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Review

Embryological development of the intestine and necrotizing enterocolitis

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Abstract

It is possible to distinguish two phases in the development and maturation of the intestine: intra-uterine and extra-uterine.

Up until the 13th week of the embryological phase, a fetus' development is not controlled by factors external to the alimentary canal. It is instead guided by the homeotic genes that control the proliferation and differentiation during the embryogenesis.

A fetus' interaction with the external environment starts with the perforation of the buccal membrane, when the fetus starts swallowing the amniotic fluid. Both in pathological and physiological conditions, the encounter with the microbiota – that surely happens at birth, but could happen before as well – furnishes to the developing intestine elements which are necessary and essential to the growth of the organ, the barrier function, and the specific and nonspecific immunity. The link between development, maturation and inflammation is very important and influences the entire intestinal homeostasis. In case of preterm birth, the immaturity of the system creates a proinflammatory environment where the tolerance of the commensal microbiota cannot be taken for granted, and the maternal milk is not always available. These grounds are preconditions for the Necrotizing Enterocolitis (NEC). NEC is a calamitous pathology for a preterm baby, able to increase mortality, morbidity and the length of hospitalization.

This review aims at understanding how to prevent NEC. It will do so by analyzing the mechanisms of the development of the inflammation at intestinal level, and at the level of its regulation. Several evidences, both clinical and experimental, show that the main form of NEC prevention is the dispensation of maternal milk. Maternal milk allows a proper growth and development of the intestine, a proper settlement of the microbiota, and control over the intestinal inflammation.

Keywords

Intestine, development, necrotizing enterocolitis, embryology.

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Introduction

The intestine's development has two distinct phases: intra-uterine and extra-uterine. The second one is induced by the encounter with the microbiota – the normal commensal flora in the process of colonizing the intestine – and with the antigens found in foods [1].

The intestine needs to encounter the microbiota only after having reached a maturity stage that allows it to tolerate the bacterial antigens evolutionarily known. Those bacterial antigens, the so-called "old friends", are able to stimulate the production of IL10 and TGFβ, which in turn are deputed to modulate the inflammatory response [2].

Evidence suggests that an early encounter with the microbiota – an encounter that happens when the intestine has not reached an optimal maturity stage – is deleterious for the short-term development of the intestinal immunity [3]. On the other hand, it is worth considering that among the germ-free animals it is possible to notice that the absence of the normal flora is source of morphological and pathological anomalies [3].

The microbiota has different functions: it influences both the inflammatory response and the innate immunity, it activates those pathways able to stimulate cell proliferation and influences the ecology of the Crypts of Lieberkühn [1, 3].

Some questions arise now, concerning – in a pattern of normal development – how the microbiota and the diet influence the intestine's maturity, what are the mechanisms that lead to the alteration to the normal development which rouse the inflammation producing the Necrotizing Enterocolitis (NEC), a calamitous pathology for a preterm baby, able to increase mortality, morbidity and the length of hospitalization [3].

In-utero intestinal development

Embryonic period

When we speak of embryonic development, we refer to both the gestational period and the Carnegie stages [4, 5], which divide the embryonic development into 23 morphogenetic steps that allows a quick comparison of the development of species with different gestational lengths.

The embryonic development begins from an undifferentiated totipotent cell, the zygote, that divides itself while maintaining identical to itself until the blastocyst stage (Carnegie stage 3, reached by humans on the 4^{th} or 5^{th} day).

The blastocyst cells are defined as "pluripotent", for they are able to originate all the tissues that form the organism, but not the organism in its integrity. When the embryo differentiates into its three germ layers and with its folding, the cells are progressively differentiated losing their initial plasticity. This process is guided by the Homeobox genes, also called homeotic genes [6].

The Homeobox are genes which encode factors for nuclear transition, and are involved in the regulation of the embryonic development. They are characterized by a sequence around 180 base pairs long, highly conserved across different species, and have been first studied in the Drosophila [7, 8]. They act by regulating the transcription of the target genes and direct the organogenesis [9]. Every organ is specified by a "cocktail" of homeotic genes products, combined in quotas which have a regional difference according to gradient of concentration which vary during the course of the time [10].

An homeotic signal triggers a series of genes able to duplicate and transform the cell. For

this reason, the same signal, under the action of the following replication, acts on a different microenvironment. It gradually stimulates the production of other factors that keep modifying it, up until the creation of a cellular environment. Here, in this protect environment, the final multipotent stem cell is able to renovate itself and to produce mature cells – in few words, to do its work [11, 12].

Thus, under the Homeobox guidance, from the germ layers are first formed the organs, and then a pool of stem, multipotent, cells. Those are organ-specific, have a limited plasticity, are able to provide for the cell renewal and the organ maturation [6].

Stem cells niches are then developed in every organ. Those niches are needed because they allow the stem cell to remain so, giving place to a differential replication: a portion of cells differentiates, the other remains stable. This is made possible also by the presence of ancillary cells. The ancillary cells, producing growth factors and protecting stem cells, are essential to their subsistence [6].

At the second week of development (11-13 days of gestation, Carnegie stage 5) the embryo's body is a disc composed by two germ layers (hypoblast and epiblast) suspended between the yolk sack and the amniotic cavity. The process called "gastrulation" takes place at Carnegie stage 6 (17 days of gestation for the human embryo) [5, 11]. The epiblast cells differentiate, and form the primitive streak, a groove that cuts in half the body of the embryo, providing a rigid axle, around which the embryonic folding occurs, and through which migrate the cells which go to great lengths under the hypoblast. At this point, a trilaminar embryo is now formed. Its body is composed by three germ layers, from the amniotic cavity to the yolk sack: ectoderm, mesoderm, endoderm.

At Carnegie stage 8 (23 days of gestation) the epiblast specifies for the mesoderm and the endoderm. It does so because of the combined action of two Homeoboxes: MIXL 1 (Mix Paired Like 1 Homeobox) and NODAL, and the activin A.

More precisely, the cells that react first at the MXL 1, react more to the activin A and NODAL, generating the endoderm of the anterior intestine, and the precursors of liver and pancreas. Those in turn express the TGFβ (Tumor Growth Factor β) and genes related to the endoderm. On the other hand, the cells that react slower and to a higher level of MIXL1 (and that express FOXH1 – Forkhead box H1 – and GSC – Goosecoid Homeobox – in order to block its action [16]) react then to NODAL stimulation, producing a mesenchymal phenotype [13-16].

The Endoderm then stimulates the mesoderm to produce the SHH (Sonic Hedgehog) which in turn stimulates the endoderm to produce WNT (Wingless Nuclear Transcription Factor). In this phase WNT, a protein which is cardinal to the preservation of a pool of stem cells in the mature intestine, promotes the proliferation of the primitive intestine and its elongation, on the cranio-caudal axis of the embryo. The primitive intestine is instead a very important structure from which most of the thoracic and abdominal organs origins [5, 10, 12, 17].

The specification of the primitive intestine to the anterior intestine begins at Carnegie stage 10, thanks to high concentrations of WNT in the cephalic area, while the prevalent BMP signal in the caudal area triggers the intestinal differentiation in the middle and posterior intestine.

At this stage, there are then 3 regions in the caudal-cranial axis: anterior, middle and posterior intestine. From the anterior intestine originate the pharynx, the upper respiratory tract, the esophagus, the stomach, the duodenum, the liver, the pancreas, and the biliary system. From the middle intestine arise the small bowel and the caecum, the appendix, the ascending colon, and the proximal traverse colon. From the posterior intestine come the other parts of the colon, the rectum, and the anus [17-19].

The mesoderm develops together with the endoderm, and while from the latter derive the supporting connective tissue, from the former the neural crest originate the enteric nervous system [17, 18]. Now, a trilaminar embryo is formed.

There are three mechanisms that not only maintain the regional identities of the portions of the primitive intestine, but also guide the process of differentiation [12]: the combination in gradient of different transcriptional factors, the cell positioning, and the cell's sensitivity to those factors that come from cells of a different germ line.

In the development of the intestine, the proliferation, differentiation and cells placement are the result of an intense exchange between the endoderm, forming the epithelium, and the underlining mesenchyme [10, 12].

Regulation of the intestinal embryogenesys

The most important signals that regulate the intestinal development are:

- Hedgehog and PDGF (Platelet Derived Growth Factor). Originating from the epithelium, those signals act on the underlining mesenchyme. These elements regulate the differentiation of the myofibroblasts and of the smooth muscle cells, and at the same time activate the cascade of transcriptional factors regulating the production of WNT and BMP [11, 12, 15].
- WNT and BMP operate on the epithelium, to regulate the differentiation and proliferation [11, 12], and are mutual antagonists because both have as a target the control of the activities of the transcriptional factor β-catenin. WNT is mainly expressed in the cephalic portion of the intestine, while the BMP I by the caudal one [11, 12].
- FGF (Fibroblasts Growth Factor) shows the same gradient as the BMP, that is to say that it grows from the cranial towards the caudal region, and regulates the transcriptional factors triggered by the retinoic acid, such as the HoxB1 (Homeobox B1) and the HoxA5 [20].

In the cranial region, every organ between the mouth and the duodenum – such as liver and pancreas – comes from the anterior intestine. The first specification step is the activation of FOXA2, by WNT and NODAL.

Other molecules, the list of which is always increasing, intervene after the FOXA2. Those molecules specify as the forerunners for the developing organs. Those are: HHEX, that specifies the liver and is activated by NODAL and WNT), SOX17 (cardinal for the pancreas' early differentiation, activates PDX1, but also – with WNT, FOXA2), CDX2 (Caudal type Homeobox 2) responsible or the specification of the middle and posterior intestine and allocated from the duodenum downward [12].

In the caudal region, that is, the middle and posterior intestine, the essential gene for the development of the intestine is CDX2. It is expressed by the junction between the anterior and the middle intestine and allows the differentiation of the progenitor cells of the intestinal epithelium. At a second moment, it is added to this signal, that of the SOX17 – Sex determining region 17 [2].

CDX2, WNT, and BMP are then important during the fetal period the maturation of the intestinal epithelium. They are important to establish the differentiation between the crypt and the villus, between the epithelial area and the intestine's proliferation and modeling, after the closing of the "pipe" [10, 12]. This process is schematized in the **Fig. 1**.

At the 12th week of gestation, in humans the macroscopic differentiation of the primitive intestine is complete, with the presence of several buds: lungs and respiratory tree, liver, pancreas

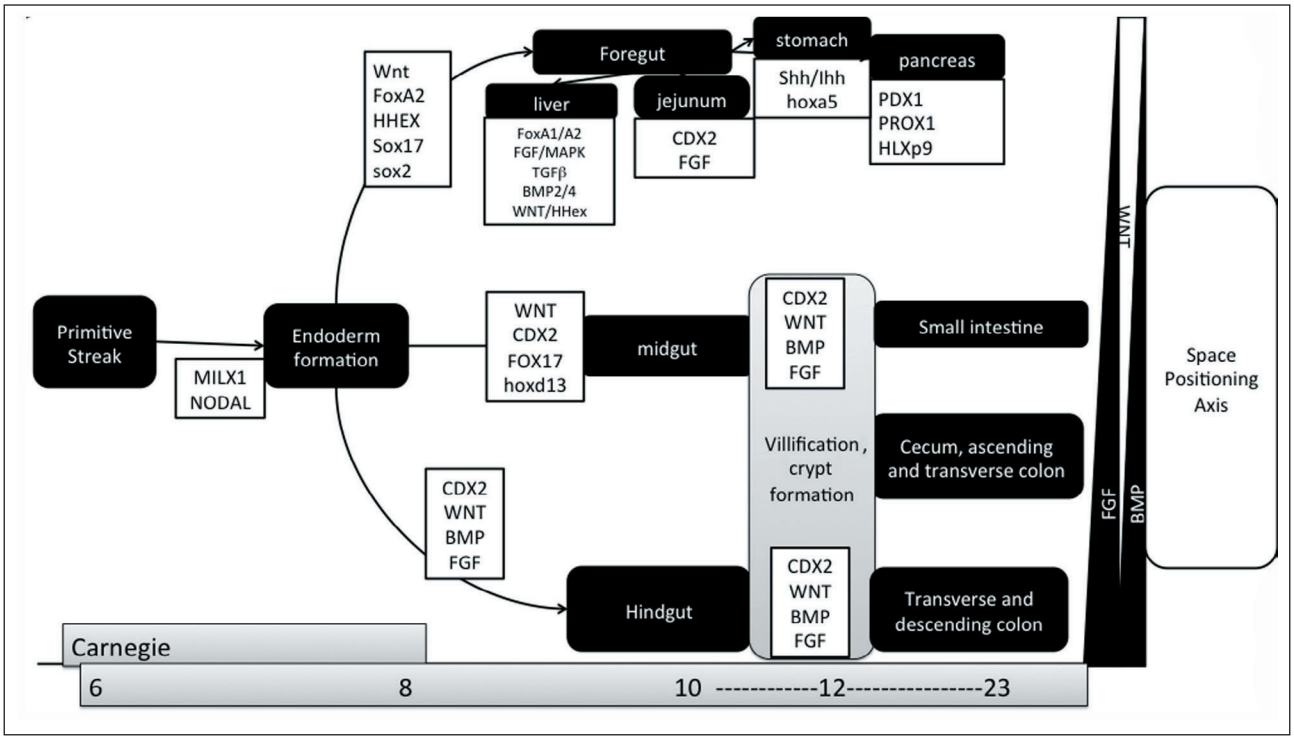

Figure 1. Intestinal embryogenesis and principal homeotic genes.

and alimentary tract [5]. The last one is a tube that goes from the mouth to the anus, and has undergone a series of bending and rotations, which have allocated it in both the thorax and the abdomen [5, 12]. Also this process of allocation is guided by the Homeobox products, the gradient of which regulates the left-right axis and the rotations both [21].

The intestinal lumen, around the 6th week is obliterated by the cellular proliferation, but subsequently – from the 7th week onwards – a process of recanalization starts, and the formation of the villi, of the crypts, and the process of cellular differentiation.

Fetal period: controlling signals for the in-utero *maturation of the intestinal epitelium*

The maturation of the intestinal epithelium happens after the end of the embryogenesis.

The last two steps of the intestinal embryogenesis are the formation of the villi, starting at around the 7th week (Carnegie stage 19) and continuing with the formation of the crypts up until the end of the 8th week (Carnegie stage 23). The secretion of the SHH from the cell of the epithelium of the primitive intestine induces in the mesenchyme the differentiation of the first level of circular muscle. It is then thus generated an elastic tension that raises the epithelium in folds [22]. This play of signals between the mesenchyme and the epithelium continues until the formation of the layer of longitudinal muscle, on the outside of both the circular muscle (which raises the zigzag folds) and the muscolaris mucosa (which induces the formation of the villus) [22].

The folding of the epithelium during the formation of the villi has a secondary effect: it concentrates the BMP signal on the villus' apex [23]. BMP, from the epithelium, induces the SHH from the mesenchyme, which in turn inhibits the WNT around the villus, leaving it on the bottom.

This way, the WNT/BMP axis is created. It divides the intestine into two areas: the crypt – the portion of active replication – and the villus – where the mature cells execute their functions [23].

The villi can also be produced in absence of the smooth muscle, but when this happens, it happens in a disorderly fashion, caused by physical reasons (because of the elastic compression from the mesoderm on the endoderm [22]) as well as by the absence of exchanges between the mesenchyme and the epithelium [23].

When the villi are formed, the crypt-villus axis is established, and the SHH, WNT, BMP start the differentiation of the cells of the endoderm [24, 25].

The cellular differentiation is schematized in **Fig. 2**.

The balance between WNT and BMP has a target: the β-catenin. The β-catenin is a nuclear transcriptional factor: it moves inside the nucleus activating the T cell factor/lymphocyte enhancer factor (TCF/LEF), which in turn activate the transcription of genes connected to the cellular proliferation. WNT has numerous final targets, mostly described in the studies on the colorectal tumors [26]. WNT, by activating NOTCH, is able to limit itself. When this happens, the stem cell rises from the bottom of the crypt and starts a process of differentiation that requires the BMP and the PI3K [24, 27, 28].

The signals that give origin to the different cells of the intestinal epithelium are synthesized in **Fig. 2**.

The crypt is a saccular pocket that stays between two villi. Because of its morphology it remains relatively protected from environmental stimuli, apoptosis and differentiation. It is surrounded externally by the pericryptal fibroblast [29] and by the mesenchyme while at the inside it is covered by an epithelium composed by 10 Paneth cells and 15 intestinal stem cells, multipotent and non-committed [29, 30]. For this reason the crypt is an anatomical structure that functions as a stem cells "reservoir", where they can be held, ready for the intestinal regeneration. The multipotent cells, commonly known as CBC (Crypt Base Columnar) are posed at the bottom of the crypt. They are characterized by the presence of the LGR5 receptor, which is able to strengthen the signal of the WNT [19, 31-33]. Normally the division of the CBCs is asymmetric: an identical daughter cell and a committed one [32], but there can also be a variation due to environmental causes such as the exposition to radiation, or to chemotherapy [29, 32].

Because the space in the crypt is limited, the CBC in active replication is pushed upwards and loses contact with the Paneth cells [34, 35]. This happens in 2-3 days, 6-7 replications. The replicating cells place themselves at the junction between the crypt and the villus, giving place to the TA cells, "transient amplifying cells" [19, 27, 29]. Those – also called +4ISC because of the position inside the crypt – are cells ready to differentiate in every mature line of the intestinal epithelium [29].

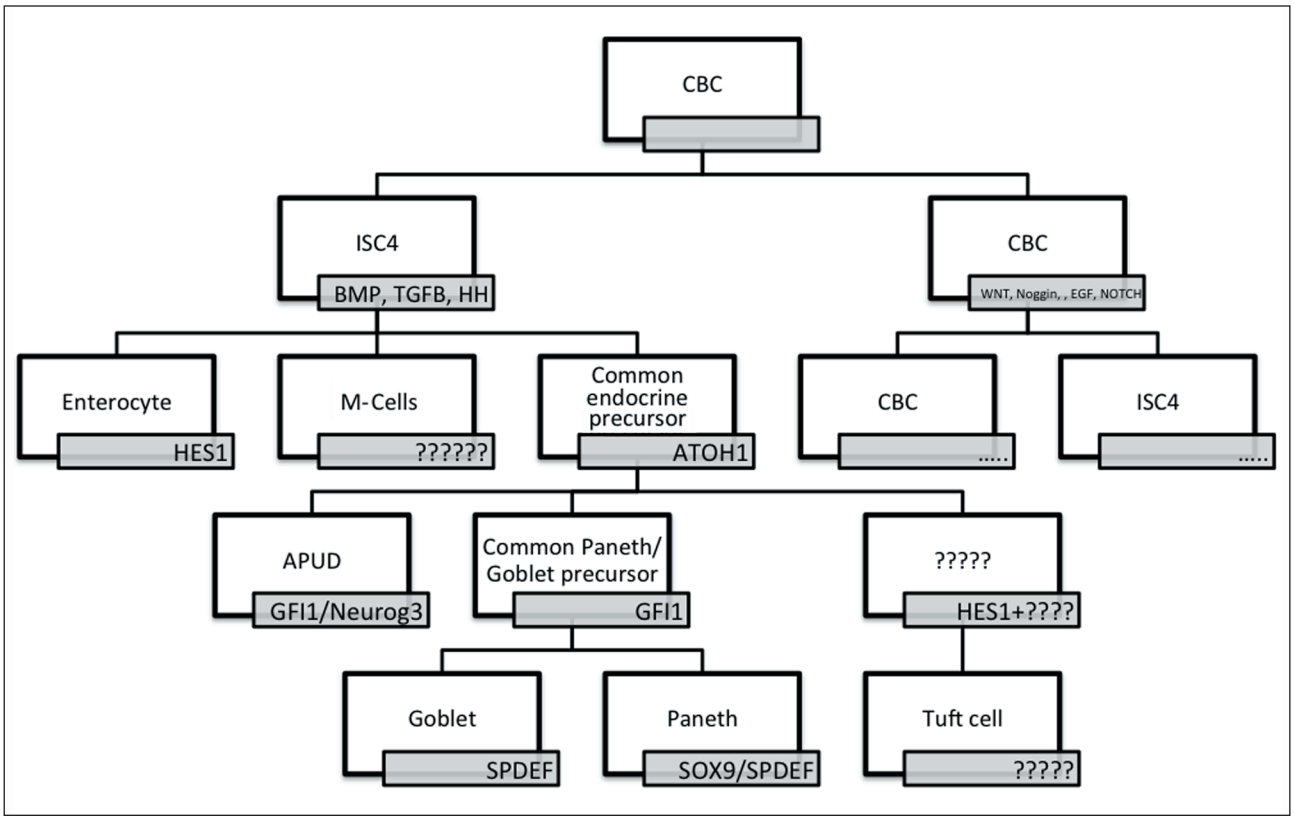

Figure 2. Epithelial cells differentiation.
Modified from Kandasamy et al., 2014 [3].

From them all the mature cells of the intestinal epithelium (**Fig. 2**), together with the Paneth cells, originate. While the formers direct themselves to reassemble the villus' axis, and are quickly (in a 3 days' time) exfoliated and substituted, the latter migrate toward the bottom of the crypt, and can remain there for up to a month. The proximity to the WNT-producing Paneth cell is the most essential requirement for maintaining the pluripotency of the CBC/LGR5+ cell, so that, when the Paneth cells are removed, also the LGR5+ are lost [30, 34-37]. The mature intestinal epithelium is composed by 90% of enterocytes with absorbing function, and for the remaining 10% by endocrines, Paneth, and M cells [3]. With the differentiation of the enterocytes, those structures are formed which guarantee to the intestine the possibility to fulfill its function of non-immunological barrier.

The term "non-immunological barrier" describes the ability of the intestinal epithelium to defend itself from the encounter with the environmental pathogens, and avoid their passage into the bloodstream.

The non-immunological barrier is made possible by the brush border of the enterocytes, by the adherence to the submucosa, by the intraepithelial tight junctions, by the mucous layer secreted by the goblet cells [19, 30, 38]. The mucous in particular contains dietary antigens, captures pathogenic bacteria. His production rapidly increases as a reaction to insults and presents multiple binding sites for chemokines, growth factors and cytokines. This barrier protects both the villus (the mature component of the intestine) and the crypt [30, 38].

The embryo begins swallowing at around the 11th gestational week [39]. The contact of the intestine with the amniotic fluid (containing cytokines regulating the immune response, stem cells, growth factor etc. [39-42]) has an impact on the maturation and on the cells growth, both at the end of the embryogenesis, and during the entire fetal period. In addition, many of the factors in breast milk are also present in the amniotic liquid. It is possible to theorize that this functional overlap between the amniotic liquid and the breast milk prepares the intestine to the passage from a secure environment of the womb to an exposed one. [43-50]. It is possible that the bioactive properties of the amniotic liquid are able to influence also the immediate postnatal development of the preterm's intestine [40]. There is much experimental evidence to support this hypothesis.

The ingestion of amniotic liquid, rich in EGF, inhibits the expression of the TLR4, responsible for the inflammatory response, in fetuses [39] and promotes the maturation and the development of the intestine thanks to the EGF and the other growth factors such as the IGF-1, FGF, HGF, and TGF-α, the same that can be found in breast milk [41].

The amniotic liquid is rich in stem cells [40]. In the presence of chorioamnionitis, the ingestion of infected amniotic liquid has a very heavy impact of the intestinal epithelium. It triggers the inflammatory cascade provoking an intraepithelial leukocyte chemotaxism, loss of the tolerance secretion of TNF and interferon γ and upregulation of Toll Like Receptors 1, 2, 4, 6 (surface receptors used for the recognition of a pattern of molecules common to different microbes).

The proliferation of the crypts diminishes during the acute phase, to grow again immediately after.

The tight junctions are inhibited, and the function of intestinal barrier is thus lost [42].

Postnatal maturation of the intestine

In order to complete its maturation the intestine needs to be colonized and nourished. This can only happen outside the utero. Let us consider the development of the different intestinal functions (as immune and non-immune barrier, motor function, or digestive) while in physiological condition. By physiological condition is intended an intestine mature enough to be able to interact with the breast milk and the microbiota maintaining a condition of homeostasis without triggering an inflammatory response.

Development of the barrier function

The microbiota

It is thought that the first encounter between the intestine and the microbiota happens during the passage through the birth canal, and with the nursing afterwards [38]. There are, however, many recent studies on the placental microbiota from which it seems that already during the fetal period the intestine is colonized, in both normal condition and in presence of chorioamnionitis [42, 51]. Anyhow, regardless of when the colonization takes place, it is thought that it permanently affects the intestinal immunity, and by reflex, the entirety of the organism [3].

The composition of the microbiota changes enormously, both among different animal species, and among individuals inside the same specie, and it is subjected to the influence of a great number of external stimuli. [52]. We can affirm that each individual lives with its own microbiota, which influences the individual phenotype [53, 54]. However, if the bacterial classification is considered, and the colonization patterns are confronted at the *Phyla* level, instead of at the *Genera* level, communal pattern of colonization will be found, which will make it possible to comprehend different phenomenon.

4 *Phyla* are predominant in the human intestine: *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria* [55]. In a newborn, some phyla are less represented [53]. The commensal microbiota is symbiotic with the human organism: it influences its health and disease, intestinal development, immunity response, and metabolism [55].

To remain commensal, and therefore symbiotic, without becoming a pathogen, the microbiota has to satisfy a couple of rules: there must be an equilibrium between the different *Phyla*, an equilibrium otherwise known as "eubiosis" [56]. Moreover, the bacteria have to remain at the level of the intestinal lumen, without for it to be possible to invade the tissues or arrive near the crypt [27].

There are only a few exceptions to this rule, and it is often discussed whether the data indicate a pathology, giving the fact that they are described in pathological as well as healthy models, and potentially trigger autoimmunity.

For example, the SFB (Segmented Filamentous Bacteria), which belong to the phylum of the Firmicutes, in the Clostridia, or do directly enter in touch with the intestinal epithelium, instead of the mucous layer. They are able to stimulate the production of the IL17 from the T lymphocytes of the own lamina, and the secretion of the IL 22 and REG3γ in the epithelium, playing a protective action over the infection by strains of enterohemorrhagic *E. coli* [57].

A second exception is represented by the "crypt-specific core microbiota" (CSCM) described in the crypt of the caecum and of the colon, where the dominant species is an aerobic, non-fermenting, bacterium that belongs to *Acinetobacter spp.* [27, 58]. It is unknown whether *Acinetobacter spp.* indicates pathology or is physiological.

Under physiological conditions the commensal microbiota acts in various ways in order to protect the body and maintain the state of homeostasis: it competes with the pathogenic flora for the same

metabolites (thus establishing a hostile environment for pathogenic elements), it produces antimicrobial peptides, receptors and ligands epithelial cells, of which the most studied are the Toll Like Receptors (TLRs) [59].

A microbiota is sensible to the diet. An excessive intake of fat and sugars, for example, triggers a disbiosis – that is, an abnormal prevalence of a phylum or of a genus inside the microbic population, source of a series of intestinal (and potentially pathologic) alteration [57, 60]. On the other hand, the neonatal immune system integrates different signals to promote the homeostasis of the system and a normal microbic colonization [27]. This is made possible by a series of mechanism both immunes and non-immunes, that together constitute the intestinal barrier function [55].

The intestine of a full-term newborn (as opposed to a preterm one) is setted in such a way as to accommodate the commensal microbiota, the so-called "old friends" [2], without reacting (the so-called concept of the immune "ignorance") [3, 59].

Probably some factors that are inside the amniotic liquid – and in the breast milk – define the early responses to the commensals, and the intestinal inflammatory response [61, 62]. Thus, the intestine is, toward the microbiota, in a state of "dynamic balance". It tries to get the maximum benefit from its presence. While on the one end it limits its expansion using sophisticated barrier mechanisms, on the other end it receives and gives stimuli, which also depend on the diet.

The intestinal barrier

The intestine has its surface covered by a thin monolayer of epithelial, multifunctional, cells with highly immunoreactive sub-mucosae. The interruption of this barrier may have numerous negative consequences, such as the systemic inflammation, autoimmune and allergic diseases [63-68]. The quantity and quality of the bacteria that arrive at the intestinal lumen is controlled by a series of barrier, both chemical and physical, located over it [69, 70]. Those barriers are: the mucous membrane surrounding the respiratory tract, which contains a series of enzymes and other substances with direct antimicrobial action, as well as microbial anti-adherence; the secretion of acid by the gastric enterochromaffin cells, bile salts produced by the liver, and the release of proteolytic enzymes from the pancreas. The antigen load that reaches the intestine is damped and controlled by all these factors, in order to prevent hematic passage and maintain the homeostasis between the intestine's inflammatory and anti-inflammatory activities. Virtually all components of intestinal epithelium combine to create the so-called "mucosal firewall" [59].

The components of the innate mucosal form a physical barrier that goes from the luminal surface to the basal membrane, passing through the cell layer, which does not allow direct contacts between epithelial cells and microbiota [71].

When in touch with the luminal surface [71] the antimicrobial peptides, produced by cells of Paneth and the enterocytes, are placed in contact with the mucous layer. Their production can be either inducible or constitutive.

The main products are lysozyme, defensins, cathelicidin and angiogenin. Cathelicidin and angiogenin are able to destroy the microbial wall (**Tab. 1**) [3, 19, 38].

IgA are initially given to the baby, for at least the first two weeks of life, via breast milk

Table 1. Antimicrobial peptides.

Production	Peptide	Constitutive stimulus	Induction by TLR/NOD	Cholinergic induction
Enterocytic	Angiogenin	x	x	x
	Cathelicidin	x	x	x
	β defensins	x	x	x
Paneth cells	α defensins	x	x	x
	PLA2	x	x	x
	Lysozime	x	x	x
Enterocytic and Paneth cells	Lectins	x	x	
	REGIIIγ	x	x	
	Collectins	x	x	
	Protease inhibitors	x		

[59]. Together with the antimicrobial peptides – produced by the epithelial cells – they cover the mucous layer and are essential in their interaction with the microbiota. They activate the complement and promote the phagocytosis [72].

Their deficiency causes dysbiosis, an increase in SFB – that is potentially pathological [55] and leads to the expansion of the bacteria associated with the mucosa such as segmented filamentous [57].

The mucous layer which is secreted by the goblet cells is located immediately below the antimicrobial peptides and IgA, and it is formed by different kind of mucins with specific functions.

Indeed there are soluble mucins, gel forming mucins, and those with the function of anchoring to the membrane [73].

The mucins present multiple binding sites for the active molecules, both for inflammatory regulation (cytokines) and the epithelial growth (growth factors).

Small nutrients pass through the mucous layer, but dietary antigens – together with pathogens – are trapped there.

The mucus production – together with its composition – changes with the postnatal age. It happens in response to the bacterial challenge, after the colonization of the commensal microbial flora and during the process of epithelial shelter [70, 73].

The cellular elements that make up the intestinal barrier are: all the cells of epithelial origin, the mast cells, the dendritic cells, the phagocytic cells (macrophages and granulocytes), the Nk cells, and the γδT cells [71]. They communicate via a tight network of signals, adhere to the submucosa through the integrins, and to one another by tight junctions [69, 70].

It is a sophisticated system of junctions, from the apex to the bottom of the cell, which ensures a certain degree of paracellular passage, which is both physiological and needed for the development of the immune system [52].

In this way it is ensured a semipermeability to the cell. This means that the passage of the bacteria into the circulation is restricted, together with that of a myriad of other luminal macromolecules. At the same time, both the transcytosis and the absorption of macromolecules generated during the normal processes of digestion are promoted. The immaturity in the composition and in the function of tight junctions, gives a reason – especially in prematures – for the increased intestinal permeability [69].

The subepithelial components of the mucosal barrier include a series of immune cells that explain the intestinal adaptive immunity. They are: macrophages (phagocitizing the commensal bacteria able to pass through the epithelial barrier, but not the pathogens [55]), dendritic cells (APC, antigen-presenting cells, necessary to regulate antibody production and the cell-mediated immunity), myofibroblasts, lymphocytes T and B.

The lymphocytes T and B, in particular, once matured (due to the contact with the antigen on the Peyer's patches and the lymphoid follicles) are placed in the basal membrane and hence secrete IgA specifications that cover the intestinal barrier [71].

Dialogue: how the microbiota influences the intestinal development

MAMPs receptors

Up to now, two classes of receptors for bacterial products are known, those present on the cell surface (TLRs) and intracytoplasmic ones (NOD). The role of the latter in the genesis of intestinal disease of prematurity is discussed [74, 75]. They are diffused on the epithelium of the villi and crypts, and on the cells of the immune system, and trigger a series of intracellular pathways, and are assigned to the recognition of the MAMPs ("microbe-associated molecular patterns") [27].

They are therefore able to activate the innate immunity, responding to the stimulus given by one or more molecules similar but not identical between the different microbes. For example, the TLR4 interacts with bacterial lipopolysaccharides (LPS) [76].

The TLRs triggers inflammation through the activation of NfκB, and then the production of antimicrobial peptides, adhesion molecules, and acute phase proteins [43, 77].

The most important TLRs for a newborn's intestine are TLR9 and TLR4 [77].

TLR4 is responsible for responding to bacterial endotoxin, long chain lipids produced by bacteria, FFA (Free Fatty Acids), and other substances, such as PAF (Platelet-Activating Factor) [78-82] and it is thought to play an essential role in the pathogenesis of NEC [76, 77, 83].

Over the intestinal mucosa, the TLR4 activates the inflammatory cascade, the NFκB signal, and the transcription of TNF and IL1β [77]. It also plays other roles that have not been studied yet [1].

TLR9 – homologous of TLR4 – recognizes the viral and bacterial DNA [76]. Once activated, it downrates the expression of TLR4 and decreases apoptosis.

The full-term infant's TLR induces a less-powerful response to known antigens than that of an adult. It is probable that this is a mechanism that promotes ignorance against commensal bacteria, as the early response to known antigens (e.g., LPS) affects the cells of the epithelium into becoming hypo-responsive to the same subsequent stimulations [59].

In preterm infants, especially in the most extreme prematurity, the response is stronger than in term newborn, because the intestinal epithelium expresses a great deal of TLR4 [76, 77]. *In utero*, starting from the 29th week the TLR4 are washed away by the contact with the amniotic fluid, and are part of the underlying reason for the proinflammatory environment of the intestine of the extremely preterm infant [39].

The Paneth cells

The presence of microorganisms in the intestinal lumen induces the production of antimicrobials peptides (such as the α-defensin) by the Paneth cells, and the enterocytes [19, 38].

Paneth cells are essential for the entire intestinal homeostasis. Defined as "sedentary neutrophils" [84], they are placed in a strategic position: the bottom of the crypt, very close to the base of the vessels that supply blood to the villi [85]. So, they are subjected to multiple stimuli from the bloodstream/hematic circle. They secrete WNT. The WNT promotes cell proliferation, and provides an essential environment for the maintenance of stem cells pool [85]. It has been demonstrated that the stem cell matures when – pushed upwards in replicative phase – it moves away from the Paneth cells [30, 32]. The Paneth cells also provide the intestine's first line of protection by secreting a number of antimicrobial peptides: lysozyme, α-defensin 5 and 6 (HD5 and HD6), phospholipase A2 (PLA2, which activates the PAF), and angiogenin. The antimicrobial peptides are constitutively produced, but their production increases with cholinergic and microbial stimulation (via TLR and NOD receptors) [52]. It is unknown whether the interaction with the bacterium occurs by direct contact, or how else. Further studies are needed. The secretion of granules containing antimicrobial peptides probably occurs under the stimulus given by the activation of the receptor NOD2 [52, 86-88] that activate NFκB. Angiogenin, induced by the microbiota, has probably a role in the capillary network development [89]. The α-defensins (HD5-6), produced via the WNT/TCF pathway, are the most specific products of Paneth cells [90, 91].

The HD5 and HD6, specific to the Paneth cells, start to appear approximately at the 24th gestational week. Under physiological conditions, in adults they are more concentrated than in the newborn [52]. They undergo a physiological increase over the weeks, and are regulated by microbial and antimicrobial stimuli [90].

For example, the administration of amoxicillin induces the down-regulation of several different genes in the rat's Paneth Cells, coding for antibacterial peptides, such as defensins, matrilysin, and phospholipase A2, MHC (Major Histocompatibility Complex) I and II [92].

The dysbiosis is also due to impaired production of HD5 and HD6. Studies on the intestinal flora in patients with Crohn's disease have shown a link between adhesion of *E. Coli* strains of the ileal mucosa, and the increased production of HD5 and HD6 [93-95]. Paneth cells are equipped with TLR4 on the cell surface. TLR4, capable of triggering the NFKB cascade (and therefore the inflammation and apoptosis), seems to have also been involved into the regulation of the cell proliferation [1]. This is a first indication that connects the regulation of the inflammation to that of cell proliferation.

Interaction between bacterical patterns and cellular surface

It is unknown how exactly the microbiota influences the development. However, from an experimental point of view it is evident that it does. Studies on germ-free mice have shown that, in absence of intestinal microbiota, intestinal morphology is generally compromised. The total surface of the intestine is significantly diminished: the villi are short and the crypts are shallow [52, 96-99]. The cell renewal is scarce [27, 100, 101], the capillary network of the villus axis does not develop [89], the maturation of intestinal B lymphocytes Th17 – as well as the ratio of Th1-2 – are compromised [55].

The same changes can be seen in the antibiotic treatment [27, 102, 103]. These changes have serious repercussions on the integrity of the mucosal barrier, and susceptibility to damages from toxic and infectious agents [52]. However,

they are able to normalize with floral colonization or re-colonization.

An important role in maintaining the intestinal barrier is played by the composition of the microbiota. The dysbiosis alters the expression of occludin and zonulin, proteins that form tight inter-epithelium junctions, allowing the passage of toxins – such as LPS – in the bloodstream. The altered permeability of the epithelium is then able to trigger an inflammation, in a vicious circle that self-amplifies and self-maintains [52, 104-110]. Similar changes are also found in mono-colonized mice [27, 89, 111].

However, alterations of the barrier function do not fully justify the profound morphological disruption that can be observed in the absence of the microbiota, or in conditions of dysbiosis. Paneth cells and enterocytes express TLR4, CBC, and NOD [27]. The presence of these cell receptors on the crypt's cells is intriguing, and makes possible to hypothesize for them a role also in the control of cell proliferation. The experimental data are still scarce. However, there is experimental evidence that by the stimulation of TLR4 and NOD – placed at the bottom of the crypt cells – it is possible to control the crypt's proliferation [1]. Depending on the examined portion of the intestine (duodenum, jejunum, ileum, colon) different genes are regulated, downstream of WNT: regulating surface proteins, cytoskeleton, enzymes, proteins, inflammation, etc. Comparing such alteration with those caused in MYD88 knockout mice (the MYD88 is part of the complex regulatory response to the TLR4), it can be seen that a much lower number of genes transcribed by WNT is enabled or disabled by the absence of this protein. This observation, combined with the fact that the MYD88 knockout mice do not show alterations in their intestinal morphology [112], makes possible to hypothesize that the microbiota regulates transcription of WNT through more than one pathway. Stimulating the microbiota with intra-cryptic LPS has an effect, also on cells expressing LGR5, namely the CBC, causing apoptosis [113].

Development of the motor function

The innervation of the gastrointestinal tract reaches gradually maturity. The motor pattern – that is, the ability to develop effective peristaltic contractions, able to advance food from the mouth to the anus – starts to mature at the 28th gestational week [18, 19, 38]. So the more preterm the baby is, the less developed are the motility and bowel tone.

In fact the intestines of a preterm infant is characterized by a decreased tone of the lower esophageal sphincter, a slow rate of contraction, slow stomach emptying and increased residual volume.

Intestinal motility is one of the major control systems of the intestinal microbiota, for it removes the excess of bacteria from the lumen. From its immaturity fecal and bacterial stagnation and dysbiosis follow, both potentially harmful [38, 114].

Development of the digestive function

The proteic digestion begins with the hydrolysis in the stomach, which is very limited in the VLBW. Those do not reach sufficiently high levels of gastric pH, and for this reason are also more exposed to invasion by pathogens. This situation improves between the first and fourth weeks of life [38].

A minor protease capacity means a greater number of antigens that reach the intestinal lumen, and thus a higher stimulation of gut immunity. Intestinal digestion of lipids by the enterokinase begins at the 24th week, and matures over time [38]. Compared to full-terms, in preterms lipids are poorly absorbed [38].

It is not known whether infants can lengthen the chain of polyunsaturated fatty acids of more than 18 carbon atoms. These are pre-formed in breast milk, but not in formula milk. They are necessary for intellectual development and have different functions. In particular, ω3 have an anti-inflammatory function and decrease the risk of NEC [38]. The digestion of carbohydrates is carried out by pancreatic amylase and intestinal lactase. This gradually increases between the 24th and the 40th week (which makes it a marker of intestinal maturation). Its activities may be insufficient in very young children. The unabsorbed carbohydrates pass the intestinal microbiota that forms gas and short-chain fatty acid (SCFA, easily absorbed) [38].

Hematic flow regulation

Proper oxygenation is an essential prerequisite for the proper development of the intestine, and limit inflammation phenomena [72]. The blood flow is influenced by metabolic factors: the voltage of oxygen and the accumulation of metabolic products, which cause vasodilatation and endogenous vasodilators, including nitroxide (NO).

It is secreted by the endothelium, with endothelin 1 (ET1, a potent vasoconstrictor). Together they are able to directly control the operation of the intestinal microcirculation, and to maintain homeostasis in stress conditions [18, 115].

The efficacy of both mechanisms is partly postnatal-age dependent [18]. ET1 is constitutively produced, and increases in response to inflammatory stimuli and hypoxic-ischemic injury. It has angiogenic and vasoconstrictor effects. It has at least two ETA receptors, which mediates the vasoconstrictor, and ETB. The ET1 tie in ETB induces the secretion of NO with which it comes in dynamic equilibrium [18, 115]. Both ET1 and NO have an effect that varies depending on the gestational age. In the newborn, the vasoconstrictor tone is predominant, and stimulation of ETB leads only to a modest NO dependent vasodilation [115]. Therefore, in all conditions where it increases the extraction of oxygen (that is under conditions of stress, but also after meals) the neonatal late response (that is an hyper-flow of blood) is less effective the lower the gestational age [18, 115].

Preterm's typical mucosal alteration

In physiological condition, an infant's intestine at birth is ready to interact with the commensal microbiota. The administration of breast milk enhances both the interaction, and the formation of a commensal, eubiotic microbial flora [19, 38, 59, 116]. However, premature birth alters these premises. With modern techniques of assistance, the fetus is viable outside the womb starting at 23 weeks of gestational age. Depending on gestational age at birth, the intestinal functions are more or less mature, and ready for the meeting with the microbiota and diet, that influences the development.

The immaturity of the general systems strongly upsets the newborn's intestine in a proinflammatory sense. In fact, the motor function and the preintestinal barrier are not properly developed: the esophageal sphincter is not competent, gastric secretions are slightly acidic, the production of digestive enzymes is short. Because of these reasons, an increased bacterial load arrives at the intestine [19, 38].

Vascular tone is set in the vasoconstrictive sense, and this does not guarantee adequate perfusion under stress conditions [115], extremely frequent in a NICU. The lower the gestational age, the greater is the production of TLR4, and the lower that of TLR9. The former are actively removed from the epithelial surface just before the end of physiological pregnancy, thanks to EGF in the amniotic fluid [39, 77, 80].

The production of cytokines is unbalanced in the proinflammatory sense [69]. The function of intestinal barrier is impaired. This is due to the immaturity of all its components: the Paneth cells are still not able to secrete a sufficient quantity of α-defensins, the mucosal layer is thin and uneven, the tight junctions not entirely effective [117]. Often, because of the frequent association of preterm labor with premature rupture of membranes and chorioamnionitis, the intestine of a preterm has already been subjected to inflammation in the uterus that altered the intestinal permeability, and enhanced the physiological proinflammatory, status. So the intestinal mucosa of a child born prematurely is often hyper-responsive to the inflammatory stimuli, even in comparison with a fetus in the uterus of the same gestational age [42, 118]. The NICU is a major challenge for the mother-child dyad, especially for what concerns the possibility of providing breast milk: all the components of breast milk so far studied have an anti-inflammatory effect, extremely valuable for a premature [116]. The formula milk, coming to our aid, promotes the development of a microbial flora completely different than the breast milk, which then does not have the same opportunity to be recognized as commensal, and is dysbiotic [119]. During hospitalization the baby is fed, at least initially, through a nasogastric tube. If he is not able to tolerate the feeding, is nourished with parenteral nutrition (routine for the preterms). Frequently, from the first days of life they are given broad-spectrum antibiotics, extremely toxic for the commensal flora [120-122]. These are all unavoidable factors, affecting bowel function. In fact, the growth of the intestine, the height of the villi, the mass of the mucosa, cell proliferation, mucosal immunity are all indices of intestinal health and tissue tropism, are significantly reduced by a total parenteral alimentation [123-126]. Often these deficits are implicated in the development of increased intestinal permeability, bacterial translocation and sepsis, and cause increased mortality and morbidity [127-129].

Generating necrotizing enterocolitis

The term Necrotizing Enterocolitis (NEC) defines a syndrome where an immature intestinal mucosa, slightly tolerant, and in some cases already damaged – that could be defined as "ready

to sparkle" – responds to stimulation given by diet, hypoxia, and by meeting with the microbiota triggering a strong inflammatory state, which initially involves only the mucosa, and later the entire thickness of the wall.

The natural history of the NEC, if not properly treated, goes to intestinal perforation [130].

Despite the association with the impairment of the fetal growth, the NEC has never been detected *in utero* [131]. There is no NEC without dysbiosis and milk [119, 132, 133].

The only consistent epidemiological risk factors for NEC are prematurity and enteral nutrition, which may include a rapid progress for diet or high osmolarity formula [134, 135]. The NEC is also rarely described in a full-term newborn child, probably with a different pathogenesis than the one of a preterm child. The onset of the disease in these children occurs within a few days after birth and is often associated with individual risk factors of hypoxia-ischemia, like in the cyanotic congenital heart disease [72, 136]. Dysbiosis plays a key role in the genesis of NEC [120-122]. The intestine of an infant – at the beginning of colonization – is inhabited by a number of bacterial species lower than in an adult subject. The NEC is associated with a severe deficiency in the diversity of the microbiota, which – favored by empirical therapies – can accentuate the impact of dysbiosis, or the single dominant microorganism.

To this regard, various data are available. [120-122]. In experimental animals, placed under stress, administration of LPS induces the NEC; moreover, the levels of LPS in the blood and in the stool are increased by the NEC [137-141]. One study reports that in the week prior to the diagnosis of NEC potential known pathogens in the feces of 79% of the children were found, but there is no specific reference to the type of pathogen recovered [142]. In another study, patients with NEC showed less diversity of bacterial species, an increase in the quota of Gammaproteobacteria, a decrease in the number of the other species, and received more antibiotics compared to controls [121]. In controls, bacteria were found from four phyla: Proteobacteria (34.97%, in relative abundance), Firmicutes (57.79%), Bacteroidetes (2.45%) and Fusobacteria (0.54%) with a 4.25% of unclassified bacteria.

In the stools of NEC patients only two phyla were found: Proteobacteria (90.72%) and Firmicutes (9.12%) with 0.16% of unclassified bacteria. Subsequent explorations indicate in every child with NEC the predominance of a single genus of Proteobacteria. The intestine of apparently healthy children was instead inhabited by different kinds of Proteobacteria, in proportions lower than 40%, except in two cases (which later developed NEC). The bacterial species most frequently placed in relation to the NEC were: *Klebsiella spp.*, *H. parainfluenzae* and *Pseudomonas spp.* [122]. Instead, the species normally unrelated are *Veillonella spp.*, *E. coli*, *Enterococcus spp.*, *Staphylococcus spp.* and *Enterobacter aerogenes*.

Some species are more immunogenic and trigger the immune response more than others [122]. Probably this difference depends on the lipid A, component of the LPS. The activation of TLR4 by the LPS depends on the acetylation of lipid A [138]. In the adult's Bacteroidetes, the lipid A is usually pentaacylated, and this leads to less powerful TLR4 activation and lower immunogenicity [140].

In Proteobacteria, lipid A is hexacylated, and this makes it a potent agonist of the TLR4. In healthy individuals – but not in inflammatory conditions –, in eubiosis conditions, the immunogenic properties of Proteobacteria are mitigated by Firmicutes and Bacteroidetes [141-143].

Dysbiosis, and the peak of Proteobacteria, may also cause late-onset sepsis (LOS), secondary to altered intestinal permeability [144]. The clinical diagnosis of chorioamnionitis, and most importantly PROM, increases significantly the NEC risk [145], as demonstrated by the association with several *in utero* inflammatory markers [146-150]. The anatomy of a newborn's intestine affected by NEC shows a full thickness coagulative necrosis [72], enterocytes necrosis, edema [83], lymphocytic infiltrate, neutrophils paucity, separation of the submucosa and lamina propria, and evidences of ischemic damage. The distal ileum and colon are more often damaged, but in the most severe cases from the stomach to the rectum [83].

The response to microbes is triggered by TLR4 [143]. The pathway NFκB, when activated, triggers mucosal damage [138], especially when prematurity, endotoxemia and hypoxia lead to a persistent upregulation of activation of TLR4 bowel [143]. In the infant's intestine the TLR4 regulates the balance between damage and repair [139], but also reduces the proliferation and migration of enterocytes, as well as the mucosal healing [140, 141, 145]. In preterms the TLR4 is upregulated, and so the response to the pathogen

exaggerated [141, 143, 145]. The administration of polyunsaturated fatty acids (found – among others – in breast milk) appears to have a protective effect on the NEC inhibiting the expression of TLR4 [116].

Animal models for the study of the NEC can give a pathogenesis' idea, even if genetic and environmental differences do not allow recreating the same condition of the humans.

The main hystopathological models, showing NEC like alterations, require a trigger for the innate immunity: the model gavage-hypoxia, the direct stimulation of TLR4 by administration of PAF or LPS, and that of Paneth cell's [85, 151]. In fact the administration of LPS activates the TLR4, which are allocated on enterocytes as well as on the Paneth cells, then pathogenic pathways between these models can be common.

The model of ischemia-reperfusion, instead, produces a severe intestinal damage, but not NEC-like. It seems that the ischemic component, always with inflammatory factors – i.e. in hearth disease, severe anemia, and transfusions – can favor some very serious types of NEC [151].

The first step that leads to the NEC seems to be the inflammatory cascade activation via the TLR4 and NFκB. It follows the secretion of TNF and IL1β and vasoactive substances (PAF, ET1, NO), the activation of the complement and coagulation cascade the vasoconstriction and coagulation necrosis [77]. It is hypothesized that the process begins with bacterial translocation, at the villus apex. However, even the Paneth cells express TLR4, and therefore can be activated by the LPS, triggering inflammation and apoptosis, and activating complement and coagulation. Moreover, they are also placed at the base of the crypt (and usually, in the pathologic findings, the bacteria settle there), and near the endothelium, and are thus capable of controlling the homeostasis, inducing the release of PAF, ET1, and NO [85].

It is assumed then that the Paneth cell dysfunction is the primary cause of the NEC [85].

In support of this hypothesis one can recall several observations: first, that the NEC develops rarely before a certain degree of adaptive and innate immunity maturation, i.e. before 30-31 weeks of gestational age. So the ELBW have the longest presentational interval in the disease [152], and it was noted that this might be related to the maturation of Paneth cells [85]. In addition, all the factors that promote Paneth cells maturation (such as lactoferrin, EGF [153-155] and corticoids [156, 157]) play a protective role against the NEC.

This hypothesis has not been shared by all researchers

For example Puiman et al. [158] show that there is no difference in the number of Paneth cells between children operated for NEC and those operated for other reasons (such as a case of intestinal atresia). In addition, the Paneth cells activity increases in response to inflammatory stimuli, and their proliferation increases in the healing phase, as can be expected given the role they play in driving the growth and intestinal tropism in response to injury [158].

It can be assumed that the physiological immaturity of the Paneth cell, rather than a pathological malfunction, can predispose to dysbiosis, and therefore to NEC. This does not mean that it is hypo-responsive to stimuli: the transcriptional activity is correct, and the granules containing antimicrobial peptides are secreted normally, as shown by Salzman [159].

The Paneth cells maturation delay is considered to be a cause of NEC only in Coutinho's work, conducted on autoptic or surgical specimen by NEC children at surgical stage, in gestational age between 32 and 42 weeks. Those are compared with babies operated for other reasons as intestinal atresia [160]. In this case it is highlighted a reduced production of lysozyme by the cell of the Paneth.

However, some doubts still remain: in this study 60% of cases are full-term NEC infants and the full-term's NEC is probably distinct from the preterm's one. In addiction, the lysozyme is not a specific marker of the Paneth cells.

Focusing on the Paneth cell for NEC pathogenesis means to assume a global involvement of the entire ecology of the crypt, and also of the stem cells, at least in the short term. It also means to assume that, promoting growth and cell refurbishment, it would be possible to cure the disease, or at least mitigate its deleterious effects.

Further studies are needed to better define and characterize the implications of these findings. However, this hypothesis opens to interesting possibilities for treatment.

For instance, by injecting stem cells from the amniotic fluid into the peritoneum of rats suffering from NEC, they integrate in the neonatal gut and improve intestinal function, control inflammation, express genes of the WNT/βcatenin pathway, increase the share in the active cell replication and reduce the apoptosis [161, 162].

The breast milk wealth

The breast milk is the ideal food for a newborn, especially for a premature one. Each component that up until now has been studied has highlighted, in addition to its nutritive properties, its anti-inflammatory and pro-maturative effectiveness [116]. Its composition is variable, according to the person and to the gestational age and varies along the week [163, 164]. It contains not only the nutrients needed for growth and development, but also numerous bioactive factors that, acting in concert, contribute to the beneficial effects of breastfeeding. The always-growing list of factors found in human milk includes hormones, growth factors, cytokines, and chemokines, which operate in network producing a cascade of effects contributing to the child's harmonious development and the general maturation of immune system functions [41, 44, 116].

Immunoglobulins, mainly IgA [44, 45], are necessary to infant, especially to a premature one, in the first weeks of life, when the endogenous production is not enough. They promote phagocytosis, and modulate local immunity [44, 47, 165]. Lactoferrin and lysozyme, antimicrobial peptides, also promoter of the Paneth cells, are also present in the breast milk [166, 167]. Breast milk is able to induce B cells to IgA production, via the IL6 pathway [48]. It also has an immunosuppressant action via IL10 [49]. Breast milk contains prebiotics, which stimulate the commensal flora, but also has its own flora, deriving from the maternal intestine with known tolerogenic features [165]. In the breast milk are also contained bacterial metabolism products, among which SCFA, stimulating the intestinal barrier function [165]. In breast milk there are a large number of growth factors, acting on the crypt cells, and promoting their proliferation: EGF, TGFβ1 and TGFβ2 promote the development function and the shelter of the gastrointestinal mucosa [19].

Finally, breast milk contains pluripotent stem cells, which have the capacity to be used by the organism [168, 169]. This particular finding is extremely interesting, because it provides an additional reason for the promotion of breastfeeding as a safe, economical, and ready-to-use mean in the prevention of inflammatory disease of the preterm's gut.

Conclusions

The promotion of intestinal health, or of the correct trophism, begins outside the uterus and requires a series of measures aimed at maintaining the physiological balance between proliferative and inflammatory function, necessary to the intestinal defense. From an histological point of view, everything passes through the cell of Paneth, which governs the health of the crypt, the inflammatory response and the intestinal proliferation.

In preterm infants, protecting gut health means investing in the general health, both in the short and long term. For its contents in probiotics, stem cells, growth factors and cytokines, breast milk is a resource not only on a nutritive point of view, but above all for active promotion of growth and trophic gut, unparalleled in nature. Numerous factors related to hospitalization of the infant cannot be changed, but the promotion of breastfeeding is the most economical, simple and effective way to protect the health of the child, and should be the first purpose of each neonatal intensive care unit.

Promising therapeutic applications, after the onset of NEC, are given both by the discovery of the content of stem cells in breast milk, and by the healing potential of amniotic fluid, whose general profile closely follows that of breast milk.

Abbreviations

Abbreviations are listed in **Tab. 2**.

Declaration of interest

The Authors declare that there is no conflict of interest.

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Table 2. Abbreviations used in the text.

Function	Name	Abbreviations
Transcription factors and Homeobox	Wingless nuclear transcriptor factor	WNT
	Bone morphogenetic protein	BMP
	Sonic Hedgehog	SHH
	Tumor growth factors β/α	TGFβ/TGFα
	Tumor necrosis factor α	TNFα
	Nuclear factor	NFκB
	Fibroblasts growth factor	FGF
	Hematopoietically-expressed Homeobox protein	HHEX
	Platelet-derived growth factor	PDGF
	Caudal type Homeobox transcription factor 2	CDX2
	Epidermal growth factor	EGF
	Insulin-like growth factor	IGF1
	Hepatic growth factor	HGF
Cellular receptors	Toll like receptor	TLR
	Nucleotide-binding oligomerization domain-	NOD
	Endothelin receptor	ETA, ETB
	Leucine-rich repeat-containing G-protein coupled receptor 5	LGR5
	Major Histocompatibility Complex I, II	MHC I-II
Citokines and others	Interleukine 10	IL10
	Interleukine 6	IL6
	α-Defensine 5, 6	HD5, HD6
	Shorty chain fatty acid	SCFA
	Bacterial lipopolysaccharides	LPS
	Endothelin 1	ET1
	Nitroxide	NO
	Platelet activating factor	PAF
Cellules	Crypt base columnar cells	CBC
	+ 4 Intestinal stem cells	+4ISC
Others	Premature rupture membrane	PROM
	Late-onset sepsis	Sepsi LOS
	Very/Extremely low birth weight	VLBW/ELBW

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