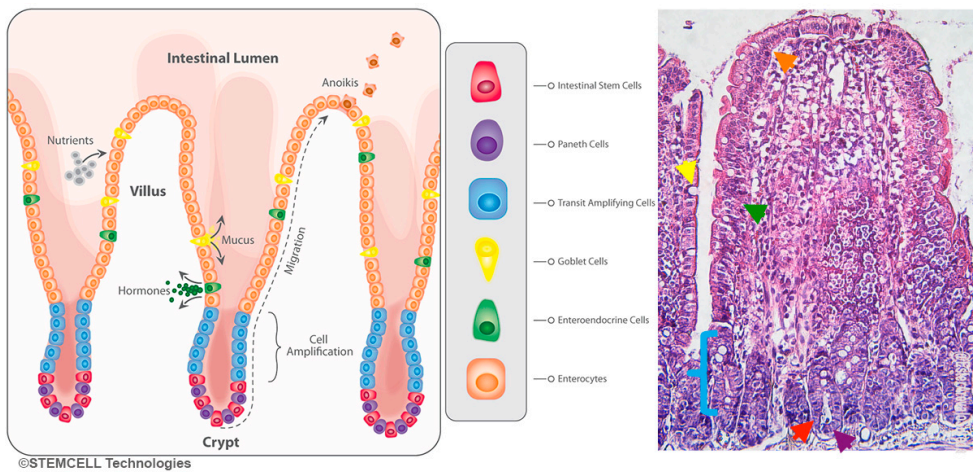


Figure 1. Intestinal epithelium structure.
Diagram of the small intestinal epithelium indicating the distribution of the main cell types (left). Photomicrograph of H&E staining representative of the small intestine epithelium (200x) (right) with the different cell types indicated with coloured arrows of the corresponding cell-type colour in the legend: orange – enterocytes, green – enteroendocrine cells, yellow – goblet cells, blue – transit amplifying cells, red – stem cells and purple – paneth cells. The small intestinal epithelium diagram on the left is used with permission, courtesy of STEMCELL technologies.

The pancreas

The pancreas is a diffuse, lobulated, glandular, accessory organ situated in the abdominal cavity, in direct contact with the stomach, the small intestine and colon portions of the GI tract, as well as the spleen. As reviewed by Wathall (2005), the pancreas has a dual function: the endocrine function, confined to the cells of the islets of Langerhans, and the exocrine function, performed by the acinar cells. The exocrine function involves the production and secretion of the digestive enzymes, as well as the chyme neutralising secretions containing bicarbonate ions into the duodenum of the GI tract, via the joint pancreatic-biliary duct in rats. The main digestive enzymes contained within the pancreatic secretions include lipase, amylase, protease and nucleases. These enzymes participate in the hydrolysis ofdietary fat, carbohydrates and proteins, as well as DNA and RNA [1].

Gut maturation

At birth, the GI system is immature and adapted to the strict milk-based diet of the young. The immature stomach secretes low levels of chymosin, the milk-clotting enzyme, and hydrochloric acid, the levels of which increase at weaning together with the secretions of pepsin, intrinsic factor and gastrin [1]. In this thesis, however, the main focus will be the small intestine and pancreas.

The small intestine

The SI intestine is immature at birth with reduced crypts, slow cell turnover, finger-shaped villi and enhanced permeability to macromolecules. At birth, the high permeability to macromolecules guarantees the absorption of bioactive molecules from maternal milk. The enterocytes in the distal SI are characterised by the presence of large supranucleolar vacuoles with high absorptive and digestive capacities [7], while in the proximal SI the enterocytes express neonatal Fc receptors. Functionally, the enzymatic activity of brush border disaccharidases is predominantly lactase.

At weaning, by the 3rd week of life in rats, the SI epithelium changes into the mature type, with the tongue-shaped villi and developed crypt structure in the mucosa and the permeability to macromolecules is drastically reduced (intestinal closure). The vacuolated enterocytes in the distal part of the SI disappear and neonatal-Fc-receptor (FcRn) expression in the proximal SI enterocytes decreases [8]. Besides, the enzymatic brush border activities switch from lactase dominance to that of maltase and sucrase. In conclusion, during weaning the digestive organs

undergo changes for the adjustment to the new solid food diet and a more selective absorption.

Differentiation to other specialised epithelial cell-types, such as goblet cells also gradually appear in the mucosal epithelium occurs with age with a drastic increase from weaning at 21 days old [9,10]. Paneth's cells are also absent at birth and with age the crypts develop and organize and ultimately Paneth cells also appear [11,12].

Pancreatic function

The pancreas, a gut accessory organ, is functionally immature at birth, and during the first few postnatal weeks, especially at weaning, undergoes a process of maturation, which includes growth and an increase in exocrine enzyme production and secretion [1]. Pancreas growth and development in foetal and postnatal rats is under the hormonal control of thyroxine and corticosteroids, with low responsiveness to secretagogues in the young, as reviewed by Morisset (2008) [13]. However, during the first few days of life, a decrease of pancreatic weight has been observed in rats, which has been suggested to be due to the release of pancreatic enzymes [14]. The secretion of pancreatic enzymes has also been stimulated in 10 day-old suckling rats with a combination of caerulein, a cholecystokinin (CCK) analogue, and secretin [15]. The increase in proteolytic enzymes in the pancreas has also been induced in suckling rats by oral administration of a protease inhibitor, camostate, in an endogenous CCK independent manner [16]. Thus, the pancreas grows exponentially during the first few postnatal weeks and is completely mature by the 4th week of age [17], with adaptation to the solid food diet, characterized by changes in the pancreatic content and enzyme composition, with a remarkable increase in proteolytic activity at weaning [18]. Also, the endocrine function of the pancreas, including the production and secretion of insulin is developed postnatally, triggered by the change in diet at weaning [19].

The gut immune system

The immune system consists of a collection of organs, scattered cells and molecules that collectively mediate the physiological function of preventing and/or eradicating infections in the organism. Immunity, or the resistance to infectious disease, in mammals can be innate, with native immunity present in all healthy individuals; or adaptive, acquired immunity depending on the life history of the individuals and the infectious agents encountered. Thus, the immune system is a network of specialised cells in continuous transit across the body via the blood and

lymphatic vessels, passing through the different organs, providing surveillance and protection, and activating the defence action mechanisms when necessary.

The immune system is composed of different cellular types originating from the bone marrow and thymus, the primary lymphoid organs. Innate immune cells, or leukocytes, originate from a common myeloid precursor in the bone marrow that further differentiate into the different cell types in circulation or in the tissues including: mast cells, dendritic cells (DCs), neutrophils, macrophages, etc. The cell lineage of the adaptive immunity originate instead from a common lymphoid precursor also in the bone marrow, where the development of B-lymphocytes takes place, with the exception of bird species where this takes place in the bursa of Fabricius. Otherwise, T-lymphocytes precursors transfer to the thymus for further development, a bilobed organ situated above the heart that it is at its maximum size per body weight at birth and involutes with age. Afterwards, all immunocompetent lymphocytes can populate the secondary lymphoid organs, including the spleen, which is the largest. The spleen is a solid organ situated in the peritoneal cavity that filters blood and creates an environment favourable for the immune cells, especially B-lymphocytes, to encounter antigens and become activated; otherwise, the activation of lymphocytes takes place in the lymph nodes spread throughout the body connected by the lymphatic vessels.

The first line of defence of an organism is the surface in immediate contact with the environment, thus the skin as well as the respiratory, the genitourinary and the intestinal mucosa. The mucosal surfaces, due to their structure represent a much larger surface than that of the skin and due to their major role in recognition of non-pathogenic and pathogenic antigens, have been defined as mucosa-associated lymphoid tissues (MALT).

In the gut mucosa, the intestine is not just a monolayer of intestinal epithelial cells (IECs), there are also members of the immune system distributed in organised structures along the GI tract. The immune system along the GI tract consists of organized immune cells or aggregates of immune cells distributed along the intestinal mucosa, the so-called gut-associated-lymphoid tissue (GALT) and can be categorized as effective or inductive. At first, solitary immune cells can be directly associated to the epithelia, intraepithelial lymphocytes (IELs); loose lymph tissue with immune cells in the *lamina propria*, and sometimes there are lymphocyte-filled villus spread along the length of the SI and intestinal lymphoid follicles (ILFs) can be found in the submucosa. All the previous have in common their function as effector sites. At last, the Peyer's patches would be the inductive site conformed by the highest organization of the aggregate lymphoid tissue in the gut [20].

The gut immune system in the young

Similarly to the digestive system, the degree of development of the immune system has been linked to gestation length in mammals [21], *i.e.* rodents vs. humans [22], with a shorter gestation associated to the a more immature immune system at birth.

Due to the low antigenic environment in utero, the immune system at birth is functionally naïve [23]. During the suckling period, maternal milk provides protection of the young by milk-borne immunoglobulins (Ig), lactoferrin and immune cells [24,25]. Thus, passive immunity transfer from the mother to the young is crucial for providing protection to the young until their own adaptive immune system is fully functional at the time of weaning [23,24,26,27]. The activation of the immune system is enhanced during the weaning period after stimulation by dietary antigens. Strikingly, the immune system of a 10 day-old rat pup is immature, but that of a 21 day-old rat is comparable to that of an adult [22] evidencing the parallelism in the activation of the immune system of the young at weaning [28-30].

The development of the immune system in the young depends on the passage of antigens across the intestinal epithelia and the stimulation of inflammatory or tolerogenic responses. In early life, during the suckling period, development of oral tolerance is of great importance for individuals not to be harmed by oral antigens, dietary or commensal microbes. Impairment in establishing a tolerogenic immune status is associated with pathologies such as allergies. The major immune cell type involved in this process are regulatory T-lymphocytes (T_{reg}), which have been shown to be dependent on interleukin (IL) – 2 for maintenance and peripheral homeostasis [31,32].

At weaning, the maturation of the immune system is characterised by the recruitment of an increased number of immune cells and subsequent epithelial crypt hyperplasia. Such activation is first associated with the up-regulation of several pro-inflammatory cytokines, *i.e.*, IL1β, IL6 and tumour necrosis factor (TNF) – α, as observed in rats and piglets [33-35] and an exponential increase in the number of lymphocytes in the intestinal wall and an increase in the mesenteric lymph nodes (MLN) [36-38]. This has been referred to as "physiological" inflammation at weaning, which is caused by the antigenic stimulation by new solid food components and microbial flora [29]. Hence, activation of the immune system and gut maturation has been described as processes that occur in parallel.

The intestinal barrier

Despite the digestive functions of the GI tract, it is also the most extensive surface in contact with the environment and hence is responsible of protecting the body and keeping it separated from the external environment, and at the same time supporting constant bidirectional communication. The barrier function has been defined as "The ability to control uptake across the mucosa and protect from damage of harmful substances from the lumen" by Keita and Söderholm (2010) [39]. The barrier functions of the gut are of physical, mechanical and chemical nature, and include the excretion of undigested macromolecules, protection from pathogenic bacteria, tolerance to the commensal microflora and dietary antigens, and biosensor of the environment. Furthermore, the loss of the intestinal barrier function has been associated with several pathologies, including inflammatory bowel disease (IBD), coeliac disease, intestinal ischemia, food intolerance, allergy and malnutrition, etc. [40-43].

Routes across the intestinal barrier

The SI epithelium is formed by a monolayer of cells constituting a tightly sealed physical barrier, which is however, permeable. This property of intestinal permeability has been defined as "a functional feature of the intestine, measurable by analysing flux rates across the intestinal wall as a whole or across wall components of defined molecules that can be adequately measured" by Bischoff et al. (2014) [44].

The uptake of macromolecules in the SI can occur by pino/endocytic vesicles, either via a selective or a non-selective pathway (Figure 2) [45,46]. The non-selective pathway consists of non-specific trancytosis within the cytoplasmic vesicular system of IECs. The selective pathway, also referred to as a specific receptor-mediated trancytosis, transports intact macromolecules bound to their receptor across the epithelia ensuring its functional arrival into the circulation [47]. Abrahamson and Rodewald (1981) showed that, both pathways of macromolecule uptake, specific and non-specific, probably occur within the same endocytic vesicles and the content is sorted during their intracellular transport. Macromolecules taken up into vesicles that are not bound to receptors are probably digested by lysosomal hydrolases in the digestive vesicles. However, receptor-bound molecules would bud off in independent coated vesicles that cross the cells, fuse with the basolateral membrane and release their content, preventing the degradation of the bound molecules [46,48].

Passage of luminal content trough the epithelium can also occur through the tight junctions, allowing water, ions and small molecules to pass. However, the paracellular route is often called leakiness, which is associated with dysfunctional

permeability under inflammatory circumstances, with loosening of the tight junctions allowing the passage of bigger molecules from the luminal content between the IECs, considered pathological.

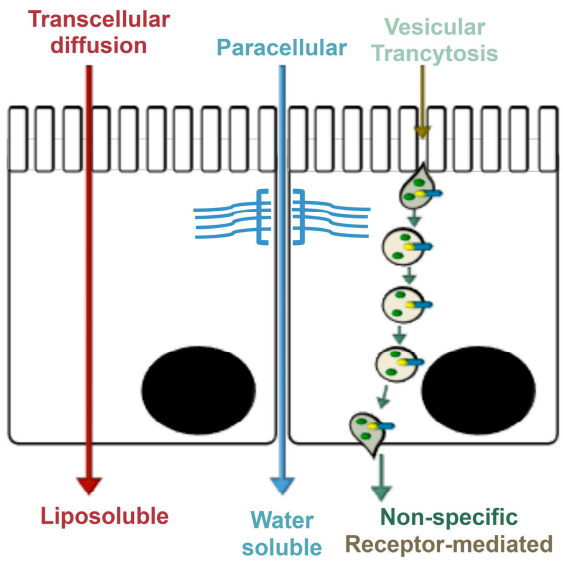


Figure 2. Routes of transport across the small intestinal epithelium.
Depending on the properties of the luminal content the transport can occur via different pathways across the intestinal epithelial cells (transcellular transport) or between the epithelial cells (paracellular transport). The transport of molecules through the epithelial cells can also be differentiated between transcellular diffusion and vesicular trancytosis. The uptake via vesicular trancytosis can be ahieved by unspecific or receptor-mediated endocytosis.

Measurement of the barrier properties

The permeability properties of the gut can be measured by the use of marker molecules of different molecular size and properties (overview in table 4, methodology section p42), *i.e.* protein markers, such as serum albumin from different species (~70kDa, bovine and human serum albumin, BSA and HSA respectively) and bovine immunoglobulin (BIgG, 150kDa), used for the evaluation of the macromolecules uptake via vesicular trancytosis. Albumin is considered to be a non-specific absorption marker whereas BIgG is considered to be a marker for the specific pathway, receptor-mediated trancytosis [49]. Therefore, BIgG could also be considered as a marker for FcRn expression and function. *In vivo* permeability studies are often susceptible to the possible digestion of the marker molecules after oral administration and thus, non-degradable markers are also available, *i.e.* fluorescein isothiocyanates (FITC) in combination with dextrans of different molecular sizes (FD). These molecules are recommended for comparison to protein markers, *i.e.* FD70 (70kDa) would be an equivalent to albumin. The paracellular transport across the intestinal epithelium can be assessed by the

permeability of small molecular weight marker molecules, *i.e.* FD4 (4kDa). Furthermore, the combination of mannitol (monosaccharide, 0.18kDa) with lactulose (disaccharide, 0.36kDa) can be used as a non-invasive method of assessment of the intestinal permeability by oral administration of the markers and subsequent measurement of their excretion in urine. The lactulose:mannitol ratio (Lac:Man ratio) is a permeability test that evaluates the relative importance of two different routes, since lactulose is believed to pass via the paracellular route, hence more prone to show changes in permeability, whereas mannitol is believed to pass by transcellular diffusion, passing through the epithelium in a more steady manner independent of any permeability changes. However, the Lac:Man ratio is recommended for use as a measurement of the health of the epithelia, rather than a marker for the specific transport routes [50].

Hence the combination of different permeability tests as well as using different cocktails containing different types of marker molecules allows for the monitoring of intestinal permeability and integrity, contributing to the evaluation of gut health and disease.

The intestinal barrier in the young

The luminal milieu

Maternal milk is not only a source of nutrition for offspring; it also provides bioactive molecules essential for offspring protection and development, which include antibodies, hormones, antibacterial compounds, etc. during the suckling period. Thus, maternal milk provides the offspring with a beneficial luminal environment within a GI system adjusted to effectively digest milk nutrients and permit milk-born bioactive macromolecules to pass undigested through the GI tract, allowing them to reach the SI intact and be absorbed [24,51-56].

Moreover, milk is not the only luminal component which confers protection to the young gut, but the gastrointestinal secretions also contribute. For instance, the stomach pH is kept less acidic allowing the bioactive molecules to pass intact. Also, the pancreatic exocrine secretions are low at first and increase within the second postnatal week, with not only digestive functions but also antibacterial.

Nonetheless, the intestinal epithelium itself can be a source of protective secretions. It has been shown that enterocytes, besides being mainly absorptive cells, can also secrete antimicrobial factors, cytokines, etc. [57]. The small intestine has a loose, unattached mucus layer which together with liquid secretions and motor activity, limit the organism to bacterial exposure by flushing [58]. However,

the mucus secreting specialised IECs, goblet cells, increase in numbers with age in the intestinal epithelia, especially in the crypt region, in newborn rats [10], and thus increasing the mucus layer above the epithelium participating in the defence from microbial colonisation. The Paneth's cells, which are also specialised IECs localised at the bottom of the crypt region that secrete antimicrobial factors such as lysozyme and defensins, also increase in numbers with age [59] in the proximal-distal axis [12,60,61].

The epithelium

Absorptive capacity

During the suckling period the distal half of the SI is mostly constituted by vacuolated enterocytes [62], which are foetal-type cells with high endocytic activity and a large supranuclear vacuole with intracellular digestive function occupying most of the cytoplasm (Figure 3)[7]. These distal absorptive cells may contribute to the uptake and transfer of intact macromolecules in a non-specific manner [63].

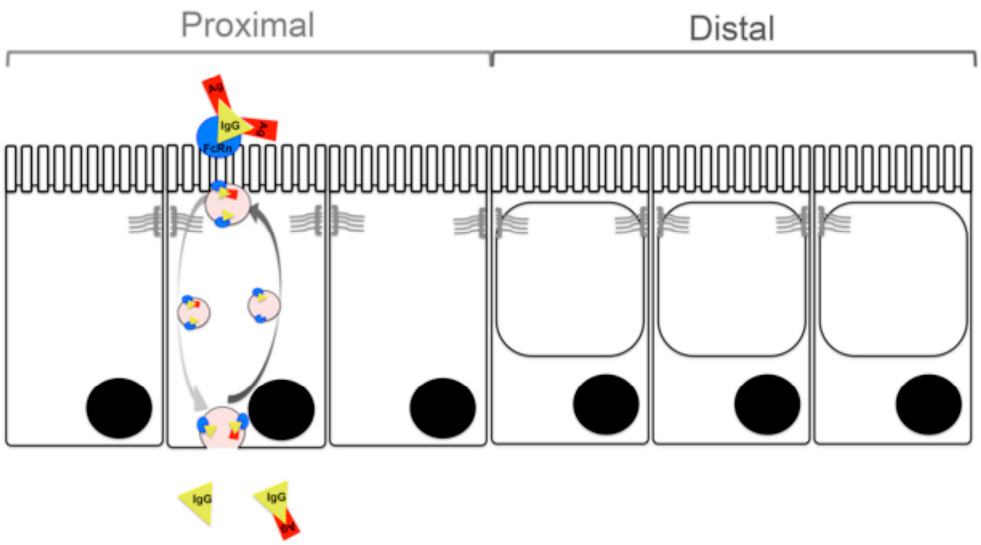


Figure 3. The proximal – distal axis in the small intestine during the suckling period in rats.
In the proximal SI the neonatal-Fc-Receptor (FcRn) binds maternal antibodies to the offspring via receptor-mediated trancytosis across the intestinal epithelial cell barrier. In the distal SI the dominant cell type are the vacuolated enterocytes with high endocytic capacity with their cytoplasma occupied almost entirely by large supranuclear vacuoles.

The transport of immunoglobulins has long been studied and attributed to a highly saturable membrane receptor with pH-dependent binding [48]. Later studies showed that binding of immunoglobulin occurs at a slightly acidic pH in absorptive

intestinal cells [47]. Finally, the IgG receptor was purified revealing a major histocompatibility complex (MHC)-class-I-related heavy chain molecule non-covalently associated to a stabilizing α-2-microglobulin forming a heterodimer, the FcRn [64-66]. FcRn is expressed by the SI epithelium and has the physiological role of transferring adaptive passive immunity from the mother to the offspring, *i.e.* protecting milk-borne IgG from proteolytic degradation during trancytosis [67].

At weaning, FcRn expression is drastically reduced in the SI epithelium and the localization pattern is altered once the development of both the gut and the immune system is complete. Additionally, it is known that the switch in FcRn expression with age is related to functional changes. The functions of FcRn in adults, as reviewed by Rath *et al.* (2012) [68], include e.g. extending IgG and albumin half-lives, and thus becoming the most abundant with the longest lifespan molecules in blood circulation [65,69].

Environmental sensors

In the epithelial cells, there is a general repertoire of receptors expressed in contact with the luminal contents, including dietary, microbial and secretions from the host, that act as sentinels of the intestinal milieu. These sensors mediate the crosstalk between the environment and the host and include innate immune receptors or pathogen-recognition receptors (PRRs) that recognise conserved structures termed pathogen associated molecular patterns (PAMPs). There are several types of PRRs grouped according to the type of PAMPs they recognise, such as the toll-like receptors (TLRs) and lectin-binding receptors (LBRs), among others. TLRs are a family of transmembrane glycoproteins that recognise bacterial antigens, *i.e.* TLR4 recognises the endotoxin characteristic of gram-negative bacteria, lipopolysaccharide (LPS) [70], and its activation often leads to signalling via the NFκB pathway which is suggested to be dependent on endocytosis of the receptor bound to the ligand [71,72]. The LBRs often act as endocytic receptors but may also be capable of signalling, possibly by direct signalling through the MAP kinase and NFκB pathways or may act indirectly by modulating TLRs.

Proteinase – activated – receptors (PARs) are a family of seven transmembrane domain receptors coupled to G proteins that are activated by proteolytic cleavage of the extracellular domain in the amino terminus, which acts as a tethered ligand. The proteases that activate this family of receptors act as signalling molecules that regulate other cellular processes, and thus, PARs have been proposed as biosensors. There are four known types of PARs, however the target of our studies is PAR2. This receptor is expressed in organs of the GI tract such are the pancreas, stomach and intestine, in the IECs [73]. The activation of PAR2 can occur by different mechanisms (Figure 4), such as canonical activation, which occurs by proteolytic cleavage, unmasking the tethered ligand domain. Non-tethered ligand activation of PAR2 can also occur by proteolytic cleavage in a different site than

that of canonical cleavage, possibly unmasking a new tethered ligand. The complete cleavage of tethered ligands can cause a proteolytic disarming of the receptor. Activation of the receptor can also be achieved with the binding of synthetic agonists to the receptor, independent of proteolytic cleavage, by binding at the site of the tethered ligand [74-76].

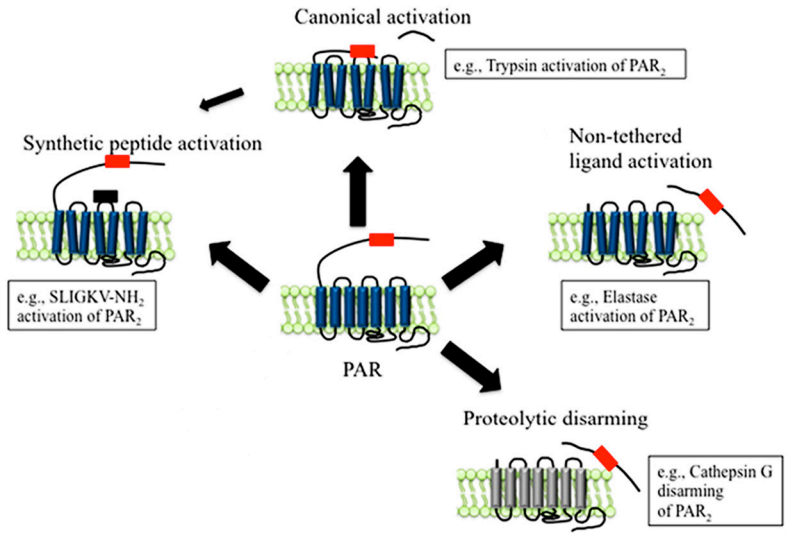


Figure 4. Protease Activated Receptors (PAR) activation mechanisms: PAR2 as an example.
Canonical activation occurs by proteolytic cleavage unmasking the tethered ligand domain. Non-tethered ligand activation occurs by proteolytic cleavage in a different site than that of canonical cleavage, possibly unmasking a new tethered ligand. The complete cleavage of the tethered ligand causes the proteolytic disarming of PAR2. Activation of the receptor can also be achieved with binding of synthetic agonists to the activation site. Adapted from Zhao P, Metcalf M, Bunnett NW. Biased Signaling of Protease-activated Receptors. Frontiers in Endocrinology. 2014;5. doi: 10.3389/fendo.2014.00067 [74].

Immune barrier function

At birth, the newborn immune system is immature and the defence of the offspring depends on the maternal transfer of passive immunity, which occurs during the suckling period in rats. Thus, from birth there is a competing balance between hostile stimuli, pro-inflammatory factors, activators of the naïve adaptive immune system and the protective mechanisms, anti-inflammatory factors, to prevent an overwhelming and dysfunctional activation of the immune system. Thus, the induction of tolerance and the activation of the immune system occur synchronously.

The sampling of antigens from the luminal content, their uptake, and the site of the encounter with antigen presenting cells (APCs) is important for the appropriate activation of the immune system. FcRn has been reported to be capable of

bidirectional transport of immune complexes (antigen-IgG-FcRn) [77,78], and therefore contributes to the activation of the naïve neonatal immune system by antigen retrieval from the luminal side as well as immune-complexes antigen-presentation to APCs, DCs and T-lymphocytes [77,79]. Maternal milk contains secretory IgA (sIgA), which restricts exposure to luminal antigens and hence prevents the maturation of intestinal T-lymphocytes [80]. It has also been proposed that sIgA-antigen immune complexes could also pass across the barrier and contribute to antigen presentation [81].

Gut microbiota in the young

Bacterial colonization is also an important change occurring at birth, when mammals go from the low antigenic milieu in utero to a hostile environment [82]. It has been reported that the first colonizing community is of urogenital maternal origin when a vaginal delivery occurs or from the skin and environment community, when delivery occurs via C-section [83,84].

During the suckling period, the milk diet favours Gram-positive bacteria, such as *Lactobacilli spp.* and *Bifidobacteria spp.*, and there is a low diversity of species. At weaning, postnatal changes in microbiota have been described to undergo a community maturation process with an increase in gram-negative bacteria, due to the cessation of milk consumption, and also an increase in diversity of species, from Firmicutes and Proteobacteria towards a Bacteroidetes dominated community, as reviewed by Jain and Walker (2015) [85].

Noteworthy, the changes in the microbial community also contribute to intestinal health via their involvement in metabolic processes and modulation of the barrier function, although not being a barrier property itself [44]. At first, tolerance must be established for the commensal community, which compete with the opportunistic and pathogenic colonizers contributing to maintaining homeostasis within the microbial ecosystem.

This change in microbiota with an increasing proportion of Gram-negative bacteria at weaning makes Toll-like receptor 4 (TLR4) an interesting candidate to be studied during gut maturation. The role of this receptor in the regulation of mucus-producing goblet cells and its importance for the development of necrotizing enterocolitis in immature mice has been shown [86,87] as well as its expression in the GI tract and accessory organs in adult rats [88]. In suckling rats the expression of TLR4 has been reported without any marked changes during intestinal development (*unpublished data*, AL Gacon).

Digestive and immune systems – developmental connections

Natural development of the gut at weaning has been suggested to be dependent on T-cell activation in rats [89-91]. Alternatively, previous studies trying to relate gut and immune system maturation in mice showed that in absence of adaptive immunity the maturation progress was temporarily delayed and that passive immunity affected the enterocytes gene expression especially in those mice with defective adaptive immunity [92].

Recently it was reported that the transcription factor Blimp1 is strongly expressed throughout the epithelium of the embryonic gut but its expression is down-regulated in mice after birth [93,94]. Among the discoveries it was described the loss of Blimp1 expression at weaning and pointed it as a key genetic program participant of gut maturation regulating element with alternative tissue-specific promoters and there could also be differences between species [95]. Moreover, the Blimp1 knockout mice were observed to have severe consequences such as postnatal mortality, reduced growth, requirement for adaptation of enterocytes to suckling, increased epithelial turnover and accelerated Paneth's cells development [93,94]. However, the physiological role of Blimp1 expressed by the immature gut epithelium remains unclear.

The suckling rat model

In mammals, the stage of intestinal maturation at birth varies among different species [45] and it correlates to the length of gestational period. Species with longer gestation periods are born more mature, such as precocious species including ungulates (pigs) and humans. Rodents, however, are altricial species, which due to the shorter gestational periods are born furless, blind, deaf, and dependent on maternal support for thermoregulation, nutrition, locomotion and the emptying of their bowels. The antenatal development of the GI tract between the third and the fifth months of pregnancy is considered to be comparable to that occurring in neonatal rats during the first three weeks of life, specifically with regards of antibody transfer (passive immunity) [96]. Thus, as an altricial species, the suckling rat is a suitable model for the study of the gut in early life as well as for biomarker identification, pathogenesis, and mechanisms; as well as for immunological studies [53,97,98]. The suitability of the suckling rat as a study model relies on general practical advantages provided by the animal models such as those related to ethical

issues, availability, handling, etc. over and above the biological similarities as well as the accumulated knowledge for their characterization.

Table 1. Speceies, rats vs humans, comparison of placentation and development timing status.

	Human	Rat
Gestation time	280 days	21-23 days
Placenta type	Hemochorial	Hemochorial
Maturation at birth	Precocious	Altricial
Weaning	1-20 weeks	19-22 days
Maturity	52 weeks	6-8 weeks

Functional and structural changes in the digestive system occur in parallel to the changes in diet that occur from birth. At first, the neonates ingest colostrum and then milk. In rats, the suckling period lasts for the first 18-21 days of life, with a 2-week period of strict milk consumption in the beginning. Afterwards, the transition from a milk-based diet to solid food starts during the third postnatal week, the weaning period, which correlates with the gut maturation process [1].

Figure 5. Timeline for the natural development of rats.
Date of birth established as day 0. Intervals of 7d (days) were studied. Diet indication at each stage of age.

Precocious induced maturation – a research tool

Despite being considered a pre-programmed process during ontogeny, the mechanisms and regulating factors involved in the gut maturation process have not yet been fully defined. Gut maturation during normal development follows a series of changes that culminate during weaning. These changes are not only structural, but also functional.

It has been shown that maturation of the SI can be induced precociously by *i.e.* early weaning [99], hormonal control (exogenous corticosteroids and thyroxin) [100], enteral administration of polyamines [101] and the lectin phytohaemagglutinin (PHA) [102]. Recently, luminal exposure to pancreatic and pancreatic-like enzymes with proteolytic activity has been shown to accelerate gut maturation in our research group (**paper I**).

Experimental models of precocious induced gut maturation have been developed in our group. These models of experimental gut provocation also performed in a splitted-litter manner, so the distribution of littermates between treatment and control groups contributes to reduce the variability between different litters. The studies on PHA-induced precocious maturation revealed the importance of oral administration since enteral PHA induced gut maturation and also affected the immune organs spleen and thymus, however, when parenterally administered PHA only had effects on the immune organs but gut maturation was not induced [103]. The original model consists of a moderate dose of 0.05 mg/g bwt of PHA and 0.6 mg/g bwt of microbial protease administered orally once per day for three days on days 14, 15 and 16 after birth, with sample collection and permeability test on day 17, 72 hours after the first treatment administration. Hence considering that natural changes are not evident until day 21 after birth.

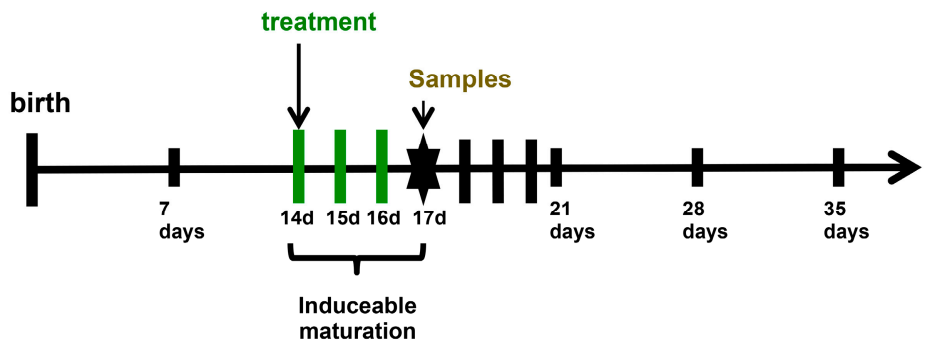


Figure 6. Experimental model of precocious induced maturation.
Birth is established as day 0. On day 14 after birth treatment by gavage with PHA or protease starts. It can either be a single dose (provocation) or 3 days once a day (14, 15 and 16 days after birth) with a lower dose. 72 hours after treatment initiation permeability test and sample collection are performed.

Furthermore, studies on induced precocious maturation revealed that it could also be achieved by a single administration of a higher dose 0.1 mg/g bwt of PHA and 0.8 mg/g bwt of microbial protease on day 14 and sample collection on day 17, 72 hours after the provocation (*unpublished data*). Thus, precociously induced maturation of the gut in the suckling rat provides a useful research tool to investigate the mechanisms behind gut development in a more defined time frame with a better control on the circumstances.

Scientific aims

Paper I – *'Involvement of pancreatic enzymes in gut maturation'*

Previous studies on PHA-induced maturation of the gut in suckling rats showed that the pancreatic function was stimulated concomitantly. Consequently, the study aimed to investigate if the pancreatic enzymes themselves were involved in gut maturation. Thus, the main pancreatic enzyme activities were studied as well as their possible role and contribution to the maturational changes of GI tract in the suckling rat model.

Paper II – *'Characterisation of the developmental changes in the intestinal epithelial barrier'*

Monitoring of a combination of changes occurring during postnatal development in the SI epithelium of suckling rats during natural and precociously induced maturation, emphasising the intestinal epithelial barrier properties, was done. The targeted parameters of the study included the expression of the FcRn receptor and the presence of endocytic vacuolated cells in the intestinal epithelium in relation to the transcriptional repressor Blimp1.

Paper III – *'Importance of the T-lymphocytes during gut maturation'*

Previous studies on gut development in suckling rats had suggested that it was dependent on T-lymphocytes. Hence, a hypothesis on the requirement of T-lymphocytes activation during the neonatal period for the initiation of gut maturation was studied in the athymic nude animal model. Therefore, the effects of luminal provocation on precocious gut maturation in thymus-derived cells T-lymphocytes deficient suckling pups were studied.

Paper IV – *'Early effects of enteral provocation resulting in gut maturation'*

Previous studies lead to the establishment of the protease- and PHA- induced precocious gut maturation experimental models in suckling rats by mimicking what occurs at weaning. However, only the effects of PHA had been investigated over time. In this study, the objective was to investigate the early effects after gut provocation with protease in comparison to PHA, with especial focus on intestinal permeability as well as exocrine pancreatic secretion stimulation. The main aim was to get a better understanding on what triggers the gut maturation process.

Methodology

Animals and ethics statement

The experiment was approved by the local Malmö-Lund Ethical Review Committee for Animal Experimentation and conducted in accordance with the European Community regulation concerning the protection of experimental animals (2010/63/EU) and the Swedish Animal Welfare Act (SFS 1988:539).

The studies were carried out using rats (*Rattus norvegicus*) of the Sprague-Dawley strain (Mol: SPRD Han; Taconic M&B, Denmark) and of the athymic T-cell-deficient (nude) strain (NIH-Foxn1^{rnu}, Charles River Laboratories International Inc.) that were bred and kept under pathogen-free conditions in the Department Animal facility at Lund University (20±1°C, 50±10 RH%, 12:12 h light-dark cycle). Before parturition, the pregnant dams were moved to separate cages (polycarbonate) with aspen wood bedding (Beekay B & K Universal AB), enriched with paper-nesting material (Sizzle-pet, Lillicobiotech). Parturition date was denominated as day 0 and litters were restricted to 10–12 pups for the study. All rat pups were kept with their dams during the experiments. The rat dams had free access to water and a rodent laboratory chow (R3, Lactamin) placed on the lid of cages. In order to prevent the pups from eating the solid chow, the cage height was increased using a 7 cm wall extender.

Experiments

The experiments were performed within experimental sets in a split-litter mode with random distribution of the rat pups from the same litter in the different groups to minimize variation. The pups were kept with their dam during the experiments or until postnatal day 21, after which the dam was separated from her litter.

– Paper I –

Induced precocious maturation was studied in groups of 14 day-old suckling rats gavaged by a soft stomach tube once a day for three days (14-16 days of age) with Creon 10000 (Abbott Products GmbH); microbial derived enzymes (Sigma-Aldrich): proteinase from *Aspergillius melleus* (type XXIII), a lipase from *Burkholderia cepacia* (Amano Lipase PS) and an α-amylase from *Aspergillius oryzae*; dissolved in water, while the control group received the vehicle (water).

– Paper II –

Natural development was studied in five age groups of litter-mates: suckling rats 7 day-old (7d) and 14 day-old (14d), on day 21 after birth, coinciding with the day of separation from their dam (21d), and two post-weaning groups at 28 day-old (28d) and 35 day-old (35d). Induced precocious gut maturation was studied on 17 day-old suckling rats that were gavaged once a day for three days (14-15-16 day-old) with microbial derived proteinase from *Aspergillius melleus* (type XXIII) or the purified lectin PHA from red kidney beans (*Phaseolus vulgaris*) [102,104], while the control group received the vehicle (water).

– Paper III –

The experiments were performed on the suckling nude pups in two different nursing sets. The first experimental set consisted of nude rats nursed by their natural dam (Nude/Nude). A second set consisted of nude pups reared by foster SD mothers (Nude/SD) from the 3rd post-natal day. Crossfostering of the nude pups was included to improve their survival and nutrition. Nude suckling rats were gavaged once a day (14 day-old) or once a day for three days (14-15-16 day-old) with the protease porcine pancreatic trypsin (Prot and Protx3) or purified PHA dissolved in water, while the control group received the vehicle (water). The experimental effects were studied at 17 day-old.

– Paper IV –

The early effects of gut provocation were studied in 14 day-old suckling rats at different time points, 1, 4, 8 and 24 hours, after the treatment. Each experimental litter included one of the treatments and controls at two different time points. The treatments administered were the protease porcine pancreatic trypsin or the purified lectin PHA and the control group received the vehicle, water.

Table2. Overview of the agents, dose and exposure period used for enteral provocation to induce gut maturation within the studies.

Treatment	Origin	Source	Dose (mg/g b.wt)	Rat Strain	Exposure period (days of age)	Time of sacrifice after treatment	Study
Creon 10000	*Sus scrofa (pancreatic porcine)*	Abbott Products GmbH	1.5	SD	14-16	72 h	Paper I
Protease	*Aspergillius melleus* (type XXIII)	Sigma-Aldrich	0.5, 0.25, 0.125, 0.0625	SD	14-16	72 h	Paper I
Lipase	*Burkholderia cepacia*	Amano Lipase PS	0.06	SD	14-16	72 h	Paper I
α-Amylase	*Aspergillius oryzae*	Sigma-Aldrich	3.33	SD	14-16	72 h	Paper I
Protease	*Aspergillius melleus* (type XXIII)	Sigma-Aldrich	0.237	SD	14-16	72 h	*Unpublished*
Papain	*Carica papaya (papaya fruit)*	Sigma-Aldrich	0.237	SD	14-16	72 h	*Unpublished*
Bromelain	*Ananas comosus (pineapple fruit)*	Sigma-Aldrich	0.237	SD	14-16	72 h	*Unpublished*
Trypsin	*Sus Scrofa (pancreatic porcine)*	Nova	0.237	SD	14-16	72 h	*Unpublished*
PHA *phytohaemagglutinin*	*Phaseolus vulgaris (red kidney beans)*	Purified in house	0.05	SD	14-16	72 h	Paper II
Protease	*Aspergillius melleus* (type XXIII)	Sigma-Aldrich	0.4	SD	14-16	72 h	Paper II
PHA *phytohaemagglutinin*	*Phaseolus vulgaris (red kidney beans)*	Purified in house	0.1	NIH-*Foxn1*^{mu}	14	72 h	Paper III
PHA (PHAx3) *phytohaemagglutinin*	*Phaseolus vulgaris (red kidney beans)*	Purified in house	0.05	NIH-*Foxn1*^{mu}	14-16	72 h	Paper III
Trypsin	*Sus scrofa (pancreatic porcine)*	Novo	1	NIH-*Foxn1*^{mu}	14	72 h	Paper III
Trypsin (Protx3)	*Sus scrofa (pancreatic porcine)*	Novo	0.6	NIH-*Foxn1*^{mu}	14-16	72 h	Paper III
PHA *phytohaemagglutinin*	*Phaseolus vulgaris (red kidney beans)*	Purified in house	0.1	SD	14	1, 4, 8, 24 h	Paper IV
Trypsin (Protease)	*Sus scrofa (pancreatic porcine)*	Novo	0.8	SD	14	1, 4, 8, 24 h	Paper IV

*Abbreviations: mg/g bwt: mg per g body weight; SD: Sprague-Dawley, NIH-Foxn1^{mu}: athymic nude rat

Animal euthanasia and samples collection

On the designated collection day, the animals were anesthetized by a subcutaneous injection of a mixture of ketamine (Ketalar®, Pfizer) and azaperone (Stresnil®, Janssen Pharmaceutica) or isoflurane (Abbott) inhalation. At first, urine was collected during administration of the anaesthesia or directly from the bladder after laparotomy. Thereafter, the abdomen and thorax were opened; 1ml of blood was collected by direct heart-puncture into a syringe containing a mixture of anticoagulant and protease inhibitor. Plasma was obtained by blood centrifugation at 3000xg for 15 min at +4ºC and stored at -20ºC. Next, the SI was dissected from the pylorus to the ileo-caecal junction, and divided into a proximal and a distal half. The luminal content was flushed out. Intestinal samples, approximately 1cm long, were taken from the middle of each half and fixed in 10% neutral buffered formalin for 24 hours at room temperature and then kept in 70% ethanol until paraffin embedding, standard procedure. The rest of SI tissue was stored at -70ºC until further analyses.

Table 3. Overview of the analysis performed within each study.

Study	Parameter	Analysis	
Paper I	Body Growth	Body Weight	
	Organ Growth	Stomach Weight and pH	
		SI Length	
		Spleen, SI Prox, SI Dist, Caecum and Liver Weight	
	Pancreatic Function	Pancreas Weight	
		Trypsin, Amylase and Lipase Activity	
		Protein	
	Small Intestine	Dissacharidases	Maltase, Sucrase, Lactase
		Protein	
		% Adult-Type Epithelium:	Dist: Non-Vacuolated Entetrocytes
	In Vivo Permeability	Plasma	BIgG, BSA
Paper II	Pancreatic Function	Pancreas Weight	
		Trypsin Activity	
		Protein	
	Small Intestine	% Adult-Type Epithelium	Prox SI: FcRn Negative Enterocytes
			Dist SI: Non-Vacuolated Entetrocytes
		SI mRNA	FcRn (Fcgrt)
			Blimp1 (Prdm1)
		SI IHC	FcRn
			Blimp1
	Barrier Function	Plasma	RIgG
Paper III	Body Growth	Body Weight	
	Organ Growth	Spleen, Stomach, SI Prox, SI Dist, Ceacum and Liver Weight	
		SI Lenght	
	Pancreatic Function	Pancreas Weight	
		Trypsin Activity	
		Protein	
	Small Intestine	Morphometry	Villi Length and Width, Crypt Depth
		% Adult-Type Epithelium	Dist: Non-Vacuolated Enterocytes
		SI IHC	CD3+ Cells
	Passive Immunity	Rat Immunoglobulins	
	In Vivo Permeability	Plasma	BIgG, BSA
Paper IV	Body Growth	Body Weight	
	Organ Growth	Spleen, SI Prox and SI Dist Weight	
		SI Lenght	
	Pancreatic Function	Pancreas Weight	
		Trypsin Activity	
		Protein	
	Luminal Content	Trypsin Activity	
		Protein	
	In Vivo Permeability	Plasma	BIgG, HSA, FD70, FD4
		Urine	Lactulose, Mannitol
		Tissue – SI	FD70

*Abbreviations: SI, small intestine; Prox, proximal; Dist, distal; BIgG, bovine immunoglobulin G; BSA, bovine serum albumin; FcRn, neonatal – Fc – receptor; Blimp1, B – lymphocyte induced maturation protein – 1; IHC, immunohistochemistry; RIgG, rat immunoglobulin G; HSA, human serum albumin; FD70, FITC – Dextran 70kDa; FD4, FITC – Dextran 4kDa

In vivo intestinal permeability

Intestinal macromolecular permeability was tested *in vivo* by gavage of a cocktail solution containing different marker molecules combinations in a volume of 0.025 ml/g b.wt via a flexible stomach tube. Three hours later, 1 ml of blood or all urine bladder content were collected during samples collection.

Table 4. Overview of the different marker molecules and their properties used for *in vivo* permeability testing within each study.

	Molecular Weight	Molecular Size (Ø, diammeter)	Biomolecule Type	Transport pathway	Dose (mg/gb.wt)	Sample	Assay	Paper
BIgG	~150kDa	~170Å	Antibody (Protein)	FcRn-mediated	0.25	Plasma	SRI	I – II III – IV
BSA	~70kDa	~100Å	Protein	Unspecific	1.25	Plasma	RocketIE	I – II – III
HSA	66.5kDa	~100Å	Protein	Unspecific	1.25	Plasma	RocketIE	IV
FD70	~70kDa	60Å	Dextran (NDS)	'Unspecific'	1.25	Plasma SI tissue	Fluorometry Microscopy	IV
FD4	~4kDa	14Å	Dextran (NDS)	Paracellular	0.1425	Plasma	Fluorometry	IV
Lactulose	342.3Da	>5Å	Dissacharide (sugar)	Paracellular	0.5	Urine	Enzymatic	IV
Mannitol	182.2Da	>5Å	Monosaccharide (sugar)	Paracellular	0.3	Urine	Enzymatic	IV

*Abbreviations: BIgG: bovine immunoglobulinG, BSA: bovine serum albumin; HSA: human serum albumin; FD70: FITC-Dextran-70kDa; FD4: FITC-Dextran-4kDa; NDS: Non-digestable sugar; FcRn: neonatal-Fc-receptor; SI: small intestine; SRI: single radial immunodifusion; RocketIE: rocket immunoelectrophoresis

Analyses

Histology and immunohistochemistry (IHC)

Small intestine sections 5 µm thick were deparaffinised and stained with haematoxylin and eosin (H&E) according to standard procedures. In addition, for immunohistochemistry analysis, antigen retrieval was performed with 10mM Citrate buffer pH 6 or 10mM Tris-EDTA buffer pH 9 in the microwave for anti-CD3 and anti-Blimp1, respectively. Afterwards, blocking of endogenous peroxidase was followed by incubation with blocking reagent to reduce background. Then, the incubation with primary antibodies, rabbit-polyclonal anti-FcRn (M-255, 1:600, Santa Cruz Biotechnology Inc.), rabbit-polyclonal anti-Blimp1 (PA5-20310, 1:40000, Invitrogen, ThermoFisher Scientific), rabbit-monoclonal anti-CD3 (SP7, 1:100, Abcam), mouse-monoclonal anti-PAR2 (SAM-11, 1:1000, Santa Cruz Biotechnology) or mouse-monoclonal anti-PCNA (PC10, 1:1500; DakoCytomation, Denmark A/S) in 1% BSA in 0.02M PBS was performed overnight at +4°C. Next day, a detection based on horse radish peroxidase (HRP)-Polymer detection (BiocareMedical, Llc.) was performed according to the manufacturer' specifications using DAB (3,3'-Diaminobenzidine) as substrate, except for PAR2 immunostaining. Finally, the sections were counter-stained with haematoxylin, dehydrated and mounted under cover slip. Imaging was performed Microscopic examination was performed using an Olympus PROVIS microscope connected to an Olympus DP50 camera (Olympus, Japan).

Table 5. Overview of the primary antibodies used for IHC.

Target	Primary Antibody	Clone	Source	Dilution	Study
FcRn	Rabbit-polyclonal	M-255	SCBT	1:600	Paper II
Blimp1	Rabbit-polyclonal	PA5-20310	Invitrogen	1:40000	Paper II
CD3	Rabbit-monoclonal	SP7	Abcam	1:100	Paper III
PCNA	Mouse-monoclonal	PC10	Dako	1:1500	*Unpublished*
PAR2	Mouse-monoclonal	SAM11	SCBT	1:1000	*Unpublished*

*Abbreviations: FcRn, neonatal-Fc-receptor; Blimp1, B-lymphocyte induced maturation protein 1; CD3, cluster of differentiation molecule 3; PAR2, protease-activated receptor 2; PCNA, proliferative cell nuclear antigen; SCBT, Santa Cruz Biotechnology Inc.

Control slides for unspecific binding of the HRP-Polymer detection kit were included. The specificity of the primary antibody anti-Blimp1 was verified by pre-incubation with the corresponding blocking peptide 30 min at RT (ratio 1:5 antibody:peptide, PEP-0430, Invitrogen, ThermoFisher Scientific Inc.) and followed the same immunostaining procedure.

Detection of PAR2 immunostaining was done with a secondary antibody fluorescent-labeled goat-anti-rabbit-DyLight®594 (Vector laboratories, Inc), with excitation in the red region in the spectrum, and the nuclei were stained with DAPI (blue) according to standard procedures. Mounting under cover slip was done with Glycerol (1:1) in PBS. Imaging was done with a confocal microscope Leica TCS SP8 (Leica Microsystems CMS GmbH).

mRNA Expression by RT-qPCR

Reverse transcription of RNA followed by quantitative polymerase chain reaction (RT-qPCR) was performed for Blimp1 (Prdm1) and FcRn (Fcgrt) mRNA. Total RNA was extracted from proximal and distal portions of the small intestine (kept at -70ºC) using the RNeasy1Mini Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was eliminated during RNA extraction by using RNase-free DNase set (Qiagen) according to instructions. Total RNA concentration was determined by using Qubit1RNA HS assay kit (Life Technologies) in a Qubit12.0 fluorometer (Invitrogen) and 50–200ng of total RNA was used for reverse transcription (RT) per reaction. The RT reactions were performed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™) according to the manufacturer's protocol. The amount of cDNA was measured with Qubit1ssDNA assay kit (Life Technologies). RT-qPCR was performed using a C1000 Touch Thermal Cycler (BioRad) on 5–10ng (2µl of a 20µl RT reaction) of first strand cDNA using SsoAdvanced™ Universal SYBR1Green Supermix (BioRad laboratories, USA) in triplicates, according to the manufacturer's instructions. The primers used were pre-designed on the rat sequence by the manufacturer (KiCqStart1 SYBR1 Green Primers, Sigma-Aldrich), and the ribosomal protein L13 (Rpl13a) was used as a housekeeping gene.

Table 6. Sequence of primers used in paper II.

Gene	Primer sequence	
	Forward	Reverse
Prdm1	ATTTTTGGCGGATCTATTCC	AGGGATAGGCTTAATAGTGTAG
Fcgrt	AAATAAATGGGACCTTCACAC	ACCAACGATATCTGTCTCC
Rpl13a	AGTTAAAGTATCTGGCCTTTC	CTCTTTTGGTCTTGTGCG

*Abbreviations: Prdm1, B-lymphocyte induced maturation protein 1 (Blimp1) gene; Fcgrt, neonatal-Fc-receptor (FcRn) gene, RPL13a, ribosomal protein 13 subunit A (housekeeping gene)

Amplification of the PCR products was preformed as follows: initial denaturing at 95°C, 3min, followed by 40 cycles (denaturing at 95°C, 15sec, annealing at 58°C, 30sec and a plate read). A melting curve for each primer was included at the end of the programfrom65°C to 95°C, with an increment of 0.5°C for 5sec and plate read. Melting curve analysis of PCR products indicated single products for each primers pair used.

Pancreatic homogenates

The pancreata were homogenized 1:10 (w/v) with ice-cold 0.2M Tris-HCl buffer + 0.05M $CaCl_2$, pH 7.8, with a glass homogenizer, centrifuged at 24000xg for 30 min at +4ºC, and the supernatants were analysed.

Enzymology

Pancreatic homogenates were analysed after activation with enteropeptidase for characterisation of proteases-specific enzymatic activity after electrophoretic separation in agarose gel. The enzymatic activity was evaluated with different specific enzymatic substrates: trypsin activity, benzoyl-DL-arginine-4-nitroanilide (BAPNA); chymotrypsin-like activity, N-acetyl-DL-phenylalanine β-naphthyl ester (APNE); and elastase-like activity, N-CBZ-L-Ala-β-naphtyl-ester (CBZ-ANE), with Diazo Blue B as the precipitating salt.

The pancreatic homogenates after enterokinase activation and electrophoretic separation were also stained for all proteins after standard fixation with Commassie brilliant blue (CBB).

Luminal Content

Luminal content was collected from each half of the small intestine (proximal and distal) by flushing with 1 ml of ice-cold 0.2M Tris-HCl buffer + 0.05M $CaCl_2$, pH 7.8. Afterwards, they were mixed by shaking and with a vortex mixer, and then centrifuged at 24000xg for 30 min at +4ºC, and the supernatants were further analysed.

Trypsin activity and protein content

Trypsin activity was determined spectrophotometrically using a microplate modification [105] of the method of Fritz *et al.* [106], after activation with enteropeptidase and benzoyl-DL-arginine-4-nitroanilide (BAPNA) as the substrate. The protein concentration was determined by the Lowry method with a modification for 96-well microplates [107] and using purified BSA as the standard.

Diamine oxidase

Plasma diamine oxidase (DAO, EC 1.4.3.6) activity in plasma was measured by an enzyme-based colorimetric method, based on two coupled reactions [108]. In the first reaction, putrescine (P7505, Sigma-Aldrich) was used as a substrate for diamine oxidase, with hydrogen peroxide production. In the second reaction, HRP (Type VI, Sigma-Aldrich) decomposed peroxide in parallel to the oxidation of the chromogenic o-dianisidine (F5803, Sigma-Aldrich), which is registered at a wavelength of 440 nm. All the plasma enzyme activities were measured at 37°C and 1 unit of activity were defined as 1 µmol substrate formed per minute.

Immunoglobulins

Rat IgG quantification in plasma was used as an accumulative marker for the intestinal barrier function and the monitoring of the maternal transfer of passive immunity. Plasma IgG was quantified by single radial immunodiffusion [109] using rabbit-anti-rat-IgG (Dako) as the precipitating antibody and purified rat IgG was used as the standard.

The BIgG concentration in plasma was also determined by single radial immunodiffusion using rabbit anti-BIgG (Sigma-Aldrich) as the precipitating antibody and purified BIgG (Sigma-Aldrich) as the standard.

Albumin

The concentrations of macromolecules marker in the blood plasma were measured in plasma collected 3 hours later and quantified by immunoassay. BSA and HSA marker concentration were measured by electroimmunoassay [110] using rabbit anti-BSA (Sigma-Aldrich) and rabbit anti-HSA (Dako), respectively, as the precipitating antibody and purified BSA (Sigma-Aldrich) and HSA (CSL Behring GmbH) as the standard.

FITC-Dextrans

Plasma

The concentration of the fluorescent markers FITC-Dextran 4 kDa and 70kDa (FD4 and FD70) (TdB Consultancy AB) in plasma was measured by fluorescent spectrophotometry with an excitation of 485nm (9nm band width) and an emission wavelength of 535nm (15nm band width), a dilution series of the corresponding FITC-dextran was used as standard. Serum from rats not administered with FITC-dextran was used to determine the background.

Small intestine

Detection of intestinal uptake of FD70 was done on intestinal sections of 5µm of thickness, deparaffinised and the nuclei were counterstained with DAPI (blue) according to standard procedures with mounting under cover slip with Glycerol (1:1) in PBS. Epifluorescence microscopy examination was performed using an Olympus microscope (Olympus, Japan) connected to a Nikon digital camera (Nikon Imaging Japan Inc.). DAPI was detected with the use of the filter within the violet range (400 – 418nm) and FD70 was detected with a blue filter (478 – 495 nm) in the spectral regions.

Lactulose and Mannitol

Urine lactulose (Meda AB) was quantified by a 3-step process oxidation of lactulose in presence of NADP to generate NADPH quantified with a spectrophotometer at a wavelength of 340 nm. Urine mannitol (Sigma-Aldrich) was measured by the conversion of mannitol to fructose and NADH measured spectrophotometrically at a wavelength of 340 nm. In both the previous assays the generation of NADPH and NADH was proportional to the initial concentration of lactulose and mannitol, respectively [111]. Analysis performed at the Department of Veterinary Clinical and Animal Sciences, University of Copenhagen. The ratio between concentrations of lactulose and mannitol (Lac:Man) were calculated.

Calculations and Statistics

All data are presented as mean ± standard deviation (SD). Organ weights were recalculated relative to body weight (mg/g b.wt) to minimize individual variation within groups. Pancreatic protein and trypsin activity were expressed relative to the pancreatic wet-weight (μg/mg pancreas and U/mg pancreas). Spectrophotometric measurements of absorbance and fluorescence were done in a 96 well plate reader SpectraMax i3x (Molecular Devices). Statistical analyses were done using Prism for Mac OS X (GraphPad Software, USA, www.graphpad.com). Microscopy images were adjusted and analysed using the ImageJ 1.49h software (Wayne Rasband, National Institutes of Health, USA; http://imagej.nih.gov/ij). Images captured of the enzymology gels were colour adjusted for distinction with Adobe Photoshop CC (2017).

Comparisons between one treatment and controls were performed with unpaired t-test. Comparisons between multiple treatments and the control group were performed with one-way ANOVA and Dunnett's multiple comparison post-test. Comparisons between multiple groups, at different ages or multiple control groups, were performed with one-way ANOVA and Tukey's multiple comparisons post-hoc test. Differences between groups were considered to be significant when $p < 0.05$ (*, §), $p < 0.01$ (**, §§), $p < 0.001$ (***, §§§), $p < 0.0001$ (****, §§§§), or non-significant (ns), respectively. Different letters were used to show significant differences ($p < 0.05$) when comparing multiple groups.

Results and Discussion

Protease–induced precocious gut maturation

In previous studies from our research group, the model of precocious PHA-induced maturation of the gut in suckling rats was established and the pancreas was suggested to play an important role during the process [102,103]. In **paper I**, oral gavage with pancreatic or pancreatic-like enzymes resulted in stimulated gut growth, increased gastric acid secretion and altered intestinal disaccharidase activity, with decreased lactase and increased maltase and sucrase activities. The foetal-type vacuolated enterocytes were replaced by the adult-type in the distal intestine and the transfer of macromolecules BSA and BIgG to the blood was declined. Protease enzyme activity was observed as the only pancreatic enzymatic activity capable of inducing precocious gut maturation in suckling rats in a dose-dependent manner. Hence, a new experimental model of induced precocious maturation of the gut was established.

Following the protease-induced gut maturation findings in **paper I**, proteases of different dietary and microbial origins were assessed to evaluate whether a variety of exogenous sources of proteolytic activity would be equally as effective in provoking precocious gut maturation. Investigation of the maturation status at 72 hours after administration of the different proteases (at the same dose) was achieved through the assessment of intestinal permeability *in vivo*, using a marker for receptor-mediated trancytosis, BIgG, and a marker for nonspecific uptake, BSA. The *in vivo* intestinal permeability results, following gavage of the marker molecules and the rat IgG are shown in Figure 7 (*unpublished data)*. From the results obtained, it seemed that proteases, in general, are potentially capable of inducing precocious gut maturation, however not all were equally effective at the tested dose.

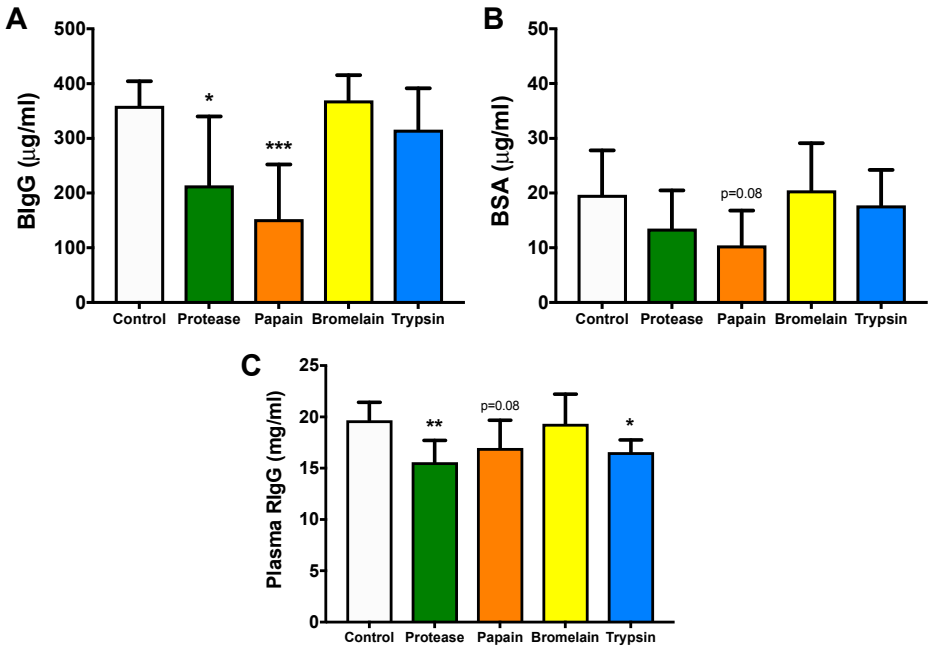


Figure 7. Intestinal *in vivo* permeability.
A) Plasma levels 3 hours after gavage of the marker molecules bovine IgG (BIgG, top pannel) and B) bovine serum albumin (BSA, lower pannel). C) Plasma levels of rat IgG (RIgG) as a cumulative marker of intestinal macromolecular permeability. The experiments were performed in 17 day old suckling rats after luminal provocation by gavage with microbial protease, papain, bromelain, trypsin or water once a day for 3 days (14-16 days-old) with the same dose for the diffent proteases. Data presented as mean±SD. Differences between each of the treatment groups and controls were analysed by one-way ANOVA with Dunnett's post-hoc test and considered significant when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****) or non significant.

The conclusions extracted from the results presented above included that of the exogenous proteolytic activity of different proteases such as pancreatic trypsin (including endogenous), or pancreatic-like enzymes of exogenous origin, such as papain from papaya fruit or bromelain from pineapple fruit as well as broad-spectrum enzymes of microbial origin from fungi, are capable of inducing precocious maturation of the gut, and hence, could be involved in triggering the process of gut maturation in neonatal rats.

Consequently, the ability to accomplish gut maturation experimentally in a defined and controlled time period by using the novel protease-induced precocious gut maturation, together with the previously described PHA-induced precocious gut maturation models in the suckling rat [102,103] and then compare the results to the naturally occurring changes in gut maturation endorsed further studies on the potential mechanisms involved in the initiation and process of postnatal gut development in suckling rats.

Maturation of the intestinal epithelium

A compilation study of parameters with potential use as markers for SI epithelial maturation was carried out in **paper II** during natural and precociously induced gut maturation. These parameters included the expression of FcRn, presence of vacuolated cells and expression of Blimp1 in the SI epithelium. As a cumulative marker for SI barrier function and absorptive capacity, plasma levels of rat immunoglobulins (IgG) were also measured. Immunostaining of FcRn showed high expression of the receptor in the enterocytes of the proximal SI of the suckling age groups, and it was markedly reduced after weaning (Figure 8A). The presence of vacuolated enterocytes only appeared in the distal SI of the suckling age groups, and they were no longer present from weaning.

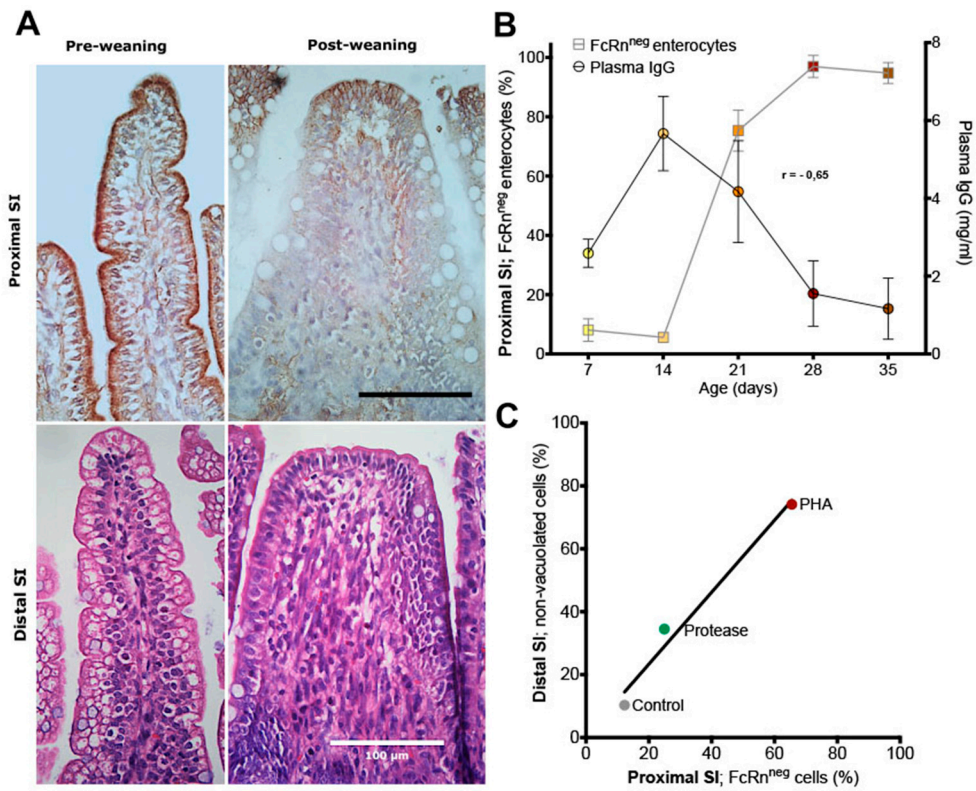


Figure 8. Maturational changes in the small intestine epithelium.
A) Immunohistochemical staining of FcRn in the proximal SI and H&E staining (vacuolated enterocytes) in the distal SI in pre-weaning (suckling) and post-weaning rats. B) Correlation between the appearance of adult-type $FcRn^{neg}$ cells in the proximal SI epitheliumin and the plasma levels of IgG. C) Correlation between the maturational appearance of adult-type epithelium in the proximal and the distal SI. Appearance of $FcRn^{neg}$ cells in the proximal SI and non-vacuolated cells in the distal SI in 17 day old rats treated with PHA or protease for 3 days, to induce precocious gut maturation, compared to control rats.

The plasma level of IgG increased during the suckling period up until weaning, after which it temporarily decreased. Also, the appearance of the $FcRn^{negative}$ cells in the proximal SI showed a negative correlation to plasma IgG absorption (Figure 8B). Finally, $FcRn^{negative}$ cells in the proximal SI and non-vacuolated enterocytes in the distal SI epithelium replaced the immature enterocytes after weaning or induced precocious maturation. This developmental switch from foetal- to adult-type SI epithelium occurred in parallel in the proximal and the distal SI, with a strong correlation between the two (Figure 8C). Hence, the measurement of the presence of FcRn and/or vacuolated cells in the SI could serve as an estimation of the degree of maturation and can be used individually or in combination as histological markers for monitoring the development of the SI in the rat.

However, the need for non-invasive parameters to assess the maturational status of the gut became evident. Thus, the plasma diamine oxidase (DAO) and the urine excretion of Lactulose:Mannitol (Lac:Man) ratio, previously described as intestinal integrity and maturity markers, were considered worthy of further investigation. Hence, an enzymatic colorimetric method for measuring the intestinally derived enzyme DAO in plasma in young rats was developed and both parameters were monitored during natural development in rats of different age groups: suckling (7 - 14 day-old), weaning (21 day-old) and post-weaning (28 - 35 day-old) (Figure 9, upper panels); as well as in 17 day-old rats, 3 days after PHA and protease gavage (Figure 9, lower panels).

The plasma levels of DAO decreased in the 21 day-old group of rats (weaning) compared to the suckling groups, whereas the 28 day-old rats exhibited a marked increase in DAO (Figure 9A). A slight increase in DAO was observed in the precociously induced maturation models, especially in the protease-treated group compared to the controls even though the increase was not significant.

It has been shown that the high DAO intestinal activity localizes at the upper region of the villi in humans and rodents and is indicative of integrity but also maturity [112]. When the intestinal epithelium is damaged the concentration of DAO in plasma is reduced, and it increases again when the damage is repaired [113,114], hence the levels of DAO in plasma reflect the size of the epithelial surface. Thus, the assessment of DAO levels as a non-invasive marker of gut health would reveal relatively low levels of DAO in suckling rats which would then be naturally lost at weaning (21 day-old) and would then further increase in post-weaning rats, compared to suckling levels. However, in 17 day-old rats it would seem that the protease treatment only had mild effects whereas the PHA-treatment had none. This could indicate that indeed during natural development in the post-weaning age groups the intestinal epithelium had undergone maturation and established epithelial integrity as well as being representative of the SI growth with age. Thus,

the increase in plasma DAO activity in the protease-treated rats could be indicative of induced maturation.

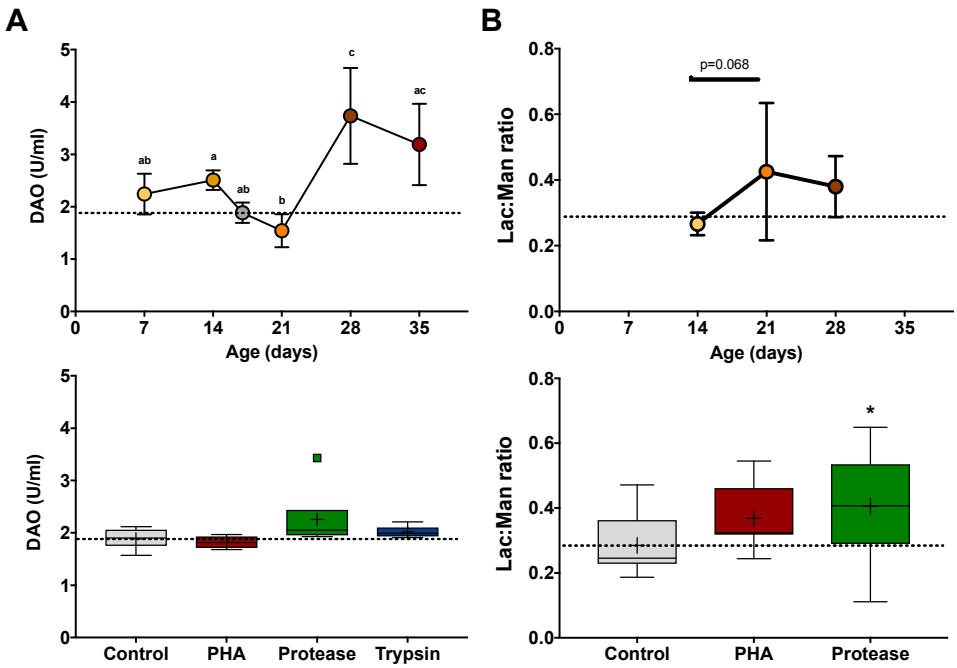


Figure 9. Intestinal integrity markers.
A) Diamine oxidase (DAO) in plasma. Plasma levels of DAO, considered a non-invasive marker for intestinal integrity. B) Lactulose:Mannitol ratio in urine. *In vivo* paracellular permeability measured as urine levels 3 hours after gavage of the marker molecules lactulose and mannitol. Both parameters were monitored during natural development (upper pannels) at 7, 14, 21, 28 and 35 day-old, with results shown as mean±SD, differences between different age groups were analysed by one-way ANOVA with Tukey's post-hoc test and indicated by different letters. Also measured, in 17 day-old suckling rats after luminal provocation (lower pannels) on day 14 by gavage with PHA, protease (microbial), trypsin or water (control); and as Tukey's boxplot ±inner limits, line indicating the median and '+' representing the mean. Differences between each of the treatment groups and controls were analysed by one-way ANOVA with Dunnett's post-hoc test. Significance was considered when $p < 0.05$ (*), or non significant. Note: Dotted lines indicating the mean of 17 day-old control for reference.

The lactulose and mannitol permeability test was performed during natural development (Figure 9B) and showed that in 14 day-old rat pups the Lac:Man ratio excreted in urine appeared to be the lowest and showed a tendency to increase at weaning. On the other hand, protease-treated 17 day-old rat pups showed a significant increase in the Lac:Man ratio, despite a high variation, and the PHA-treated group seemed to not be much affected.

The ratio of lactulose and mannitol excretion in urine is used to assess gut health, as a marker for dysfunctional permeability or leakiness [115]. The purpose of using the Lac:Man ratio is mostly due to the compensation for incomplete sample collections, as well as gastric retention or bacterial degradation. However, it

assumes the premise that mannitol is absorbed from the intestines by passive diffusion, independent of any changes in gut permeability, and lactulose passes via the paracellular route, which is susceptible to changes in intestinal permeability [44,116].

Moreover, the quantification of the total amounts of lactulose and mannitol excreted in the urine can also be informative, and reduced excretion of mannitol can be indicative of a reduction in epithelial surface [117]. The results obtained indicate that the increased Lac:Man ratio observed in the 21 day-old rats was accompanied by decreased amounts of total mannitol excreted, as well as decreased amounts of total lactulose (Table 7). Differences in the urine volumes were also observed with lower production in the 21 day-old rats compared to that of the 14 and 28 day-old pups.

Table 7. Total amount of Mannitol (mg) and Lactulose (mg) excreted in urine during 3 hours after gavage in rats at 14, 21 and 28 days of age during natural development.

	14 days old	21 days old	28 days old
Mannitol (mg)	0.83±0.18 [a]	0.20±0.17 [b]	0.84±0.29 [a]
Lactulose (mg)	0.22±0.03[a]	0.08±0.06[b]	0.30±0.05[c]
Urine (ml)	0.57±0.09 [ab]	0.29±0.17 [a]	2.66±0.54 [b]

Differences between different age groups were analysed by one-way ANOVA with Tukey's post-hoc test, signifcant differences indicated by different letters.

In the induced precocious maturation groups, PHA and protease also caused a decrease in the total amount of mannitol excreted in the urine (Table 8). However, total amounts of lactulose and the urine volumes were not significantly affected when comparing 17 day-old treated rats to controls.

Table 8. Total amount of Mannitol (mg) and Lactulose (mg) excreted in urine during 3 hours after gavage in 17 day-old suckling rats after luminal provocation by gavage with PHA, protease (microbial), or water (control) at 14 days of age.

	Control	PHA	Protease
Mannitol (mg)	1.37±0.40	0.91±0.34**	0.93±0.34*
Lactulose (mg)	0.38±0.16	0.31±0.13	0.40±0.15
Urine (ml)	0.41±0.18	0.44±0.25	0.37±0.17

Differences between each of the treatment groups and controls were analysed by one-way ANOVA with Dunnett's post-hoc test, considered significant: $p < 0.0001$ (****) or non significant.

Thus, it would seem that both PHA and protease decreased the total amounts of mannitol excreted in the urine, however the Lac:Man ratio was only significantly increased in the protease-treated group. The reduced mannitol excretion observed in the PHA and protease-treated groups corresponded to those levels obtained in the 21 day-old rat pups and thus could indicate some sort of dysfunctional permeability, or gut leakiness. Additionally, the increase in SI weight was

accompanied by a reduction in the total amount of SI epithelia. However, use of the Lac:Man ratio as an adequate parameter for estimating the maturational status should be further studied, since even though the protease-treated group of rats showed significant differences there was high variability within groups. Besides, disease diagnosis in humans by this method has often proven to be difficult as no reference is available for comparison, due to the high variability between individuals [118].

Taken together, the changes in plasma levels of DAO and urine excretion of Lac:Man both indicated that the weaning process could implicate a temporary decrease in integrity of the intestinal epithelium (damage) involving a natural (controlled) process of inflammation. This naturally occurring inflammation at weaning would also be supported by the Lac:Man ratio measurements with increased leakage after protease-treatment and reduced total amounts of mannitol excretion in 21 day-old, as well as PHA- and protease treated groups.

Nonetheless, the methods for measuring plasma DAO and urine Lac:Man in young rats showed limitations that should be further evaluated, in order to improve the sensitivity of the method.

Exocrine pancreatic function during development

In previous studies on PHA-induced maturation from our research group, the pancreatic exocrine function appeared to be affected, hence, the question of the possible role of the pancreas during gastrointestinal maturation arose. Thus, further investigations on changes in pancreatic function and pancreatic proteolytic activity expression were conducted during natural and precociously induced gut maturation.

The pancreatic function was monitored during natural development in suckling rats (Figure 10). Pancreatic weight increased markedly relative to body weight with age in a gradual manner. The pancreatic protein content levels started slightly higher in the suckling groups (7 and 14 day-old) and were maintained at similar levels with age, however at 35 days old there was a slight tendency to decrease. Pancreatic trypsin activity was initially higher, with a decrease noted at 14 days old, and then a slight increase with age, that seemed to reach a plateau at weaning.

The ratio of trypsin:protein in the pancreas was calculated to estimate the maturity of pancreatic function. This ratio showed that 7 day-old rats have accumulated exocrine enzymes and the subsequent decrease in 14 day-old rats indicates that secretion was stimulated. Afterwards, the pancreatic trypsin:protein ratio increased with age. Overall, it could be perceived that the changes in pancreatic protein and

trypsin content were not major, however, the increase in pancreatic weight relative to body weight, which was especially marked from weaning (21 day-old), indicates that the total amount of protein and trypsin activity increased.

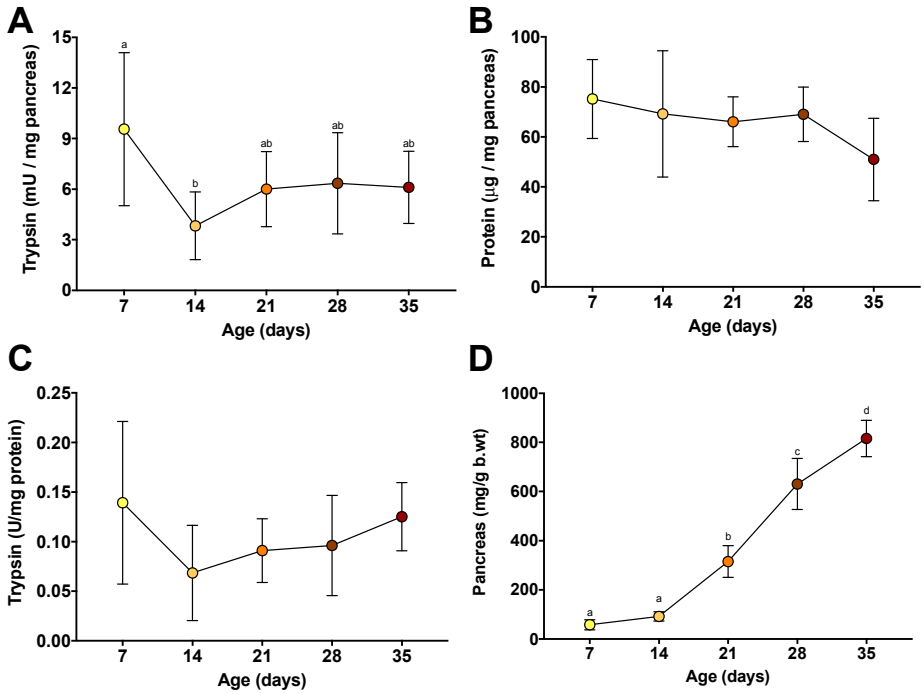


Figure 10. Pancreatic function during natural development in young rats.
Changes in pancreatic function during natural development: suckling period (7 and 14 days old), at weaning (21 days old) and post weaning ages (28 and 35 days old). A) pancreatic trypsin (U/g b.wt), B) protein content (mg/g b.wt) C) trypsin:protein ratio (U/mg protein) and D) pancreatic growth (mg pancreas/g b.wt). All data presented as mean±SD. Differences between different age groups were analysed by one-way ANOVA with Tukey's post-hoc test, significance indicated by different letters.

Furthermore, the changes in enzymatic activity in the pancreas during development were studied using a qualitative method, which made use of a variety of substrates that were either more or less specific. The results obtained showed major differences in the electrophoretic pattern of different enzymatic activities in pancreatic homogenates (Figure 11, *unpublished data*).

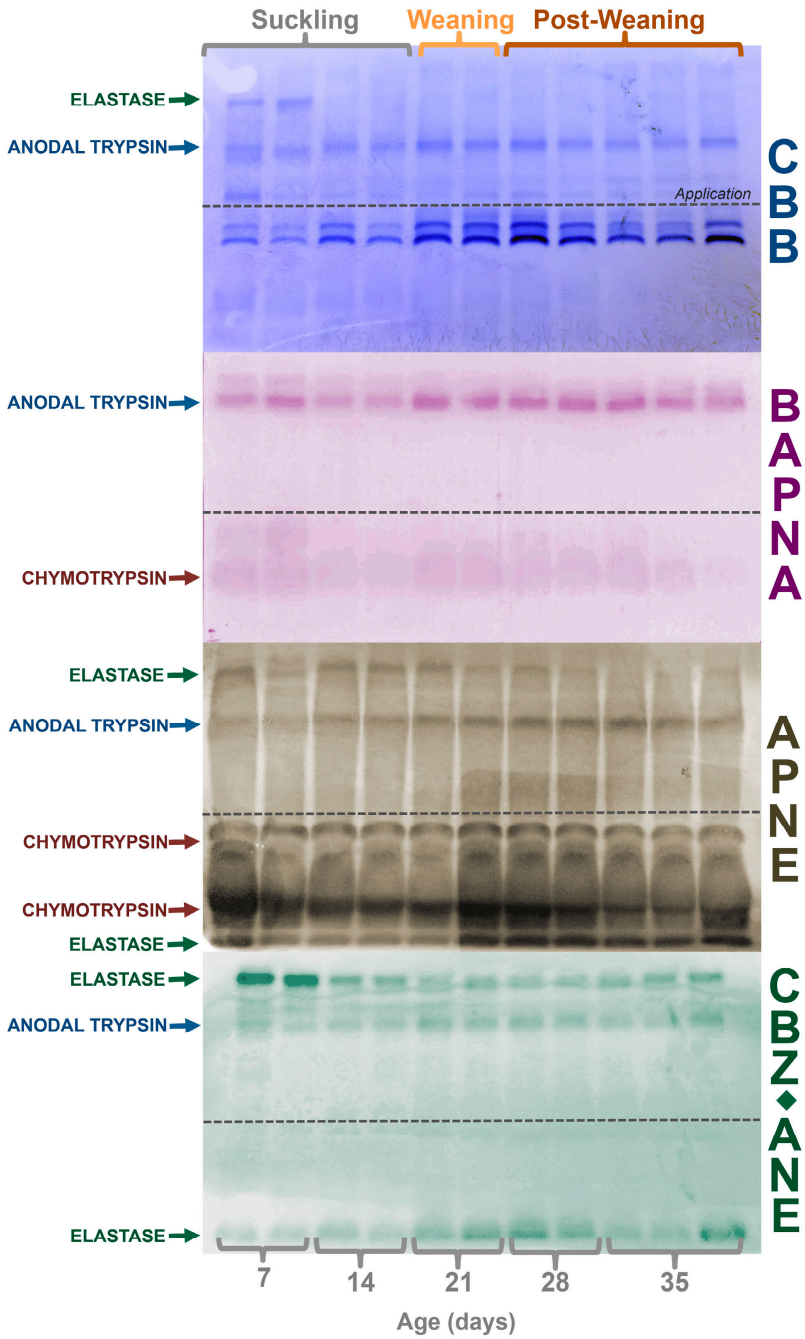


Figure 11. Enzymatic activity characterisation of pancreatic proteases during natural development in rats. Protein pattern after staining with CBB (Coomassie Brilliant Blue) and specific enzymatic activity in pancreas homogenates analysed after electrophoretic separation in agarose gel with the specific chromogenic substrates: BAPNA (trypsin-like activity), APNE (chymotrypsin-like activity), and CBZ-ANE (elastase-like activity).

The proteolytic activity in pancreas homogenates showed changes in pattern during postnatal development in rats, and staining for all proteins with CBB was used as reference for electrophoretic mobility. Trypsin-like activity, detected by BAPNA substrate, appeared with a band of anodal trypsin that increased with age. Chymotrypsin-like activity, detected with APNE substrate, revealed a cathodal chymotrypsin band with decreasing activity with age. At last, elastase-like activity, revealed with CBZ-ANE specific substrate, revealed a band mostly present in 7 day-old suckling rats.

In brief, the most notorious difference observed between the different age groups was the band with anodal elastase-like activity and it was confirmed by enzymoblotting (not shown). However, due to the slight changes in proteolytic activity patterns and the qualitative character of the technique with its limitations, further investigations would be interesting to pursue.

Precociously induced gut maturation

Pancreatic function has previously shown to be increased after PHA gavage [102]. More recently, exposure to proteolytic pancreatic or pancreatic-like enzymes also promoted pancreas growth as well as increased amylase and trypsin production in rat pups (**paper I)**. Furthermore, the results showed that protease and PHA could also increase the pancreatic trypsin production in athymic nude rat pups (**paper III)**. Hence, PHA and protease exposure in both rat strains, conventional and immunodeficient, caused not only maturation of the gut but also stimulated maturation of endogenous exocrine pancreatic function [18,24,102]. Thus, the increase in exocrine pancreatic enzyme production, especially that of proteinase, may be essential for initiation of the maturation process as an endogenous stimuli responsible for initiating gut maturation after release of the accumulated enzymes into the lumen. However, changes in pancreatic proteolytic enzymatic activity were not analysed during induced precocious maturation in suckling rats. Hence, more studies are needed to investigate the role of proteases with different proteolytic activities in the gut during development, and elastase could perhaps also be considered as a potential study target.

Due to the suggested importance of proteolytic enzymes in the gut, immunostaining of the protease-activated receptor–2 (PAR2) in the SI during natural development in suckling rats was performed. The results showed high expression of the receptor in both proximal and distal parts of the SI during natural development in rats (Figure 12, *unpublished data*) with epithelial localisation in both apical and basolateral sides of the enterocytes, especially during the suckling period. PAR2 was also localized in solitary cells in the *lamina propria*, in increasing numbers with age, presumably immune cells.

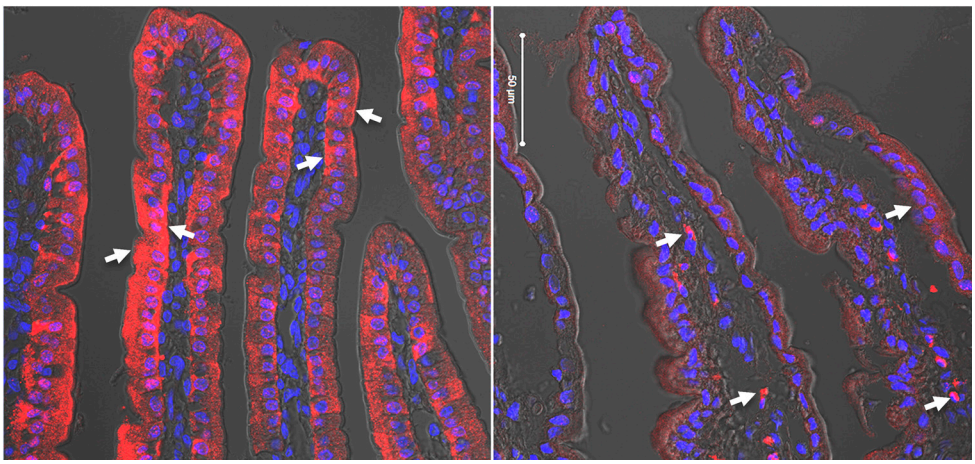


Figure 12. Confocal microscopy imaging of PAR2 immunostaining in the small intestine of young rats. Immunohistochemical staining of PAR2 (red) and nuclei (DAPI, blue) in the small intestine of 14 day-old (left) and 21 day-old (right) rats. Note (white arrows): epression of PAR2 apical and basolaterally along the epithelia in 14 day-old rats, in 21 day-old rats positive PAR2 staining in cells in the lamina propria as well as in the epithelium. (scale bar 50µm).

Early effects of the provocative agents

Precocious gut maturation can be induced 3-4 days prior to the natural temporal frame, on postnatal day 21, by enteral provocation with the previously mentioned PHA [102] and proteases (**paper I**). The effects of PHA- and protease- induced precocious gut maturation models can be differentiated in an early phase, within the first 24 hours after provocation, and a late phase, from 24 up to 72 hours after treatment administration, when the time allows for a complete renovation of the intestinal epithelia [119,120] (*unpublished data*, J Wang). The progression of the PHA induced provocation has been previously studied and revealed that during the early phase PHA binds to the brush border of the epithelia instigating a mucosal disturbance, seen as villi atrophy and decreased permeability, increased plasma corticosterone and pancreatic secretion, as well as intensified recruitment of lymphocytes to the gut mucosa [119,120].

The early effects of protease and PHA within 24 hours after enteral provocation were further investigated in **paper IV**. Both provocative agents caused weight loss and diarrhoea, and even though the effects of protease gavage on growth and performance were more severe, the pups recovered faster than those that were treated with PHA. Both treatments showed stimulation of gut growth within 24 hours, and pancreatic function was immediately enhanced as evidenced by the pancreas homogenates and luminal content analyses. Noteworthy, in the PHA

treated group, trypsin activity in the luminal content was initially depressed compared to that of the controls during the first 4 hours after gavage, thereafter trypsin started to increase coinciding with decreasing trypsin activity in the pancreas, indicating a stimulation of pancreatic secretion by the PHA treatment, albeit somewhat delayed [121-123].

Intestinal barrier function

The SI epithelium in young rats has high permeability until weaning, which is an important property to allow the passage of milk-borne bioactive molecules, *i.e.* milk-borne IgG, lactoferrin, etc. However, it is also essential for intestinal permeability to be highly regulated in order to preserve the barrier function, preventing the passage of potentially harmful dietary or microbial molecules [45].

Hence, the passage across the intestinal epithelium is highly regulated and susceptible to changes. During development, the gut undergoes the characteristic shift in the SI epithelia from the highly endocytic foetal-type enterocytes, expressing the FcRn in the proximal SI and containing supranuclear vacuoles in the distal SI, to adult-type enterocytes, which are $FcRn^{negative}$ and non-vacuolated respectively, and display a marked decrease in the absorptive capacity to macromolecules (**paper II)**. Thus, at weaning the so-called "closure" process takes place, providing an increased barrier function when the protection of the maternal factors is no longer available.

Thus, intestinal permeability can be used to monitor not only the barrier function properties in young rats but also as a parameter for estimating the maturational status induced by provocative agents. In previous studies, it has been shown that PHA and proteases (**paper I**) induce precocious gut maturation and closure of the small intestine to the passage of intact macromolecules, such as BIgG and BSA, 3 days after exposure. The effects on permeability could also be observed in the measured cumulative marker of RIgG, which was also diminished within the 3 days after exposure to PHA and protease. Additionally, the Lac:Man ratio obtained from permeability tests performed on those suckling rats indicated that the PHA and protease treatments, as well as natural weaning, involved changes in the intestinal barrier properties. PHA exposure has been shown to severely decrease permeability immediately [119], but studies on the early effects of proteases on intestinal permeability were still needed.

Hence, **paper IV** was designed to further investigate the early effects of proteases on intestinal permeability by monitoring the different absorptive routes over time after exposure, and compare them with those induced by PHA. At first, a marker cocktail containing BIgG, HSA and FD4 was used. Both treatments rapidly and markedly decreased plasma BIgG levels, indicating that FcRn receptor-mediated

endocytosis was severely affected by PHA and protease exposure. The loss of absorptive capacity via the receptor-mediated pathway was not restored within 24 hours after either of the treatments. The absorption of marker HSA was prominently lower already 1 hour after gavage in the PHA treated group, while in the protease treated group it decreased progressively and became significantly lower at 8 hours after provocation. This indicated different effects of the two treatments on the nonspecific endocytosis of macromolecules, and specifically HSA absorption seemed to be less sensitive to the effects of protease provocation. FD4 was used to trace the paracellular permeability [124]. In the PHA treated group no effect on the FD4 plasma level was observed compared to control. However, the protease treatment caused an increase in FD4 within the blood circulation early after provocation and decreased gradually up until 24 hours after provocation. The dysfunctionality of the intestinal barrier can be estimated by the measurement of paracellular permeability; hence protease treatment caused an instant increase in the leakiness of the intestinal barrier, which seemed to be overcome within 24 hours.

The IgG uptake takes place via the FcRn receptors expressed by the enterocytes in the proximal SI [125] (**paper II**), and non-specific endocytosis mostly takes place in the distal SI by the vacuolated enterocytes with high endocytic capacity [7,63]. However, the absorption of Ig via the non-specific pathway cannot be excluded. These foetal-type epithelial cells are replaced by FcRn-negative and non-vacuolated adult-type enterocytes, respectively, during postnatal maturation in suckling rats [119] (**paper I**), but it is known that the complete renewal of the gut mucosa takes 3-5 days in young rats [5,6]. Hence, the provocative agents likely affect the intestinal absorption of different macromolecules via different mechanisms. PHA binds to carbohydrate structures in the brush-border of the intestinal epithelium in the suckling rat [119], evidently blocking all intestinal absorptive capacity. Then, PHA would cause reduced permeability due to physical impediment for the absorptive cells to reach the molecules in the luminal content due to the binding of the lectin to the epithelial surface. Protease provocation, however, provoked a dysfunctional permeability, with increased leakiness of the gut barrier for the first 24 hours after provocation, which was considered worthy of further investigation.

Consequently, re-evaluation of the different permeability routes was done with a new cocktail containing lactulose and mannitol, as tracers of the paracellular route, as well as the non-degradable fluorescence-labelled dextran FD70, to track the unspecific macromolecular uptake, together with the BIgG and HSA markers as references. The results obtained showed an increased Lac:Man ratio excreted into the urine, which reassured the increased paracellular permeability and thus, dysfunctional permeability or leakiness of the gut. The plasma level of FD70 increased already 1 hour after provocation with protease, indicating that even

though having a comparable molecular weight to HSA, it was not absorbed in a similar way. The pattern of absorption of FD70, instead was more similar to that of the markers used to monitor the paracellular passage, such as FD4 and the Lac:Man ratio.

Even though the molecular weight of FD70 is similar to that of albumin, the molecular diameter differs between the two molecules, being ~100Å for HSA whereas that of FD70 is ~60Å. The study of the epithelial uptake of FD70 by fluorescence microscopy provided additional information showing a rapid decrease in the endocytic uptake in the proximal SI while the uptake in the distal SI increased, which supported the increased plasma level of FD70. Thus, the microscopic analysis revealed epithelial uptake of FD70 via both transcellular, including the large supranuclear vacuoles, as well as paracellular routes, however further investigation is needed to improve and confirm these results due to the limited resolution of the method used.

It has been reported that activation of PAR2 by proteases can induce changes in paracellular permeability by disassembling the intestinal tight junctions, causing loosened pore diameter, allowing molecules of bigger sizes to pass [126-128]. Proteins such as claudins, zonulin and occludin form the intestinal tight junctions; which regulate paracellular permeability and can indicate dysfunctional barrier function, as reviewed by Bischoff *et al.* (2014) [44], and specifically zonulin (haptoglobin-related protein) has been associated with PAR2 activation [127,129] Moreover, despite the lack of studies on immune cell recruitment in the protease-induced gut maturation, it is also known that proteases can cause the activation of pro-inflammatory pathways via the PAR2 receptor.

Involvement of the immune system in gut maturation

A relation between maturation of the digestive and immune systems was shown in mice when the absence of adaptive and/or passive immunity temporarily delayed the maturation progress and affected developmentally regulated genes in the enterocytes during suckling and weaning [92]. Furthermore, natural weaning has been associated with a 'physiological inflammatory' process with immune cell recruitment to the gut due to the release of chemokines and cytokines by the enterocytes [130]. Previous studies from our lab have shown that PHA-induced maturation also involves initial inflammation followed by recruitment of T- and B-lymphocytes to the SI mucosa [120]. Thus, further studies were engaged on the involvement of the immune system in the maturation of the GI tract in neonatal rats.

Involvement of Blimp1

The transcription factor B lymphocyte induced maturation protein-1 (Blimp1) plays an important immune role in differentiation of B-lymphocytes to plasma cells [131], as well as T-lymphocyte homeostasis and function [132], mainly effector T-cells, including the suppression of IL2 production [133].

However, Blimp1 expression has been described in the postnatal intestine epithelium with a drastic decreased in expression at weaning in mice [93,94]. These studies suggested that the transcription factor Blimp1 was a key regulating-element in the gut, keeping the enterocytes adapted to suckling. Also, Blimp1 knockout mice developed more severe colitis due to their role in inhibiting T-lymphocytes, and hence a role in intestinal mucosa homeostasis was attributed [134]. More recently, another study showed that the loss of Blimp1 expression in the intestine at weaning was coincident with a marked increase in expression of MHC class I in the SI, in relation to antigen processing [135].

Hence, Blimp1 was included in the **paper II** study, in which its expression was monitored in parallel to the expression of FcRn and the presence of supranuclear vacuoles in IECs. Blimp1 gene expression (Prdm1 mRNA) was higher in the distal SI and then decreased at weaning, which would fit with the decrease at weaning previously found in mice. In the proximal SI, the expression of Blimp1 was found to be lower but more constant than in the distal part. However, the immunostaining of the proximal and distal parts of the SI showed that Blimp1 was highly expressed in the nuclei of the immature enterocytes in the villi of the SI and underwent intracellular redistribution, from nuclei to cytoplasm, during weaning or induced maturation.

Furthermore, Blimp1 immunostaining was also observed in both the proximal and the distal parts of the SI in all age groups not only in enterocytes but also in crypt-region cells; epithelium-associated cells, presumably IELs; cells in the *lamina propria* and the submucosa, most likely immune cells and in cells between the muscular layers, potentially cells of the myenteric plexus (enteric nervous system). The distribution and localization of Blimp1 in the gut during development seems to suggest that cells susceptible to inflammation could express Blimp1.

Blimp1 has been described as a reciprocal antagonist with Bcl6 in lymphocytes [136,137], using Blimp1 as a marker for maturation and Bcl6 as a marker for proliferation. In the intestinal epithelium, proliferation occurs in the stem cells and "+4 cells" in the crypts. In early life, slow migration rate has been described at the same time as high proliferation in the intestinal epithelium, leading to the speculation that perhaps maturation and proliferation could be key regulators of cell fate in the SI. Proliferation was also investigated by immunostaining of the proliferative cell nuclear antigen (PCNA) in the SI, and the results (*unpublished*

data, Figure 13) showed that vacuolated foetal-type cells in the distal SI in 7 day-old rats were $PCNA^{+}$. Hence, Blimp1 could be suggested as a repressor of differentiation in proliferative cells (transit amplifying cells) but an apoptotic/cell fate label in highly mature and differentiated cells.

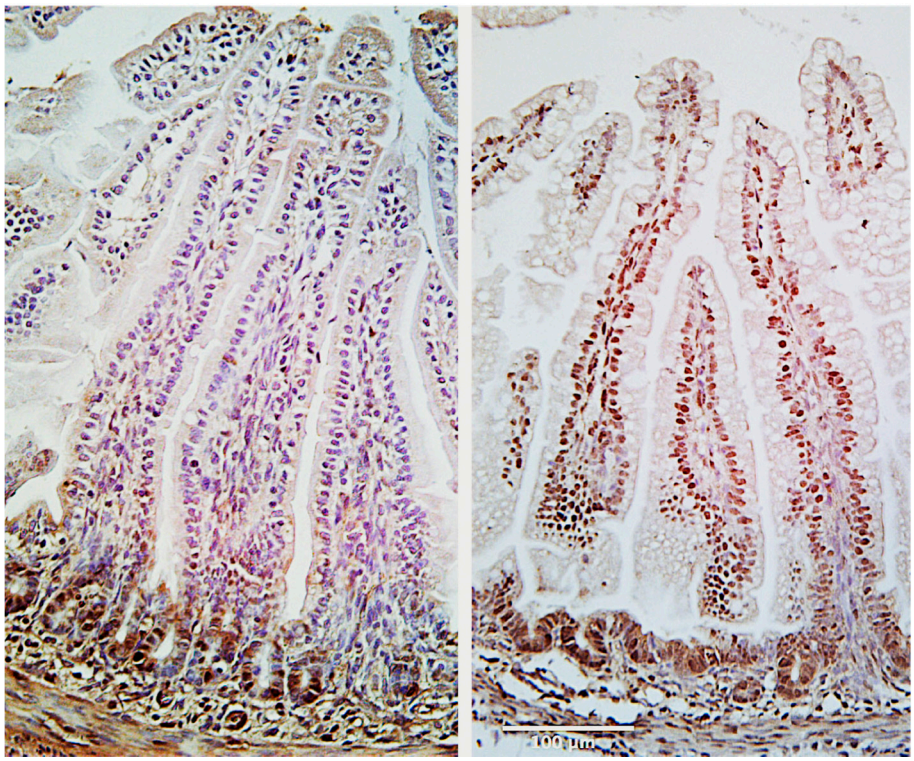


Figure. 13 Proliferative cells in 7 day-old suckling rats
Immunohistochemical staining of PCNA (brown) with haematoxylin counterstaining (blue) in the proximal (left) and the distal (right) parts of the villi in the small intestine of 7 day-old suckling rats (magnification of 200x, scale bar 100µm).
Note: positive immunostaining in the nuclei of polifereative vacuolated foetal-type enterocytes in the distal SI.

In summary, despite the differences found in Blimp1 expression in the intestinal epithelia between neonatal mice and rats, the participation of the transcriptional repressor in the gut could still be preventing/delaying the replacement of the foetal-type for adult-type epithelium until weaning, and therefore more studies are needed to elucidate the role of Blimp1 as a regulating factor of gut maturation in neonatal rats. Nonetheless, the expression of Blimp1 in the gut mucosa contributed to emphasise the importance of the immune system in the gut and the potential involvement in gut maturation.

Role of mucosal T–lymphocytes

The importance of T-cell recruitment to the neonatal mucosa is essential for gut maturation and immune system activation, including the establishment of oral tolerance in early life [31,32]. It is known that $CD3^+$ IELs can already be found on the first day after birth and progressively increase during the suckling period [36]. In neonatal nude rats, mature T-lymphocytes cannot develop and $CD3^+$IELs and LPLs do not appear until between four to six months of age [138]. Recruitment of T- and B-lymphocytes to the gut mucosa has previously been observed in the PHA-induced precocious gut maturation model in suckling rats[120]. Additionally, studies using the immunosuppressant drug Cyclosporine A, which is an inhibitor of IL2 transcription and results in reduction of T-cell activity, showed a decrease in intestinal growth with signs of delayed gut maturation [90] and could also block PHA-induced precocious maturation [139].

Thus, induced precocious gut maturation after exposure to protease and PHA in the immunodeficient athymic nude rat model was investigated in **paper III**. Provocation with both, PHA or protease, resulted in increased gut growth and precocious maturation, with epithelial replacement of foetal- by adult-type non-vacuolated enterocytes in the distal SI, decreased intestinal macromolecular permeability (gut closure) and increased pancreatic function in the treated pups.

The immunostaining of $CD3^+$cells, marker for T-cell lineage, of the intestinal mucosa in the nude rats was performed and included in **paper III**. The results obtained showed the presence of $CD3^+$cells in the gut of the nude pups, but much fewer compared to conventional SD pups, which remained unaltered by induced maturation. Interestingly, nurturing of nude rats by immunocompetent SD dams increased macromolecular absorptive capacity in the nude rats.

The presence of mature T-lymphocytes in the conventional and nude suckling rat pups, albeit in low amounts, could be explained by the ability of milk-borne immune cells, including maternal T lymphocytes, to translocate to the intestinal mucosa, where they can be activated by luminal antigens, during the early postnatal period as found in rabbits, mice, and humans [25,140,141]. The role of the maternally transferred immune cells on intestinal maturation has not yet been elucidated. However, in the present study mucosal $CD3^+$ cells were investigated in athymic nude rat pups nurtured by nude or euthymic dams, but no differences in the amounts of $CD3^+$ cells were observed.

This study showed that gut maturation is independent of thymus-mediated regulation despite previous studies suggesting that gut maturation was T-cell mediated. However, nude rats are not totally depleted of bone-marrow derived T-cell precursors and mucosal T-lymphocytes ($CD3^+$cells) were present and unaffected by enteral provocation to induce precocious gut maturation. The

extrathymic development of T-lymphocytes has been reported to take place in the MLNs from where they migrate and accumulate in the intestine mostly as IELs [142], as well as in the gut mucosa [143,144]. Moreover, other studies in young nude rats have shown the presence of activated mature T-lymphocytes in the MLNs during weaning in approximately the same amount as those found in euthymic young rats [141]. Hence, thymus-derived T-lymphocytes are not indispensable for gut maturation but non-thymus derived T-lymphocytes cannot be underestimated. Furthermore, the immune system during the neonatal period shows adjustments to the T-cell deficient function in nude rats, with an increase in alternative cell types that can compensate for the essential roles of T-cell signalling [145]. Suitable candidates, besides the T-lymphocytes, with shared cytokines signalling could be natural killer cells, mast cells and activated DCs as reviewed by Boyman and Sprent (2012) [146].

It has been proposed that *lamina propria* T helper cells (T_h) are mediators of IECs differentiation and they contribute to the maintenance of the epithelial barrier function [147]. It is also known that T_h cells in the neonatal intestine in mice are inhibited by T_{reg} and IgA-mediated antigen translocation [80]. However, increasing studies on the novel role of innate lymphocytes (ILCs) in the GI tract have recently been published and have provided evidence that ILCs would be the counterpart of T-lymphocytes in the innate immunity [148], with a role as integrative glial-cell-derived factors and as the receivers and regulators of multisystem signalling in the gut [149]. Furthermore, a specific type of ILCs (type 3) are described as the first immune cells to colonize the neonatal intestine [150], and to express MHC class II in the absence of the co-stimulatory signals, with a proposed mechanism of immune response inhibition [151]. Thus, ILCs could play the role which has for a long time been attributed to T-lymphocytes, as regulators of developmental processes especially those occurring in the GI tract.

The conclusions extracted from **paper III** included that of the ability of proteases to induce precocious gut maturation in the nude rat model suggests proteases, endogenous or exogenous, could be the stimuli triggering gut maturation. Hence, indicating that the adaptive immune system, especially T-lymphocytes, has more of a supporting role during gut maturation than as an instigator, and perhaps it is the extrathymic T-lymphocytes that might be of importance. However, more studies are required to elucidate the full mechanism of intestinal maturation in mammals.

Possible mechanisms for initiating gut maturation

Gut provocation – Induced precocious maturation

Phytohaemagglutinin

It is known that PHA rapidly binds directly to the small intestine epithelial surface after gavage [24,102,119,152], and it has been shown to stimulate the release of cholecystokinin (CCK), the stimulatory hormone of exocrine pancreatic secretion [121,153]. However, the stimulation of pancreatic growth and secretion by PHA (**paper IV**) is only partially regulated by a CCK–dependent mechanism [123]. Besides, CCK receptors have been shown to be less expressed and thus less sensitive to secretagogues during the suckling period compared to at weaning in the rat [154,155]. Nevertheless, in a study by Linderoth, *et al.* (2006) it was shown that PHA stimulates the secretion of pancreatic enzymes 3 hours after gavage [119]. More importantly, it has also been shown that for PHA to induce precocious gut maturation it is essential that it is administered orally [103], reflecting the importance of the enteral stimulation in early life for an appropriate gut development.

The binding of PHA to the epithelial surface and the interaction with the brush-border molecules of the IECs could activate or cross-activate receptors such as PARs, TLRs, LBRs, etc. the activation of all of which leads to the signalling of the inflammatory NFκB pathway. Moreover, PHA has been shown to pass across the intestinal barrier and induce immune cell recruitment to the intestinal mucosa [156], and thus, stimulation of the release of immune proteases and activation of PAR2 endogenously, not only in the epithelia but also in the enteric nervous system [157]. PAR2 has also been found in enteric nervous system cells, such as myenteric and submucosal neurons, exposed to endogenous immune proteases, PHA antigenic presence in the mucosal site could also stimulate neuronal pathways and consequently, pancreatic stimulation.

Proteases

Exogenous proteases of diverse origin such as pancreatic-like enzymes from dietary and microbial sources can accelerate gut maturation after luminal exposure (**paper I, paper III,** and Figure 7). However, the intestinal milieu during the suckling period in rats is characterised by the presence of maternal milk-borne protease inhibitors, together with an immature pancreatic function, and thus low proteolytic activity in the intestinal lumen [49]. At weaning, the change of milk diet to solid food may stimulate the secretion of exocrine pancreatic enzymes, gradually increasing the proteolytic activity in the lumen from the 3rd postnatal week. Moreover, the pancreatic function not only increases its proteolytic activity content (trypsin activity) but also a change in the specific enzymatic pattern was

preliminary shown (Figure 11). PAR2 is expressed in the GI tract, especially in the enterocytes in the SI (Figure 12) and in pancreatic acinar cells [73]. Interestingly, the most evident change in the pancreatic enzymology during development was an increased elastase-like activity in 7 day-old rat pups (Figure 11). Furthermore, elastase activity has been shown to have a diminishing effect on PAR2 receptors, *i.e.*, disarming them by enzymatic cleavage, in the respiratory epithelium [158]. Thus, the high activity of pancreatic elastase found during the early suckling period might play a similar role, inactivating PAR2 and postponing SI maturation.

PAR2 mediated gut maturation – a hypothesis

The experimental protease- and PHA-induced precocious gut maturation models used in the studies included in this thesis seem to indicate that PAR2 might be a suitable candidate for further investigations as a trigger mechanism to provoke gut maturation.

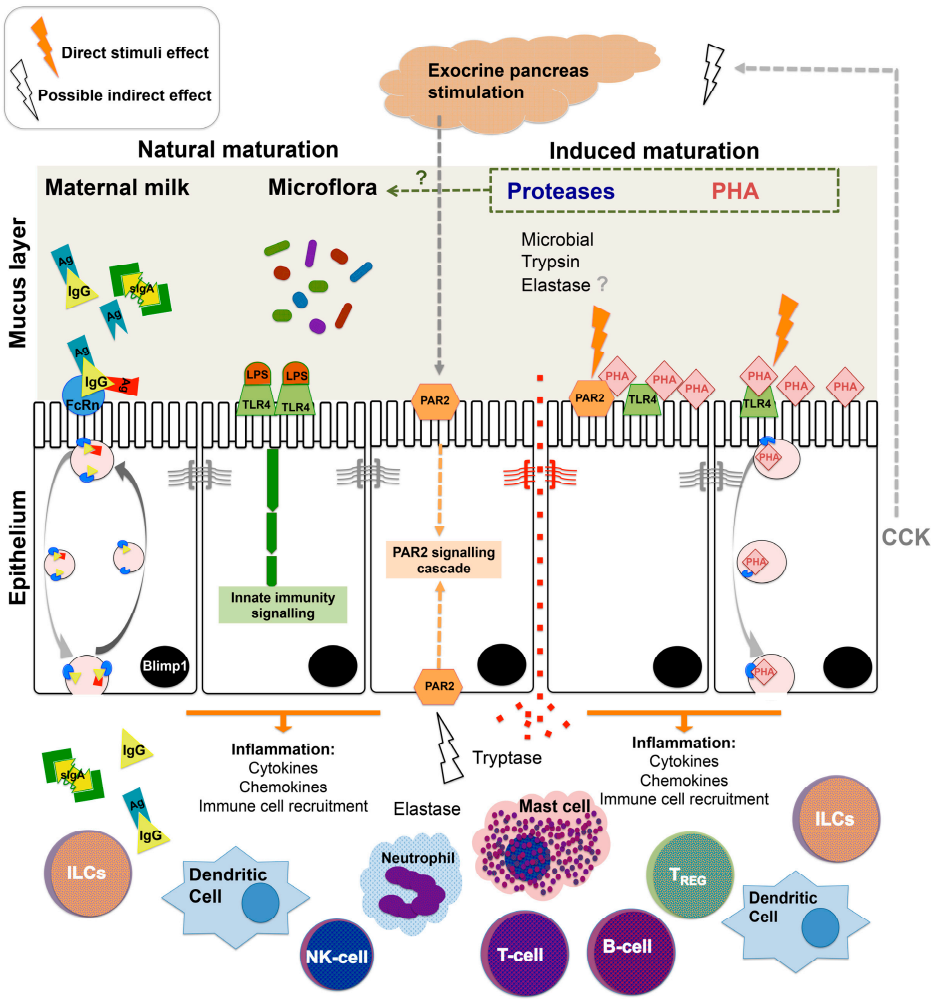
It has been shown that PAR2 is expressed in the apical and basolateral sides of SI enterocytes of adult [73] and newborn rats (Figure 12). Thus, the distribution of PAR2 in the intestinal epithelium makes it an optimally distributed sensor of both the exogenous or luminal proteases from pancreatic, dietary or microbial origins, as well as endogenous immune-derived proteases available in the gut mucosa, such as mast cell tryptase [159] or neutrophil derived elastase. For instance, the change in microbiota with increasing gram-negative bacteria at weaning due to the dietary transition from milk to solid food and neonatal exposure to LPS affects pancreatic secretion in adult rats [160], and the activation of TLR4 has been indirectly linked to PAR2 [161,162]. Moreover, PHA-induced gut maturation showed recruitment of immune cells to the gut mucosa after exposure, in support of PAR2 activation from the basolateral side, however immune-cells recruitment during protease-induced gut maturation has not yet been fully elucidated.

The activation of the PAR2 receptor has been associated with different functions, such as signalling to enterocytes, inflammatory cells or the enteric nervous system [163] and immune cell recruitment [164,165]. PAR2 receptor activation has also been reported to affect gut health, triggering of pro-inflammatory responses in the SI epithelia [73], mucus secretion [166], changes in paracellular permeability [126,127,129] as well as proliferation and differentiation of epithelial cells. A model of PAR2 knockout mice showed a delayed onset of gut inflammation [165], but it never has been studied in relation to the maturational changes occurring in the gut.

Thus, PAR2 could be involved in gut maturation by coordinating the intestinal epithelial replacement from foetal-type to adult-type (**paper I and III**), the pancreatic function maturation (**papers III and IV**; Figure 10) and the immune system activation that occurs during postnatal development in rats. It would be

plausible to relate the activation of PAR2 with the initiation of gut maturation in the young, occurring at weaning, which is associated with vast changes in the SI mucosa that lead to the concept of 'physiological inflammation' [130]. Thus, PHA recruitment of immune cells to the gut mucosa and the decrease in intestinal integrity (DAO, Lac:Man ratio, FD4, FD70) caused by enteral proteases (**paper IV** and Figure 10) could similarly mimic the naturally occurring inflammatory events.

The presented hypothesis proposes gut maturation as the result of the crosstalk between environmental changes (dietary and microbial), the intestinal barrier, the pancreatic function and immunity and suggests that gut maturation could be mediated via PAR2. Thus, further studies are needed for the elucidation of more details.



Conclusions

– Paper I –

- Pancreatic and pancreatic-like enzymes with proteolytic activity were found to provoke gut maturation in neonatal rats, while other enzyme activities had no effects.
- An alternative model of induced precocious maturation by enteral exposure to proteases in the suckling rat was established.

– Paper II –

- The developmental switch from foetal- to adult-type SI epithelium, with decreased FcRn expression in the proximal SI and replacement of vacuolated enterocytes in the distal SI, are correlated to and can be used for monitoring gut maturation in neonatal rats.
- The timing of the maturational changes with the intracellular redistribution of the transcription factor, Blimp1, indicates involvement in the regulation of SI maturation and supports the idea that postnatal development of the digestive and the immune systems of the gut are connected.

– Paper III –

- Precocious gut maturation can be induced by oral provocation in immunodeficient athymic nude rat pups, similarly to in conventional neonatal rats, indicating that gut maturation is independent from thymus-derived T-lymphocytes.
- Involvement of extrathymic T-lymphocytes in gut maturation might be a possibility, since few mucosal $CD3^+$ cells were found in neonatal athymic rats.

– Paper IV –

- Enteral PHA or protease exposure hampered the high intestinal macromolecular permeability (endocytosis) in neonatal rats while protease exposure also temporarily increased the intestinal paracellular leakiness.
- The different early effects on intestinal permeability of the two provocative agents after enteral exposure indicate that they initiate gut maturation via different mechanisms.

– General –

- Using the suckling rat as a model provides us with evidence that postnatal gut maturation, naturally at weaning or precociously induced by enteral provocation, is initiated by luminal cues affecting the intestinal epithelium involving an activation of the immune system and pancreatic function.
- Contributing to an increased understanding of the initiating cues and the mechanisms of maturation of the intestinal barrier in young mammals, the knowledge could perhaps be applied in translational studies to improve strategies for the prevention or treatment of gut-related complications often affecting premature infants.

Future studies and perspectives

Our results indicate that the protease-sensitive receptor PAR2 in the gut may be a possible common mediator of exogenous stimuli and endogenous signalling occurring during gut maturation, natural or in both models of experimentally induced precocious maturation, by exposure to proteases and PHA.

- Further characterisation of the pancreatic enzymatic profile during gut development could provide us with a better of understanding of the possible role of pancreatic function in inducing gut development.
- Assessing whether the exocrine pancreas plays a role in facilitating or preventing postnatal gut maturation, which could be done by stimulation of pancreatic secretion to induce precocious gut maturation.
- Assessing whether the luminal proteases in general play a role in facilitating or preventing postnatal gut maturation, which could be done by using PAR2 agonists and antagonist molecules in our experimental induced precocious maturation models.

- The potential role of endogenous proteases, such as mast cell tryptase, in activation of PAR2 could be investigated by the use of mast cell degranulation or stabilizing (blockers) compounds in natural or experimentally induced precocious gut maturation models.

The role of the immune system, with a special interest in innate lymphocyte cells (ILCs), would be worthy of further investigations on their presence in both, natural and experimentally induced gut maturation models, as well as in the T-cell deficient rat model, athymic nude rats.

- The identification for classification of the $CD3^+$ cells found in the neonatal athymic nude rats and their potential ILC character would be of interest due to the sensitivity of these cells to dietary cues. Afterwards, the study of the defined cell type in conventional rats would be included.
- A study on possible mediator cross-linking signals, including cytokines and chemokines and microbial products, during natural and experimentally induced maturation could help in elucidating the mechanism by which the digestive and the immune systems intercommunicate.

At last, comparison of different species, such as rodents, mice and rats, as well as pigs, related to humans could help in finding the conserved pathways in comparison to those more species-specific, and thus finding the elements more genetically hard-wired from those more subject to environmental regulation, and thus, manipulation.

- Pigs are a precocious species, physiologically similar to humans. Thus studies on the gut developmental changes in piglets could bring more understanding of the gut maturation mechanisms, getting closer to possible translational applications.

Popular summary

The rat is a mammalian species with a short gestation period and thus born very immature. It is totally dependent on the mother for many functions, such as nourishment, thermoregulation, urination and defecation, since they are naked, blind and deaf at birth. During the suckling period the digestive function of the gut is fully adapted to the milk diet. The barrier function in the gut is also immature and allows for passage of undigested milk-borne bioactive molecules to reach the blood circulation in suckling rats. The function of the pancreas, which secretes the digestive enzymes, is also low contributing to the uptake of intact bioactive compounds from the milk. This uptake of maternal molecules, such as antibodies, hormones and antimicrobial components, is essential for providing the offspring with protection against infections (passive immunity) until their own immune system has matured.

The passage of macromolecules across the immature small intestine takes place by a selective receptor-mediated uptake after antibody binding to the neonatal-Fc receptor (FcRn) highly expressed in the first proximal half of the small intestinal epithelium during the suckling period, which correlates to the passive immunity transfer (paper II). Simultaneously, cells with a high absorptive capacity conform the most of the epithelium in the second distal half of the small intestine, being distinctive by a big cellular vacuole taking almost all the cytoplasmic space (paper II).

At weaning, the transition from milk to a solid food diet, the maturation of the gut accelerates. The changes occurring include the replacement of the immature-type intestinal epithelial cells by more mature-types, characterised by a decreased expression of the antibody receptor and disappearance of the highly absorptive vacuolated cells. The barrier function is also drastically increased at ‘gut closure’, and the loss of the absorptive capacity to macromolecules (papers II, III and IV). The function of the pancreas at weaning is also promoted (unpublished data) and the secretion of pancreatic enzymes is stimulated as also shown in our induced gut maturation models (papers III and IV).

This natural maturation process can be also experimentally induced and we have developed two different models of induced gut maturation in suckling rats by experimental feeding a lectin from red kidney beans, phytohaemagglutinin (PHA), or proteases, protein digestive enzymes (paper I). These experiment models of precocious gut maturation mimic the changes that occur during natural development at weaning in a more defined time frame and controlled conditions.

The early effects after feeding the two provocative agents provided us with a better understanding of the triggering mechanisms leading to gut maturation. PHA

showed an immediate blockage of all routes of macromolecular passage across the intestinal wall. Protease blocked the specific and unspecific vesicular transport pathways, however, increased the paracellular leakage, causing dysfunctional permeability in the distal part of the small intestine (paper IV). The results indicate that the two agents have different mechanisms for provoking gut maturation.

The immune system has also been related to the maturation of the gut in rats. T-lymphocytes have been proposed as key modulators and recently, an immune-cell related transcription factor (Blimp-1) was appointed as a key regulator of the intestinal epithelial cells maturation in mice. Thus, Blimp1 was studied in the small intestine in rats and the results showed decreased expression, timing with the maturation process (paper II). The connection of the immune system, especially T-cells, to gut maturation was also studied by means of the use of and an immunodeficient nude rat model, lacking the organ (thymus) for T-cell development. The nude rats appeared to be normally susceptible to feeding provocation and precocious gut maturation was also induced in these rats (paper III). The results suggest that maturation of the gut is independent on thymus-derived T-cells, but there could be other immune cell types involved in the process.

Furthermore, the developmental changes that occur at weaning have been related "physiological inflammation" with recruitment of immune cells to the gut. It was previously shown that induced precocious maturation involves the recruitment of lymphocytes, but more studies are still needed. In fact, in support we found an increased barrier leakage early after feeding provocation with proteases, which indicates that an inflammatory process occurs (paper IV).

The present thesis high-light the postnatal development of the gut using the neonatal rat model with focus on the intestinal barrier function, the pancreatic function, and the relation to gut immune factors, especially the early possible triggering mechanisms. The parameters were studied during natural and experimentally induced precocious maturation using the feeding provocation models developed in our lab. The findings of the present thesis have contributed with new knowledge and a better understanding on what triggers intestinal barrier maturation in neonates and may help improve or develop new strategies for the treatment of gut-related complications often affecting premature infants.

Populärvetenskaplig sammanfattning

Råttan är en däggdjursart med en kort dräktighetstiden och föds därför mycket omogen. Den är vid födelsen naken, blind och döv och är helt beroende av sin moder för många funktioner, såsom näringstillförsel, termoreglering, urinering och tarmtömning. Under diperioden är råttans mag-tarmsystem och matsmältning helt anpassad till modersmjölkdieten. Bland annat är tarmens barriärfunktion omogen, vilket möjliggör en passage av intakta bioaktiva mjölkmolekyler till blodcirkulationen hos diande råttor. Bukspottkörteln funktion, som utsöndrar matsmältningsenzymer, är också låg vilket underlättar upptaget av intakta bioaktiva makromolekyler från mjölken. Detta upptag av, t.ex., antikroppar, hormoner och antimikrobiella komponenter, är viktigt för att ge avkomman ett skydd mot infektioner (passiv immunitet) tills det egna immunsystemet har mognat.

Passagen av makromolekyler över den omogna tunntarmen sker genom ett selektivt receptor-medierad upptag av antikroppar efter inbindning till den neonatala Fc-receptorn (FcRn) vilken uttrycks i den första proximala halvan av tunntarmens epitel (arbete II). Celler med en hög absorptionsförmåga, som särskiljs med en stor cell-vakuolen som upptar nästan hela cellens volym, utgör samtidigt det mesta av epitelet i den andra distala halvan av tunntarmen, (arbete II). Dessa egenskaper i den omogna tarmens slemhinna korrelerar till överföringen av den passiva immuniteten under diperioden.

Vid avvänjningen, d.v.s. vid övergången från mjölk till en diet med fast föda, accelererar mognaden av tarmen. De förändringar som sker, inkluderar ett utbyte av de omogna tarmepitelcellerna med mer mogna typer, som kännetecknas av ett minskat uttryck av antikroppsreceptorn och frånvaro av de hög-absorptiva vakuoliserade cellerna. Tarmens barriärfunktion ökar också drastiskt vid ”gut closure” med en förlust av den upptagskapaciteten för makromolekyler (arbete II, III och IV). Dessutom ökar bukspottkörtelns funktion vid avvänjningen (opublicerad data) och utsöndringen av pankreasenzymer stimuleras vilket visas i våra modeller med inducerad tarmmognad (arbete III och IV).

Tarmmognad kan också induceras experimentellt och vi har utvecklat två olika modeller av inducerad tarmmognad i diande råttor genom att experimentell mata, dels med ett lektin från röda kidneybönor, fytohemagglutinin (PHA), och dels med proteaser, protein-nedbrytande enzymer (arbete I). Dessa experiment modeller med brådmognad av tarmen efterliknar de förändringar som naturligt sker vid avvänjning, men under en mer kontrollerad tidsram och förhållanden.

De tidiga effekterna orsakade av behandling med de två provokativa substanserna har gett oss en bättre förståelse av de utlösande mekanismerna ledande till

tarmmognad. Behandling med PHA gav en omedelbar blockering av all passage av makromolekyler över tarmväggen. Proteas-behandling blockerade både, den specifika och ospecifika vesikulära transportvägen, men skapade också ett läckage mellan cellerna, resulterande i en ökad permeabilitet i den distala delen av tunntarmen (pappers IV). Dessa resultat tyder på att de två substanserna har olika mekanismer för att provocera tarmmognad.

Tarmens immunsystem har också satts i samband med mognaden av tarmen hos unga råttor och T-lymfocyter har föreslagits som viktiga modulatorer. Nyligen har en immuncells-relaterad transkriptionsfaktor, Blimp-1, utpekats som en viktig hämmare av tarmepitelceller mognad hos möss. Därför studerades Blimp1 i tunntarmen hos råtta och resultaten visade ett minskat uttryck, vältajmat med ökad mognad (papper II). Effekter av immunsystemet, särskilt T-celler, på tarmmognaden studerades också med hjälp av en modell med nakna råttor, vilka har ett nedsatt immunförsvar eftersom de saknar tymus, organet där T-celler utvecklas. De nakna råttorna verkade vara normalt känsliga för tarmprovokation eftersom brådmognad av tarmen kunde även induceras i dessa råttor (arbete III). Resultaten tyder därför på att mognad av tarmen är oberoende av tymus T-celler, men det kan finnas andra typer immuncell inblandade i processen.

De utvecklingsmässiga förändringar som sker vid avvänjningen har också satts i samband s.k. "fysiologisk inflammation", med rekrytering av immunceller till tarmen. Det har tidigare visats att inducerad brådmognad av tarmen medför en rekryteringen av lymfocyter men fler studier behövs. Som stöd för detta fann vi ett ökat barriärläckage efter provokation med proteaser vilket tyder på att en inflammatorisk process sker (arbete IV).

Min avhandling belyser tarmens utveckling efter födseln, m.h.a en modell med nyfödda råttor, med fokus på tarmens barriärfunktion, bukspottkörtelns funktion, och i relation till tarmens immunsystem, med speciellt focus på de tidiga utlösande mekanismerna. Detta studerades, både vid naturlig och experimentellt inducerad brådmognad av tarmen, med provokationsmodeller som utvecklats i vårt labb. Resultaten som framkommit bidrar med ny kunskap och en bättre förståelse för vad som utlöser mognad av tarmbarriären hos nyfödda och kan förhoppningsvis bidra till att förbättra eller utveckla nya strategier för behandling av tarmrelaterade komplikationer som ofta uppkommer hos förtidigt-födda barn.

Resum de divulgació científica

La rata és un mamífer amb un període de gestació curt i, per tant, al néixer és nua, cega, sorda, i totalment dependent de la mare, per a funcions com ara l'alimentació, la termoregulació, orinar i defecar.

Durant el període d'alletament, la funció digestiva està totalment adaptada a la lactància. La funció de barrera a l'intestí també és immadura i permet el pas de les molècules amb activitat biològica de la llet sense digerir per arribar intactes a la circulació sanguínia de les rates lactants. La funció del pàncrees, que segrega els enzims digestius, és també baixa, fet que contribueix a l'absorció dels compostos bioactius de la llet sense digerir. Aquesta transferència de molècules de la llet materna, com ara anticossos, hormones i components antimicrobians, és essencial per a poder proporcionar a la descendència protecció contra infeccions (immunitat natural passiva) fins que el seu propi sistema immunitari hagi madurat.

El pas de les macromolècules a través de l'intestí prim immadur succeeix per transport selectiu dels anticossos a través del receptor Fc-neonatal (FcRn), altament expressat en l'epiteli de la primera meitat de l'intestí prim durant el període d'alletament, que es correlaciona amb la transferència d'immunitat natural passiva (paper II). Alhora, cèl·lules amb una alta capacitat d'absorció conformen la major part de l'epiteli intestinal de la segona meitat de l'intestí prim, distingibles per tenir una gran vacuola cel·lular ocupant gairebé tot l'espai citoplasmàtic dels enteròcits (paper II).

Durant el deslletament, el temps de transició entre la lactància estricta i la introducció progressiva d'altres aliments a la dieta, l'intestí madura ràpidament. Els canvis que es produeixen inclouen la substitució de les cèl·lules epitelials de l'intestí, que es caracteritzen per una expressió reduïda del receptor d'anticossos i la desaparició de les cèl·lules vacuolades amb gran capacitat d'absorció. La funció de barrera també s'incrementa dràsticament amb la pèrdua de la capacitat d'absorció de macromolècules (papers II, III i IV), o tancament de l'intestí. La funció digestiva del pàncrees també s'incrementa durant el deslletament (dades no publicades) juntament amb l'estimulació de la secreció d'enzims pancreàtics, com succeeix en els nostres models de maduració intestinal induïda (papers III i IV).

Aquest procés de maduració natural de l'intestí prim durant el deslletament pot ser induït experimentalment. En el grup, s'han desenvolupat dos models diferents de maduració intestinal induïda en rates lactants per mitjà de l'alimentació forçada d'una lectina extreta de mongetes vermelles, fitohemaglutinina (PHA), o proteases, enzims que digereixen proteïnes (paper I). Aquests models experimentals de la maduració intestinal precoç imiten els canvis que succeeixen durant el

desenvolupament natural del deslletament en un marc de temps més definit i unes condicions més controlades.

Els efectes causats just després d'administrar els agents provocatius per tub estomacal han proporcionat una millor comprensió dels mecanismes d'acció de cada un dels tractaments. La PHA va resultar en un bloqueig immediat de totes les rutes de transport a través de la paret intestinal. La proteasa va bloquejar les vies de transport vesicular, tant l'específic com l'inespecífic, però va augmentar el pas paracel·lular de la barrera intestinal causant una alteració en la permeabilitat de l'epiteli a la part distal de l'intestí prim (paper IV). Aquest resultats indiquen que els tractaments actuen a través de diferents mecanismes per provocar la maduració intestinal.

El sistema immunitari també s'ha relacionat amb la maduració de l'intestí en rates, amb els limfòcits T proposats com a mediadors i el factor de transcripció de les cèl·lules immunes (Blimp-1) designat com un regulador clau de la maduració de les cèl·lules epitelials de l'intestí en ratolins. L'expressió de Blimp1 a l'intestí prim es va estudiar també en rates i els resultats van mostrar una disminució de l'expressió sincronitzada amb el procés de maduració (paper II).

La connexió del sistema immune, especialment les cèl·lules T, es va estudiar també en un model de rata immunodeficient sense tim, l'òrgan on es desenvolupen els limfòcits T. Les rates atímiques van resultar ser susceptibles a la provocació intestinal amb PHA i proteasa, i es va poder induir la maduració precoç de l'intestí (paper III). Els resultats suggereixen que la maduració de l'intestí és independent de cèl·lules T derivades del tim, però altres cèl·lules del sistema immunitari podrien estar implicades en el procés. D'altra banda, els canvis que es produeixen en l'intestí durant el deslletament s'han relacionat amb un procés de "inflamació fisiològica". A més, s'ha demostrat prèviament que la maduració precoç induïda per PHA implica el reclutament de limfòcits i el fet que les proteases causin una alteració de la permeabilitat paracel·lular indica també un procés inflamatori (paper IV).

Així doncs, durant aquest projecte s'ha investigat el desenvolupament de l'intestí en el model de rata després del naixement amb especial atenció en la funció de barrera de l'intestí, la funció pancreàtica, i la relació amb factors immunològics, així com l'aclariment dels possibles mecanismes d'activació. Els resultats obtinguts contribueixen a una millor comprensió dels desencadenants de la maduració de la barrera intestinal en nadons i podrien ajudar a millorar les estratègies per a la prevenció i el tractament de les complicacions relacionades amb els intestins que sovint afecten els infants prematurs.

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