LUMEN ILLUMINATED

Intestinal defense mechanisms in the neonate

Patrycja Jolanta Puiman

The studies as presented in this thesis were financially supported by Danone Research BV and Nutricia Research Foundation. The grant supplier had no involvement whatsoever in the study design, in the collection, analysis, and the interpretation of data, and in the decision to submit the reports for publication.

The printing of this thesis was financially supported by Nutricia Nederland B.V., Danone Research B.V., Nestlé Nutrition and Stichting Sanguin Bloedverziening.

ISBN: 978-90-8559-271-6

Layout: Optima Grafische Communicatie, Rotterdam, The Netherlands

Cover: Photograph: Tunnel, © Serknor, www.dreamstime.com

Printed by: Optima Grafische Communicatie, Rotterdam, The Netherlands

Copyright: P.J. Puiman, The Netherlands, 2010

All rights reserved. No part of this book may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording, or otherwise, without the prior written permission of the holder of the copyright.

LUMEN ILLUMINATED

Intestinal defense mechanisms in the neonate

HET LUMEN TOEGELICHT

Intestinale verdedigingsmechanismen in de pasgeborene
Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus

Prof. Dr. H.G. Schmidt

En volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 22 december 2010 om 13.30 uur

door

Patrycja Jolanta Puiman
Geboren te Velp

Promotiecommissie:

Promotoren:	Prof. Dr. J.B. van Goudoever
	Prof. Dr. H.J.G. Boehm
Co-promotor:	Dr. I.B. Renes
Overige leden:	Prof. Dr. A.J. van der Heijden
	Prof. Dr. P.J.J. Sauer
	Prof. Dr. H.N. Lafeber

Table of contents

Chapter 1.	General introduction and outline of the thesis	7
Chapter 2.	Animal models to study neonatal nutrition in humans	21
Chapter 3.	Dietary influence on colitis-development in Muc2-deficient mice: *diet matters!*	33
Chapter 4.	Intestinal threonine utilization for protein and mucin MUC2 synthesis is decreased in preterm pigs fed formula.	51
Chapter 5.	Intestinal threonine uptake routes for mucin MUC2 synthesis in preterm pigs and infants	67
Chapter 6.	The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection.	85
Chapter 7.	Modulation of the gut microbiota with antibiotic or probiotic treatment suppresses body urea production and stimulates mucosal mucin production in neonatal pigs.	105
Chapter 8.	Paneth cell hyperplasia and metaplasia in necrotizing entero-colitis	127
Chapter 9.	Enteral arginine does not increase superior mesenteric arterial blood flow but modestly increases mucosal growth in neonatal pigs.	143
Chapter 10.	General discussion	163
Chapter 11.	Summary	181
Chapter 12.	Nederlandse samenvatting	187
Dankwoord		195
List of affiliation of co-authors		199
List of publications		201
Curriculum vitae		203
Portfolio		205

Chapter 1

General introduction and outline of the thesis

Prematurity and NEC

Preterm births constituted 7.6% of live births in 2007 in the Netherlands (http://www.perinatreg.nl). In the United States, premature infants comprised 12.8% of live births and the incidence of premature live births is rising because of the improved perinatal care[1]. With the rising incidence of preterm births and the improving survival rates of (extremely) very low birth weight neonates, efforts to decrease morbidity concerning short and long term outcome remain a challenge in the neonatal intensive care unit (NICU).
Necrotizing enterocolitis (NEC) is the most common surgical emergency involving the gastrointestinal tract of preterm neonates and affects 2-7% of all premature infants[2-3]. Both the incidence of NEC and its fatality rate are inversely related to birth weight and gestational age[4].
Treatment is still limited to immediate restriction of enteral feeds and broad-spectrum antibiotics. Although most cases of NEC are managed medically, an estimated 20-40% of infants undergo surgery[5-7]. Mortality rates from NEC range from 15-30% but mortality rates for infants requiring surgery are as high as 50%, and are highest for the smallest, most immature infants[2,4]. Survivors of NEC are at increased risk for complications such as short bowel syndrome and impaired neurodevelopment[8-9]. Stoll and colleagues[8] reported that between 18 and 22 months of corrected gestational age, infants who recovered from NEC in the postnatal period were at high risk for adverse outcomes, including poor growth, cerebral palsy, vision and hearing impairment, and decreased neuromotor development. Furthermore, infants who are surgically treated are more likely to have growth impairment and adverse neurodevelopmental outcomes than infants who were treated medically[10].

Risk factors for NEC

The etiology of NEC is unknown, however riskfactors identified for the development of NEC are prematurity and low birth weight, enteral (formula) feeding, and bacterial colonization[2,11]. Prematurity is the one risk factor that is most consistently recorded[2]. NEC occurs rarely in full term infants, and is different from NEC in preterm infants because of the association with underlying disorders such as perinatal asphyxia, polycythemia, and congenital heart disease[12].

The use of formula as substitute for mother's milk poses a risk for developing NEC. A study performed in 1990 showed that NEC was six times more likely to occur in preterm infants fed formula than those exclusively fed their own mother's milk or donor human milk[13]. Since then, more studies have shown a risk reduction in NEC when infants were fed human milk compared to formula[14-17]. However, one should note that the NEC incidence in the formula fed infants was high in these studies. The exact mechanism behind the suggested protective effect of human milk on NEC still remains to be elucidated.

However, several growth factors, cytokines, bacteria, and oligosaccharides are present in breast milk and their activities in the neonatal gut are likely to affect maturation of the epithelium and immune system, and to improve gut barrier function[18-19]. Also, a potential disruptive effect of cow's milk protein on the intestinal epithelium cannot be disregarded. These potential mechanisms and interactions require further study, as they might have major implications for the future development of improved infant formulae.

The bacterial flora is also thought to play a role in the development of NEC. At birth, the neonatal intestine is instantly challenged with the introduction of microbiota.
Commensal bacteria interact symbiotically with the mammalian intestine to regulate the expression of genes important for barrier function and digestion[20]. Interestingly, commensals have shown to be able to inhibit inflammatory signaling in the intestinal epithelium[21]. Delay in enteral feeding and frequent use of antibiotics results in inadequate and delayed colonization by commensal bacteria[22], and could possibly cause hyperactive inflammation in preterm infants. Furthermore, premature infants are especially susceptible to intestinal colonization by pathogens because of their daily exposure to nosocomial flora and the likelihood of exposure to antibiotics during their admission to the NICU[23]. Human milk may decrease the incidence of NEC by decreasing pathogenic bacterial colonization, promoting growth of nonpathogenic flora, stimulating maturation of the intestinal barrier, and ameliorating the pro-inflammatory response[24]. Recently, probiotics are immerging as a promising therapy for prevention of NEC. Probiotics are living micro-organisms which, when administered in adequate amounts, confer a health benefit to the host[25]. It has been hypothesized that probiotics can decrease the incidence of NEC by reducing enteric pathogens, improving gut structure and function, and enhancing the mucosal barrier[26]. Different meta-analyses reported a significant reduction of NEC incidence by administration of probiotics, but call for a confirmatory trial because the data could not be extrapolated to extremely low birth weight infants[27-30]. Moreover, further research is necessary to determine the optimal dose, duration, and the type of probiotic strains to use for supplementation in preterm infants. Furthermore, specific mechanisms by which probiotics confer their protective effect to the intestinal epithelium have yet to be illuminated.
Prebiotics, non-digestible dietary oligosaccharides that selectively promote proliferation of beneficial enteric bacteria, might confer another strategy to prevent NEC. Prebiotic supplementation of infant formula has shown to establish a beneficial bifidogenic flora and reduce the presence of pathogens[31-33]. Recently, an infant formula with prebiotic supplementation has been developed[34]. However, the function of the complex oligosaccharides present in human breast milk is still under investigation to improve our understanding of the prebiotic effects on the intestine. The findings from these studies will offer opportunities to improve infant formulae.

Pathophysiology of NEC

Despite extensive research, and the recognition of certain important risk factors, the pathogenesis of NEC remains poorly understood. Preterm infants are at increased risk for NEC because of their immature gastro-intestinal functions, in particular motility and digestive ability, circulatory regulation, intestinal barrier function, and immune defense[2].

The intestinal epithelium

The intestinal epithelium consists of four principal epithelial cell lineages that rise from the multipotent crypt stem cells (Figure 1). Absorptive enterocytes make up more than 80% of all small intestinal epithelial cells. Goblet cells produce a variety of mucins, while enteroendocrine cells export peptide hormones. Paneth cells are specialized secretory cells that play an important role in innate immunity by the secretion of endogenous antimicrobial peptides. Each small intestinal crypt supplies cells to several adjacent finger-shaped villi. Enterocytes, goblet cells, and enteroendocrine cells differentiate as they migrate up towards the villi, whereas precursor Paneth cells migrate and differentiate towards the base of the crypts.

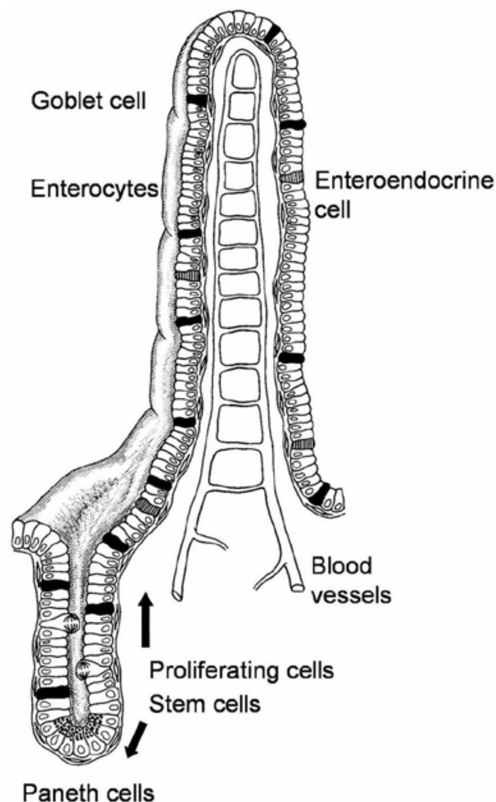


Figure 1. Schematic representation of the small intestinal epithelium adapted from (34).

Motility and digestive ability of the preterm intestine

Fetal studies in humans and animal models suggest that development of gastrointestinal motility begins in the second trimester, and matures in the third trimester[35-37]. Intestinal motility has shown to have an immature pattern in preterm infants compared to full-term infants, but that enteral feeding can mature these responses[38-40]. In addition to impaired intestinal motility, the intestinal digestive and absorptive processes are only partially available before 26 weeks of gestation, whereas gastric and pancreatic secretion is only basal[41]. Enzymatic hydrolysis of disaccharides by disaccharidases such as lactase, sucrase, isomaltase, maltase, and glucoamylase necessary for monosaccharide uptake, takes place at the brush border membrane of the enterocytes in the proximal small intestine. Between 26 and 34 weeks of gestation the level of lactase is 30% of the newborn level, while levels of sucrase-isomaltase, maltase, and glucoamylase reach 70% of the newborn level[42]. Thus, impaired digestion of nutrients, coupled with delayed transit time, could result in injury of the intestines with immature barrier and immune defenses.

Intestinal barrier function and the mucin layer

The intestinal barrier regulates transport and host defense mechanisms at the mucosal interface with the outside world. If either the structural or biochemical component of the intestinal epithelial barrier is not fully developed, bacteria could gain access to deeper tissues and cause inflammation. An important feature of gut barrier function is the mucus layer that overlies the intestinal epithelium. Goblet cells synthesize and secrete large gelforming glycoproteins, called mucins of which MUC2 is the most predominant secretory mucin in the human intestinal tract[43-44]. Within the mucus gel, other components including water, electrolytes, sloughed epithelial cells, and secreted defensins and immunoglobulins reside. Together these factors produce a physical and chemical barrier that protects the epithelium from luminal pathogens and toxic substances that pose a threat to the mucosa[45-46]. Preterm infants might have immature goblet cells. Developmental expression of mucin genes changes throughout the intestine and seems to mimic adult pattern expression between 23 and 27 weeks of gestation[47]. A diminished mucus layer decreases gut barrier function and causes intestinal inflammation and mucosal eruption facilitating bacterial translocation[48]. In combination with an immature immune system, this renders the preterm infant at particular risk for the development of intestinal inflammation, sepsis, and NEC[48]. The peptide backbone of MUC2 is particularly rich in the essential amino acid threonine constituting ~30% of the total amino acids in MUC2[49-53]. Threonine availability is known to impact protein mucosal synthesis and mucin synthesis in pigs and rats[54-57]. Therefore, threonine might be of critical nutritional importance in maintaining the protective mucus layer and hence adequate barrier function.

Immune defense of the preterm intestine

Paneth cells, named after Joseph Paneth[58], are an important feature of the innate immune system by the secretion of bactericidal products such as lysozyme, phospholipase A2, and human defensins (HD) 5 and 6[59-62]. Paneth cells secrete their products in response to microbial stimuli and regulate the composition and distribution of bacterial populations[63-64]. Paneth cell numbers and HD5 and -6 mRNA expression are lower in premature infants at 24 weeks of gestation compared to term infants, and up to 200-fold lower than in adults[65]. In the premature infant, who is often exposed to nosocomial pathogens and has delayed colonization with beneficial commensals, this phenomenon could result in higher susceptibility to bacterial infection and inflammation.

Circulatory regulation in the preterm intestine and hypoxic-ischemic injury

Immature regulation of intestinal circulation might lead to intestinal hypoxia-ischemia in response to feeding or to the presence of pathogenic bacteria. Nitric oxide (NO) is a signaling molecule that plays a central role in regulating vascular resistance and hence blood flow in the newborn intestinal circulation[66]. Reduced endothelial production of NO might result in a predisposition to ischemic injury. Arginine, an essential amino acid in neonates, is the sole physiological precursor for NO[67]. Clinical studies have shown low levels of plasma arginine in preterm infants which is associated with an increased incidence of NEC[68]. Low circulating levels of arginine or arginine precursors might result in shortage of NO and a decrease in intestinal blood flow, and subsequently may contribute to the development of NEC. Interestingly, arginine administration has also been evaluated in a randomized, double-blind, placebo-controlled study where arginine supplementation to infants less than 28 weeks gestation increased plasma arginine levels and significantly decreased the incidence of NEC[69]. However, whether arginine increases neonatal intestinal blood flow and subsequent mucosal growth needs to be investigated.

Consideration

In summary, immaturity of the neonatal gut makes the preterm infant vulnerable for abnormal colonization, feeding intolerance, and hypoxic-ischemic injury that potentially lead to the development of NEC as presented below (Figure 2).

However, the exact mechanism behind the development of NEC remains inconclusive. Given the limited therapeutic options, high mortality rates, and the increased risk of adverse long term outcome, this warrants extensive research to increase our understanding of NEC development. Therefore, different aspects within the lumen need to be illuminated such as the impact of enteral formula feeding on the intestinal barrier and which factors regulate intestinal barrier function. Furthermore, gaining insight in the effect of arginine supplementation on intestinal blood flow and gut growth might

provide evidence for the potential decrease of NEC by arginine. Finally, can we offer clues for stimulation of intestinal innate defense mechanisms by investigating Paneth cell presence and functions in preterm infants recovering from NEC? Answers to these questions should ultimately lead to optimized nutritional support of the preterm neonate in need of adequate intake of especially proteins for growth and improved development. In short, we need to understand what and how to feed the very young premature infant at risk for NEC.

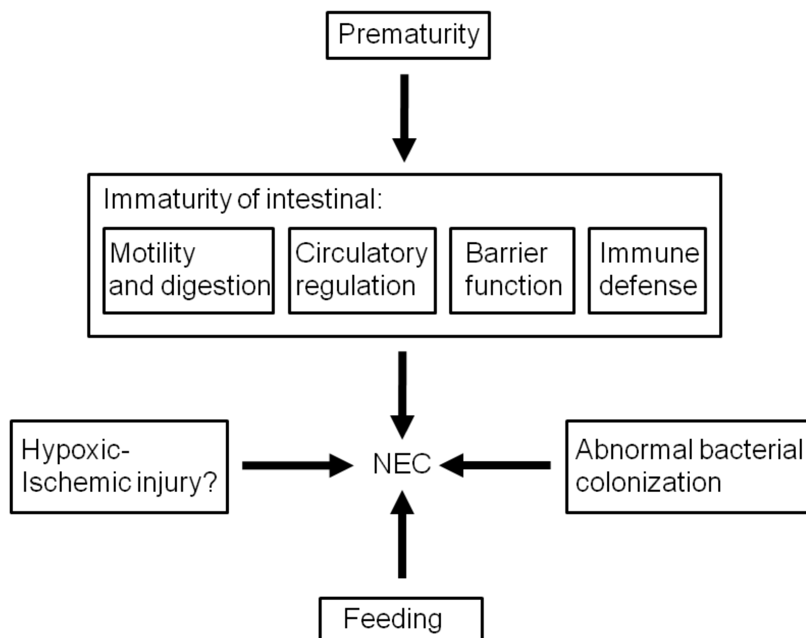


Figure 2. Pathophysiology of necrotizing enterocolitis adapted from (2).

Aims and outline of the thesis

The overall aim of the work presented in this thesis was to determine to which extent various nutritional factors and the intestinal microbiota impact intestinal defense mechanisms and gut barrier function. The ultimate goal of this thesis was to achieve a better understanding of factors regulating intestinal defense using *in vitro* studies, studies performed in various established animal models, and studies undertaken in preterm infants. The knowledge gained from the studies presented in this thesis, will provide insights for the improvement of nutritional care of the preterm infant at risk for NEC.

Chapter 2 of this thesis highlights the use of animal models as an invaluable tool to study human neonatal nutrition and related diseases such as NEC. In recent years, mice, rats, and pigs have become the most frequently used animal models to study human neonatal nutrition. Mice have great potential for mechanistic and genomic research in postnatal nutrition and related diseases. The piglet model most closely resembles the human infant intestinal growth and function, and is invaluable to study acute and chronic effects of (par)enteral nutrition on intestinal and whole-body metabolism.

Chapter 3 describes the dietary impact on intestinal inflammation. The aim of this chapter was to determine the effect of standard rodent chow versus a purified diet, and dietary supplementation of probiotics, on growth and intestinal inflammation in wild type and Muc2-deficient mice.

Chapter 4 describes a study performed to determine the dietary impact of infant formula compared to bovine colostrum in preterm pigs. The aim of this project was to determine intestinal threonine utilization for protein and mucin synthesis in preterm pigs fed either infant formula or colostrum. Second, we aimed to provide a plausible theory for the impact of nutrition on the development of NEC in the preterm neonate.

Chapter 5 presents a study showing the preferential site of threonine uptake for the production of mucin MUC2 in both preterm pigs and preterm infants. The aim of this study was to test the hypothesis that type of enteral nutrition as well as the route of nutrition, i.e. enteral or parenteral, is important for synthesis of MUC2.

Chapter 6 elucidates the regulation of intestinal *MUC2* expression by short-chain fatty acids. We hypothesized that short-chain fatty acids, fermentation products of the intestinal microbiota, affect *MUC2* expression and hence alter epithelial protection. The aim of the study was to investigate the mechanisms that regulate butyrate-mediated effects on MUC2 synthesis.

Chapter 7 describes the impact of an altered intestinal microbiota on amino acid and protein metabolism. We hypothesized that modulation of the intestinal microbiota by antibiotics or probiotics would impact amino acid metabolism. In this study we aimed to investigate the impact of the gut microbiota whole body nitrogen and amino acid turnover in neonatal pigs receiving no treatment (control), antibiotics, or probiotics. We quantified whole body urea kinetics, threonine fluxes, and threonine disposal into protein, oxidation, and tissue protein, and more specifically MUC2 synthesis.

Chapter 8 gives insight into Paneth cell presence and function in NEC. Paneth cells are a major component of intestinal innate defense and enhance the gut barrier by secretion of endogenous antibiotic peptides. We aimed to investigate Paneth cell presence, protein expression, and developmental changes in preterm infants with NEC. Furthermore, we determined Paneth cell products and their antimicrobial capacity in ileostomy outflow fluid.

Chapter 9 describes the effect of dietary arginine supplementation on intestinal blood flow and intestinal protein synthesis in neonatal pigs. The aim of this study was to investigate whether enteral arginine supplementation is a specific stimulus for neonatal intestinal blood flow and mucosal growth under conditions of total parenteral and partially enteral nutrition.

Chapter 10 highlights and discusses the most important findings of this thesis. Moreover, we give our recommendations for future research projects to further illuminate the mechanisms of intestinal defense, and to study gut barrier function in order to further unravel the predisposition of the preterm neonate to NEC.

Chapters 11 and 12 summarize the main results of the studies described in this thesis.

References

1. Martin JA, Hamilton BE, Sutton PD, et al. Births: final data for 2006. Natl Vital Stat Rep 2009;57:1-102.
2. Lin PW, Stoll BJ. Necrotising enterocolitis. Lancet 2006;368:1271-83.
3. Holman RC, Stoll BJ, Curns AT, Yorita KL, Steiner CA, Schonberger LB. Necrotising enterocolitis hospitalisations among neonates in the United States. Paediatr Perinat Epidemiol 2006;20:498-506.
4. Blakely ML, Lally KP, McDonald S, et al. Postoperative outcomes of extremely low birth-weight infants with necrotizing enterocolitis or isolated intestinal perforation: a prospective cohort study by the NICHD Neonatal Research Network. Ann Surg 2005;241:984-9; discussion 9-94.
5. Guthrie SO, Gordon PV, Thomas V, Thorp JA, Peabody J, Clark RH. Necrotizing enterocolitis among neonates in the United States. J Perinatol 2003;23:278-85.
6. Luig M, Lui K, Nsw, Group AN. Epidemiology of necrotizing enterocolitis--Part II: Risks and susceptibility of premature infants during the surfactant era: a regional study. J Paediatr Child Health 2005;41:174-9.
7. Sankaran K, Puckett B, Lee DS, et al. Variations in incidence of necrotizing enterocolitis in Canadian neonatal intensive care units. J Pediatr Gastroenterol Nutr 2004;39:366-72.
8. Stoll BJ, Hansen NI, Adams-Chapman I, et al. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. Jama 2004;292:2357-65.
9. Petty JK, Ziegler MM. Operative strategies for necrotizing enterocolitis: The

prevention and treatment of short-bowel syndrome. Semin Pediatr Surg 2005;14:191-8.
10. Hintz SR, Kendrick DE, Stoll BJ, et al. Neurodevelopmental and growth outcomes of extremely low birth weight infants after necrotizing enterocolitis. Pediatrics 2005;115:696-703.
11. Hsueh W, Caplan MS, Qu XW, Tan XD, De Plaen IG, Gonzalez-Crussi F. Neonatal necrotizing enterocolitis: clinical considerations and pathogenetic concepts. Pediatr Dev Pathol 2003;6:6-23.
12. Maayan-Metzger A, Itzchak A, Mazkereth R, Kuint J. Necrotizing enterocolitis in full-term infants: case-control study and review of the literature. J Perinatol 2004;24:494-9.
13. Lucas A, Cole TJ. Breast milk and neonatal necrotising enterocolitis. Lancet 1990;336:1519-23.
14. Schanler RJ, Shulman RJ, Lau C. Feeding strategies for premature infants: beneficial outcomes of feeding fortified human milk versus preterm formula. Pediatrics 1999;103:1150-7.
15. Sisk PM, Lovelady CA, Dillard RG, Gruber KJ, O'Shea TM. Early human milk feeding is associated with a lower risk of necrotizing enterocolitis in very low birth weight infants. J Perinatol 2007;27:428-33.
16. McGuire W, Anthony MY. Donor human milk versus formula for preventing necrotising enterocolitis in preterm infants: systematic review. Arch Dis Child Fetal Neonatal Ed 2003;88:F11-4.
17. Sullivan S, Schanler RJ, Kim JH, et al. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. J Pediatr 2010;156:562-7 e1.
18. Chirico G, Marzollo R, Cortinovis S, Fonte C, Gasparoni A. Antiinfective properties of human milk. J Nutr 2008;138:1801S-6S.
19. Field CJ. The immunological components of human milk and their effect on immune development in infants. J Nutr 2005;135:1-4.
20. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science 2001;291:881-4.
21. Neish AS, Gewirtz AT, Zeng H, et al. Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. Science 2000;289:1560-3.
22. Westerbeek EA, van den Berg A, Lafeber HN, Knol J, Fetter WP, van Elburg RM. The intestinal bacterial colonisation in preterm infants: a review of the literature. Clin Nutr 2006;25:361-8.
23. Stoll BJ, Gordon T, Korones SB, et al. Early-onset sepsis in very low birth weight neonates: a report from the National Institute of Child Health and Human Development Neonatal Research Network. J Pediatr 1996;129:72-80.
24. Claud EC, Walker WA. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. Faseb J 2001;15:1398-403.
25. WHO. Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. In; 2001.
26. Penna FJ, Peret LA, Vieira LQ, Nicoli JR. Probiotics and mucosal barrier in children. Curr Opin Clin Nutr Metab Care 2008;11:640-4.
27. Hammerman C, Kaplan M. Probiotics and neonatal intestinal infection. Curr Opin Infect Dis 2006;19:277-82.
28. Alfaleh K, Bassler D. Probiotics for prevention of necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev 2008:CD005496.
29. Deshpande G, Rao S, Patole S. Probiotics for prevention of necrotising enterocolitis in preterm neonates with very low birthweight: a systematic review

of randomised controlled trials. Lancet 2007;369:1614-20.
30. Alfaleh K, Anabrees J, Bassler D. Probiotics reduce the risk of necrotizing enterocolitis in preterm infants: a meta-analysis. Neonatology 2010;97:93-9.
31. Boehm G, Lidestri M, Casetta P, et al. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. Arch Dis Child Fetal Neonatal Ed 2002;86:F178-81.
32. Knol J, Boehm G, Lidestri M, et al. Increase of faecal bifidobacteria due to dietary oligosaccharides induces a reduction of clinically relevant pathogen germs in the faeces of formula-fed preterm infants. Acta Paediatr Suppl 2005;94:31-3.
33. Knol J, Scholtens P, Kafka C, et al. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. J Pediatr Gastroenterol Nutr 2005;40:36-42.
34. Boehm G, Stahl B, Jelinek J, Knol J, Miniello V, Moro GE. Prebiotic carbohydrates in human milk and formulas. Acta Paediatr Suppl 2005;94:18-21.
35. Sase M, Lee JJ, Park JY, Thakur A, Ross MG, Buchmiller-Crair TL. Ontogeny of fetal rabbit upper gastrointestinal motility. J Surg Res 2001;101:68-72.
36. Sase M, Miwa I, Sumie M, et al. Gastric emptying cycles in the human fetus. Am J Obstet Gynecol 2005;193:1000-4.
37. Sase M, Miwa I, Sumie M, Nakata M, Sugino N, Ross MG. Ontogeny of gastric emptying patterns in the human fetus. J Matern Fetal Neonatal Med 2005;17:213-7.
38. al Tawil Y, Berseth CL. Gestational and postnatal maturation of duodenal motor responses to intragastric feeding. J Pediatr 1996;129:374-81.
39. Berseth CL. Gut motility and the pathogenesis of necrotizing enterocolitis. Clin Perinatol 1994;21:263-70.
40. Berseth CL. Gastrointestinal motility in the neonate. Clin Perinatol 1996;23:179-90.
41. Lebenthal E, Lee PC, Heitlinger LA. Impact of development of the gastrointestinal tract on infant feeding. J Pediatr 1983;102:1-9.
42. Antonowicz I, Lebenthal E. Developmental pattern of small intestinal enterokinase and disaccharidase activities in the human fetus. Gastroenterology 1977;72:1299-303.
43. Van Klinken BJ, Tytgat KM, Buller HA, Einerhand AW, Dekker J. Biosynthesis of intestinal mucins: MUC1, MUC2, MUC3 and more. Biochem Soc Trans 1995;23:814-8.
44. Tytgat KM, Buller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J. Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology 1994;107:1352-63.
45. Specian RD, Oliver MG. Functional biology of intestinal goblet cells. Am J Physiol 1991;260:C183-93.
46. Hecht G. Innate mechanisms of epithelial host defense: spotlight on intestine. Am J Physiol 1999;277:C351-8.
47. Buisine MP, Devisme L, Savidge TC, et al. Mucin gene expression in human embryonic and fetal intestine. Gut 1998;43:519-24.
48. Anand RJ, Leaphart CL, Mollen KP, Hackam DJ. The role of the intestinal barrier in the pathogenesis of necrotizing enterocolitis. Shock 2007;27:124-33.
49. Gum JR, Jr., Hicks JW, Toribara NW, Rothe EM, Lagace RE, Kim YS. The human MUC2 intestinal mucin has cysteine-rich subdomains located both upstream and downstream of its central repetitive region. J Biol Chem 1992;267:21375-83.
50. Lien KA, Sauer WC, Fenton M. Mucin output in ileal digesta of pigs fed a protein-free diet. Z Ernahrungswiss 1997;36:182-90.

51. Mantle M, Allen A. Isolation and characterization of the native glycoprotein from pig small-intestinal mucus. Biochem J 1981;195:267-75.
52. Allen A, Hutton DA, Pearson JP. The MUC2 gene product: a human intestinal mucin. Int J Biochem Cell Biol 1998;30:797-801.
53. Toribara NW, Gum JR, Jr., Culhane PJ, et al. MUC-2 human small intestinal mucin gene structure. Repeated arrays and polymorphism. J Clin Invest 1991;88:1005-13.
54. Faure M, Mettraux C, Moennoz D, et al. Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. J Nutr 2006;136:1558-64.
55. Nichols NL, Bertolo RF. Luminal threonine concentration acutely affects intestinal mucosal protein and mucin synthesis in piglets. J Nutr 2008;138:1298-303.
56. Law GK, Bertolo RF, Adjiri-Awere A, Pencharz PB, Ball RO. Adequate oral threonine is critical for mucin production and gut function in neonatal piglets. Am J Physiol Gastrointest Liver Physiol 2007;292:G1293-301.
57. Faure M, Moennoz D, Montigon F, Mettraux C, Breuille D, Ballevre O. Dietary threonine restriction specifically reduces intestinal mucin synthesis in rats. J Nutr 2005;135:486-91.
58. Paneth J. Ueber die secernirenden Zellen des Dünndarm-Epithels. Arch Mikroskop Anat 1888:113— 91.
59. Erlandsen SL, Parsons JA, Taylor TD. Ultrastructural immunocytochemical localization of lysozyme in the Paneth cells of man. J Histochem Cytochem 1974;22:401-13.
60. Jones DE, Bevins CL. Paneth cells of the human small intestine express an antimicrobial peptide gene. J Biol Chem 1992;267:23216-25.
61. Jones DE, Bevins CL. Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel. FEBS Lett 1993;315:187-92.
62. Kiyohara H, Egami H, Shibata Y, Murata K, Ohshima S, Ogawa M. Light microscopic immunohistochemical analysis of the distribution of group II phospholipase A2 in human digestive organs. J Histochem Cytochem 1992;40:1659-64.
63. Salzman NH, Hung K, Haribhai D, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol 2010;11:76-83.
64. Salzman NH, Underwood MA, Bevins CL. Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. Semin Immunol 2007;19:70-83.
65. Salzman NH, Polin RA, Harris MC, et al. Enteric defensin expression in necrotizing enterocolitis. Pediatr Res 1998;44:20-6.
66. Nankervis CA, Giannone PJ, Reber KM. The neonatal intestinal vasculature: contributing factors to necrotizing enterocolitis. Semin Perinatol 2008;32:83-91.
67. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336 (Pt 1):1-17.
68. Becker RM, Wu G, Galanko JA, et al. Reduced serum amino acid concentrations in infants with necrotizing enterocolitis. J Pediatr 2000;137:785-93.
69. Amin HJ, Zamora SA, McMillan DD, et al. Arginine supplementation prevents necrotizing enterocolitis in the premature infant. J Pediatr 2002;140:425-31.

Chapter 2

Animal Models to Study Neonatal Nutrition in Humans

Patrycja Puiman

Barbara Stoll

Current Opinion in Clinical Nutrition and Metabolic Care 2008, 11:601–606

Abstract

Purpose of review

The impact of neonatal nutrition on the health status of the newborn and incidence of disease in later life is a topic of intense interest. Animal models are an invaluable tool to identify mechanisms that mediate the effect of nutrition on neonatal development and metabolic function. This review highlights recently developed animal models that are being used to study neonatal human nutrition.

Recent findings

In recent years, mice, rats, and pigs have become the most frequently used animal models to study human neonatal nutrition. Techniques for rearing newborn mice, preterm rats and preterm pigs have been developed. Neonatal mice have great potential for mechanistic and genomic research in postnatal nutrition and related diseases. The neonatal pig model is valuable to study acute and chronic effects of parenteral and enteral nutrition on whole-body metabolism as well as specific tissues. To date, a wealth of information from studies with neonatal pigs has been applied to humans.

Summary

Further development of neonatal animal models related to nutrition is required for the advancement of research in early postnatal nutrition. Improvement of nutritional support during this critical period of development will enhance immediate clinical outcomes and possibly prevent diseases later in life.

Introduction

Le choix intelligent d'un animal…est souvent la condition essentielle du succèss d'une expérience et de la solution d'un problème physiologique très important.
(The success of the enterprise clearly depends upon selecting a suitable animal for the investigation)
(Claude Bernard, 1865)

The study of nutritional influences on neonatal growth and functional development are important because of the critical stage of development and potentially long-lasting impact of intervention. Factors that limit the clinical investigation of human neonates besides the obvious ethical constraints include small sample size, methodological difficulties and genetic heterogeneity as well as differences in disease and (intensive care) treatment. Therefore, animal models are an invaluable tool to study physiologic, metabolic and cellular events related to early nutrition in both neonatal and later life under highly controlled circumstances. Studies of neonatal animals have led to the improvement of nutritional intervention and advanced care of the human neonate. However, limitation of in vivo animal experimentation lies in the observation that embryology, physiology and anatomy in animals do not completely resemble that in humans and that frank disease is not easily reproducible. Hence, the purpose of this review is to describe neonatal animal models suitable for studying early postnatal nutrition and to provide guidance for investigators who are new to this field of research.

Neonatal animal models

Animal models used for human neonatal nutrition include mice, rats, rabbits, guinea pigs, dogs, pigs and nonhuman primates. Traditionally, studies in this field have been dominated by rodent species like the rat and the mouse and to a lesser extend the rabbit, guinea pig, pig, and baboon. The latter species, i.e. nonhuman primates are a good model to study postnatal nutrition in humans because of their close homology in several organ systems compared to humans. They have been used to study short and long-term effects of pre- and postnatal (parenteral) nutrition in term as well as preterm neonates, but expensive housing, lifespan and ethical considerations limit their use[1-8]. In recent years, techniques to artificially rear rodents and methods to investigate the effects of neonatal pig nutrition in health and disease have evolved. At present, rodents and pigs are widely used to study human neonatal nutrition and therefore will be the focus of this review.

Neonatal rodent models

In the current state of neonatal intensive care, with increasing numbers of premature babies and their rates of survival, neonatal nutrition has to be tailored to specific newborn populations differentiated by their gestational age and health status. To prevent nutritional deficits in this critical developmental period and because neonatal nutrition may determine health in later life, it is important to have appropriate animal models to study the underlying mechanisms involved[9**]. Although rodents differ from humans in many ways including developmental, anatomical and physiological characteristics they are highly related in terms of similarity of genes and biochemical pathways. Rodent models have the advantage of a relatively uniform genetic background on which environmental effects during gestation or early postnatal life into adulthood can be studied. Low cost and advanced breeding programs in rodents, and especially mice, allow studies that enable the investigation of mechanistic pathways in genetically modified animals. The length of gestation for rodents, like the rat and mouse, is short (19–22 d), and the pups are born very immature with respect to the stage of gut and brain development. In newborn pups the gut matures gradually during lactation (0-21 d) and a particularly rapid maturation takes place during the short period of transition from milk to solid food (weaning) [9**,10] (Fig.). This maturation includes anatomical, functional and immunological aspects of the gut. Considering this developmental pattern, rodents represent animal species that are quite different from human infants whose intestinal

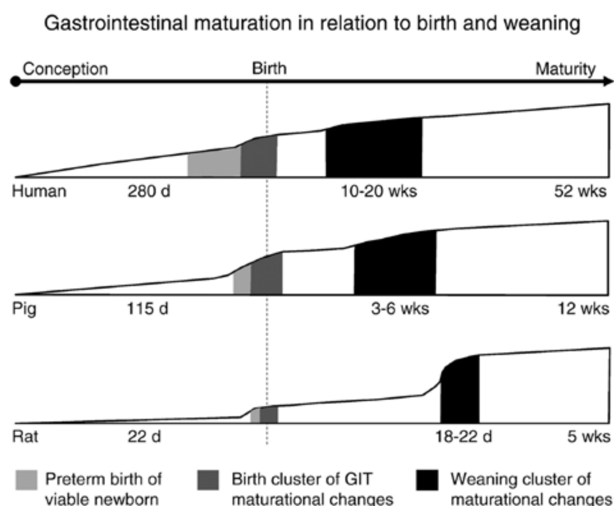


Figure. Timing of gut maturation in three different groups of mammalian species[9].
In humans and other primates, gastrointestinal development is slow and maturation starts early (in fetal life). Gut maturation in pigs is intermediate (i.e., maturation is rapid during the period from shortly before birth to shortly after weaning). In most small rodents and carnivorous species, the developmental changes occur relatively quickly and late (postnatally around weaning). Around birth (dark grey areas) and weaning (black areas), maturation is particularly rapid, resulting in a birth and weaning cluster of maturational changes. Birth of viable preterm neonates occurs over a wider range of gestational ages in humans compared to pigs and rats (light grey areas).

tract is more mature at birth[9**] (Fig.). Therefore, the immature rodent gut resembles more that of a human infant born prematurely. Furthermore, the rapid development of the intestinal tract in rodents has the advantage that nutritional effects can be studied within a relatively short time.

The goal in the clinical management of premature and low birth weight infants is to achieve growth at a rate that approximates intra-uterine growth; however cumulative deficits in energy and protein intakes are still a concern in many neonatal intensive care settings. Limited early nutrition as a result of delayed adequate nutritional support and the inability to meet the high metabolic demand leads to extra-uterine growth restriction. This may have devastating short-term consequences, such as increased susceptibility to infection and lack of organ growth, as well as poor neurodevelopmental outcome[11]. Since the seminal studies in newborn animals of Widdowson[10] and McCance[11], it has become clear that nutrition in early life affects postnatal growth and development. This work applied a straightforward approach to investigate the effects of over- and undernutrition on growth and development by manipulating litter size, and is still used today[12]. However, precise control of volume and nutrient intake can only be obtained by artificial rearing. To date, most studies using artificial rearing to investigate nutrient intake and specific effects on biochemical mechanisms have been performed in rats. One of the most effective approaches is the so-called ‘pup in a cup’ model, which has been used more extensively in recent nutritional neonatal research. This model, first described by Hall[13], has recently been applied to mouse pups and is a useful adaptation given the greater availability of transgenic and knockout mice[14**]. Mouse pups for this model are taken from the dam at postnatal day 5 and put individually in Styrofoam cups that float in a temperature-controlled water bath, hence the name “pup in a cup”. Subsequently, intragastric feeding tubes are placed into the pups that allow for regular infusion of rodent milk substitute (RMS). Recently, a hand-feeding technique using a surrogate nipple for artificial rearing of mouse pups has been developed[15]. Although more time consuming, this technique enables studying pups shortly after birth and prevents physical injury. Furthermore, hand feeding permits the use of a nursing box housing multiple pups, and mimics natural feeding, that both stimulates physical activity and reduces stress. Newly developed RMS formulas accomplished a comparable weight gain between artificially reared mouse pups and breastfed littermates, although differences in organ weight, precocious gut maturation, and altered immune development have been observed[14,16-18].
In our opinion, artificial rearing of mouse pups provides a means to study the effects of specific nutrients, food composition and energy intake on gut development and metabolism within the relatively immature gut. Furthermore, rearing mice from birth onwards offers the opportunity to investigate whether early nutrition can change expression of genes and alter epigenetic markings. So far, only maternal nutrition has

shown to induce epigenetic changes in offspring and it is unknown if early neonatal nutrition can introduce or modify maternal epigenetic alterations[19,20].

Neonatal piglet model

Compared to rodents, the neonatal pig has more similarities in anatomy, physiology, immunology, and metabolism with the human neonate. The piglet has a gestational length of ~115 days and, compared to humans is slightly less mature at birth in several aspects including digestive system, and body composition (low fat content). However, during the neonatal period protein deposition is very rapid, and due to similarities of postnatal nutrition and intestinal development to humans, the piglet can be viewed as an accelerated model of postnatal growth and development[9**]. As breast-feeding is initiated and maintained, the intestine continues to develop and adapt to enteral food and bacterial colonization. The changes are primarily reflected in rapid intestinal growth and changes in functional parameters like digestive enzyme activity, nutrient absorption and immune function. Later on, gut development is more gradual than around birth and probably reflects the slow transition from milk-based nutrition towards solid food. The pig is also appropriate for modeling liver function and metabolism as it has similar hepatic features as humans and, unlike rodents, the presence of a gallbladder. More importantly, however, due to its body size the piglet model allows extensive surgical manipulation, repeated blood sampling, and long-term dietary treatment protocols[21-24].

The piglet model is a long established model used for both enteral and parenteral nutritional studies. We and others have used this model combined with stable isotopic tracer techniques to investigate postnatal intestinal, splanchnic and whole body nutrient metabolism[25-28,29**,30]. The indicator amino acid oxidation method (IAAO), initially developed to measure amino acid requirements in growing pigs also has been applied to enterally and parenterally fed piglets[31*]. Importantly, these studies have provided a wealth of information that formed the conceptual basis for similar studies to be conducted in neonates, children and adults[29**,31**,32-38] (Table). A series of studies in neonatal pigs investigated the mechanisms by which feeding stimulates neonatal protein synthesis, a response that decreases with development, particularly in skeletal muscle. These studies led to the development of the hyperinsulinemic-euglycemic-euaminoacidemic clamp technique to examine the role of insulin in the regulation of protein synthesis, independent of changes in circulating amino acids and glucose[39,40*]. The piglet also has been proven to be a useful representation of the human neonate when studying lipid nutrition[41,42] including the effect of long-chain n-3 polyunsaturated fatty acids on protein metabolism in the neonate during growth[43*].
In human neonates, total parenteral nutrition (TPN) is a lifesaving therapy when enteral nutrition cannot be provided. Besides the pig, neonatal rabbits[44,45], guinea pigs[46,47], and

Table. Splanchnic utilization rates of dietary amino acids and glucose as percentage of intake in piglets, human neonates and human adults[29, 33, 36].

Amino Acid	Piglets	Neonates	Adults
Leucine	42	42-48	21
Phenylalanine	51	-	29
Lysine	43	18	32
Methionine	39	-	33
Threonine	71	70	-
Glutamine	-	53	64
Glutamate	92	75	96
Glucose	40	32	-

dogs[48] have been used to study effects of TPN. However, the prominence of the piglet in studies concerning multiple aspects of human neonatal nutrition reflects the thought that the pig is most similar to humans compared to other animals and therefore the preferred model. The TPN model has been applied to study specific effects of TPN on intestinal growth, blood flow, digestion, absorptive function, epithelial integrity and gut barrier function[49-52]. TPN-associated liver injury in piglets resembled that seen in human neonates[53,54*]. These studies suggest that TPN-induced hepatic steatosis is influenced by the source of lipid used[55]. Whether TPN-administration during the neonatal period affects later health is unknown. Recently, development of insulin resistance and diabetes in adult life has been linked to not only being small for gestational age, but also prematurity alone[56*,57]. The mode of nutrition in this critical window of development may be an underlying factor of this phenomenon. In this respect, TPN in early postnatal life might have a role in metabolic programming. In support of this idea, in our current studies TPN induced insulin resistance, hepatic steatosis, and greater fat deposition in TPN-fed compared to formula-fed piglets[58].

Most recently, the piglet model has been advanced to the delivery of viable premature piglets that can be studied using enteral and parenteral feeding protocols[59,60]. In comparison to mammals with a long gestation, preterm birth of viable offspring is possible only with a maximum of 10-12 days reduction in pigs (10%) and 1-2 days for rodents (Fig.). Considering their overall immaturity at full-term birth, preterm delivery of piglets at 90% of gestation translates into a relatively more premature animal. Preterm piglets have physiological similarities with human preterm infants and thus, are suitable for studying preterm gut function, effects of parenteral and enteral nutrition and immunity using novel in vivo experimental approaches.

Rodent and piglet models related to necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is the most common gastro-intestinal disease in premature infants and is associated with a high rate of morbidity and mortality. The exact

etiology is unknown, but major risk factors include intestinal immaturity, enteral feeding and bacterial colonization. Most NEC models are neonatal rat models in which intestinal injury is induced by (over)feeding with formula, induction of pathogenic bacteria or endotoxins (LPS), or exposure to stress via hypoxia and/or hypothermia[61-67]. More recently these experimental approaches have been extended to mouse models of NEC that have examined the impact of specific gene products in neonatal NEC, namely toll-like receptor 4[68,69*]. Asphyxiated rats and piglets have been studied for the role of hemodynamic and vascular changes in the intestine that could lead to NEC[70-71]. However, asphyxia is not believed to be the primary cause in the development of NEC. Studies performed in a new premature piglet NEC model, solely based on prematurity and formula feeding, have shown to mimic pathological changes in the gut similar to that observed in human infants[72**]. This model will enable the use of more invasive experimental approaches to investigate the role of parenteral nutrition, blood flow, and digestive capacity for specific nutrients.

Conclusion

In conclusion, neonatal animal models are an effective and valuable tool in understanding the impact of nutrition administered early in life on short- and long-term functional development and metabolism. Rodents have the advantage of their low cost and provide a mean of using genetically modified animals to study mechanistic pathways. Using (preterm) piglets as a neonatal model favors translation to the human neonate with respect to gut development, nutritional requirements and neonatal disease. In general, careful choice of an animal model is critical in the design of any study attempting to answer relevant questions.

References

1. Motta GE, Jackson EM, Klein ML, et al. Programming of initial steps in bile acid synthesis by breast-feeding vs. formula-feeding in the baboon. Lipids. 2003 Dec;38(12):1213-20.
2. Stabler SP, Morton RL, Winski SL, et al. Effects of parenteral cysteine and glutathione feeding in a baboon model of severe prematurity. Am J Clin Nutr. 2000 Dec;72(6):1548-57.
3. Golub MS, Hogrefe CE, Germann SL. Iron deprivation during fetal development changes the behavior of juvenile rhesus monkeys. J Nutr. 2007 Apr;137(4):979-84.
4. Diau GY, Hsieh AT, Sarkadi-Nagy EA, et al. The influence of long chain polyunsaturate supplementation on docosahexaenoic acid and arachidonic acid in baboon neonate central nervous system. BMC Med. 2005;3:11.
5. Hsieh AT, Anthony JC, Diersen-Schade DA, et al. Biochemical and white blood cell profiles of baboon neonates consuming formulas with moderate and high

dietary long-chain polyunsaturated fatty acids. J Med Primatol. 2008 Apr;37(2):81-7.
6. Hsieh AT, Anthony JC, Diersen-Schade DA, et al. The influence of moderate and high dietary long chain polyunsaturated fatty acids (LCPUFA) on baboon neonate tissue fatty acids. Pediatr Res. 2007 May;61(5 Pt 1):537-45.
7. Kothapalli KS, Anthony JC, Pan BS, et al. Differential cerebral cortex transcriptomes of baboon neonates consuming moderate and high docosahexaenoic acid formulas. PLoS ONE. 2007;2(4):e370.
8. Sarkadi-Nagy E, Wijendran V, Diau GY, et al. Formula feeding potentiates docosahexaenoic and arachidonic acid biosynthesis in term and preterm baboon neonates. J Lipid Res. 2004 Jan;45(1):71-80.
9. ** Sangild PT. Gut responses to enteral nutrition in preterm infants and animals. Exp Biol Med (Maywood). 2006 Dec;231(11):1695-711.
   This review describes in detail developmental similarities with preterm infants that make the pig an attractive animal model to study neonatal nutrition and clinical complications related to preterm birth.
10. Widdowson EM. Food intake and growth in the newly-born. Proc Nutr Soc. 1971 Sep;30(2):127-35.
11. McCance RA. Food, growth, and time. Lancet. 1962 Oct 6;2(7258):671-6.
12. Xiao XQ, Williams SM, Grayson BE, et al. Excess weight gain during the early postnatal period is associated with permanent reprogramming of brown adipose tissue adaptive thermogenesis. Endocrinology. 2007 Sep;148(9):4150-9.
13. Hall WG. Weaning and growth of artificially reared rats. Science. 1975 Dec 26;190(4221):1313-5.
14. ** Beierle EA, Chen MK, Hartwich JE, et al. Artificial rearing of mouse pups: development of a mouse pup in a cup model. Pediatr Res. 2004 Aug;56(2):250-5.
   This is the first report of artificially rearing mouse pups. This article describes in detail accompanied by illustrations the development of this technique.
15. Hoshiba J. Method for hand-feeding mouse pups with nursing bottles. Contemp Top Lab Anim Sci. 2004 May;43(3):50-3.
16. Yajima M, Hoshiba J, Terahara M, Yajima T. Reduced thymic size and numbers of splenic CD4+ and CD8+ cells in artificially reared mouse pups. Biosci Biotechnol Biochem. 2007 Oct;71(10):2420-7.
17. Dvorak B, McWilliam DL, Williams CS, et al. Artificial formula induces precocious maturation of the small intestine of artificially reared suckling rats. J Pediatr Gastroenterol Nutr. 2000 Aug;31(2):162-9.
18. Yajima M, Kanno T, Yajima T. A chemically derived milk substitute that is compatible with mouse milk for artificial rearing of mouse pups. Exp Anim. 2006 Jul;55(4):391-7.
19. Waterland RA. Epigenetic mechanisms and gastrointestinal development. J Pediatr. 2006 Nov;149(5 Suppl):S137-42.
20. Mathers JC. Early nutrition: impact on epigenetics. Forum Nutr. 2007;60:42-8.
21. Moughan PJ, Birtles MJ, Cranwell PD, et al. The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. World Rev Nutr Diet. 1992;67:40-113.
22. Stoll B, Burrin DG, Henry J, et al. Phenylalanine utilization by the gut and liver measured with intravenous and intragastric tracers in pigs. Am J Physiol. 1997 Dec;273(6 Pt 1):G1208-17.
23. Wykes LJ, Ball RO, Pencharz PB. Development and validation of a total parenteral nutrition model in the neonatal piglet. J Nutr. 1993 Jul;123(7):1248-59.
24. Shulman RJ. The piglet can be used to study the effects of parenteral and enteral

nutrition on body composition. J Nutr. 1993 Feb;123(2 Suppl):395-8.
25. Burrin DG, Stoll B. Key nutrients and growth factors for the neonatal gastrointestinal tract. Clin Perinatol. 2002 Mar;29(1):65-96.
26. Stoll B, Burrin DG. Measuring splanchnic amino acid metabolism in vivo using stable isotopic tracers. J Anim Sci. 2006 Apr;84 Suppl:E60-72.
27. Riedijk MA, Stoll B, Chacko S, et al. Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract. Proc Natl Acad Sci U S A. 2007 Feb 27;104(9):3408-13.
28. Urschel KL, Rafii M, Pencharz PB, Ball RO. A multitracer stable isotope quantification of the effects of arginine intake on whole body arginine metabolism in neonatal piglets. Am J Physiol Endocrinol Metab. 2007 Sep;293(3):E811-8.
29. ** Riedijk MA, van Goudoever JB. Splanchnic metabolism of ingested amino acids in neonates. Curr Opin Clin Nutr Metab Care. 2007 Jan;10(1):58-62.
This comprehensive review describes the application of the piglet model combined with stable isotope technique to human term and preterm neonates to investigate gastrointestinal amino acid metabolism.
30. Stoll B, Henry J, Reeds PJ, et al. Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. J Nutr. 1998 Mar;128(3):606-14.
31. ** Elango R, Ball RO, Pencharz PB. Indicator amino acid oxidation: concept and application. J Nutr. 2008 Feb;138(2):243-6.
This review describes the indicator amino acid oxidation (IAAO) method that has been applied in piglets and humans to measure amino acid requirements during enteral and parenteral nutrition.
32. van Goudoever JB, van der Schoor SR, Stoll B, et al. Intestinal amino acid metabolism in neonates. Nestle Nutr Workshop Ser Pediatr Program. 2006;58:95-102; discussion -8.
33. Riedijk MA, de Gast-Bakker DA, Wattimena JL, van Goudoever JB. Splanchnic oxidation is the major metabolic fate of dietary glutamate in enterally fed preterm infants. Pediatr Res. 2007 Oct;62(4):468-73.
34. Riedijk MA, van Beek RH, Voortman G, et al. Cysteine: a conditionally essential amino acid in low-birth-weight preterm infants? Am J Clin Nutr. 2007 Oct;86(4):1120-5.
35. Riedijk MA, Voortman G, van Beek RH, et al. Cyst(e)ine requirements in enterally fed very low birth weight preterm infants. Pediatrics. 2008 Mar;121(3):e561-7.
36. van der Schoor SR, Wattimena DL, Huijmans J, et al. The gut takes nearly all: threonine kinetics in infants. Am J Clin Nutr. 2007 Oct;86(4):1132-8.
37. Elango R, Ball RO, Pencharz PB. Individual amino acid requirements in humans: an update. Curr Opin Clin Nutr Metab Care. 2008 Jan;11(1):34-9.
38. Ito Y, Doelle SM, Clark JA, et al. Intestinal microcirculatory dysfunction during the development of experimental necrotizing enterocolitis. Pediatr Res. 2007 Feb;61(2):180-4.
39. Burrin DG, Davis TA. Proteins and amino acids in enteral nutrition. Curr Opin Clin Nutr Metab Care. 2004 Jan;7(1):79-87.
40. Davis TA, Suryawan A, Orellana RA, et al. Postnatal ontogeny of skeletal muscle protein synthesis in pigs. J Anim Sci. 2008 Apr;86(14 Suppl):E13-8.
41. Corl BA, Mathews Oliver SA, Lin X, et al. Conjugated linoleic acid reduces body fat accretion and lipogenic gene expression in neonatal pigs fed low- or high-fat formulas. J Nutr. 2008 Mar;138(3):449-54.
42. Odle J. New insights into the utilization of medium-chain triglycerides by the neo-

nate: observations from a piglet model. J Nutr. 1997 Jun;127(6):1061-7.
43. * Bergeron K, Julien P, Davis TA, et al. Long-chain n-3 fatty acids enhance neonatal insulin-regulated protein metabolism in piglets by differentially altering muscle lipid composition. J Lipid Res. 2007 Nov;48(11):2396-410.
The findings of this study provide new evidence of the potential role of LCn-3PUFAs in the regulation of protein metabolism and insulin sensitivity in the neonate.
44. Hata S, Kamata S, Nezu R, et al. A newborn rabbit model for total parenteral nutrition: effects of nutritional components on cholestasis. JPEN J Parenter Enteral Nutr. 1989 May-Jun;13(3):265-71.
45. Yamanouchi T, Suita S, Masumoto K. Non-protein energy overloading induces bacterial translocation during total parenteral nutrition in newborn rabbits. Nutrition. 1998 May;14(5):443-7.
46. Chessex P, Friel J, Harrison A, et al. The mode of delivery of parenteral multivitamins influences nutrient handling in an animal model of total parenteral nutrition. Clin Nutr. 2005 Apr;24(2):281-7.
47. Lavoie JC, Chessex P, Rouleau T, et al. Shielding parenteral multivitamins from light increases vitamin A and E concentration in lung of newborn guinea pigs. Clin Nutr. 2007 Jun;26(3):341-7.
48. Owens L, Burrin DG, Berseth CL. Minimal enteral feeding induces maturation of intestinal motor function but not mucosal growth in neonatal dogs. J Nutr. 2002 Sep;132(9):2717-22.
49. Kansagra K, Stoll B, Rognerud C, et al. Total parenteral nutrition adversely affects gut barrier function in neonatal piglets. Am J Physiol Gastrointest Liver Physiol. 2003 Dec;285(6):G1162-70.
50. Niinikoski H, Stoll B, Guan X, et al. Onset of small intestinal atrophy is associated with reduced intestinal blood flow in TPN-fed neonatal piglets. J Nutr. 2004 Jun;134(6):1467-74.
51. Burrin DG, Stoll B, Chang X, et al. Parenteral nutrition results in impaired lactose digestion and hexose absorption when enteral feeding is initiated in infant pigs. Am J Clin Nutr. 2003 Sep;78(3):461-70.
52. Burrin DG, Stoll B, Jiang R, et al. Minimal enteral nutrient requirements for intestinal growth in neonatal piglets: how much is enough? Am J Clin Nutr. 2000 Jun;71(6):1603-10.
53. Zambrano E, El-Hennawy M, Ehrenkranz RA, et al. Total parenteral nutrition induced liver pathology: an autopsy series of 24 newborn cases. Pediatr Dev Pathol. 2004 Sep-Oct;7(5):425-32.
54. * Wang H, Khaoustov VI, Krishnan B, Cai W, Stoll B, Burrin DG, et al. Total parenteral nutrition induces liver steatosis and apoptosis in neonatal piglets. J Nutr. 2006 Oct;136(10):2547-52.
This article shows that TPN-induced steatosis in newborn piglets may serve as a novel animal model to assess the pathogenesis of fatty liver and apoptosis-mediated liver injury in infants
55. Hyde MJ, Amusquivar E, Laws J, et al. Effects of lipid-supplemented total parenteral nutrition on fatty liver disease in a premature neonatal piglet model. Neonatology. 2008;93(2):77-86.
56. ** Hofman PL, Regan F, Cutfield WS. Prematurity--another example of perinatal metabolic programming? Horm Res. 2006;66(1):33-9.
This mini review presents data demonstrating that premature infants have reduced insulin sensitivity similar to those observed in term, small for gestational age children. The authors speculate that this metabolic perturbation occurs during the neonatal period and might be a result of nutritional and other perinatal factors.
57. Hovi P, Andersson S, Eriksson JG, et al. Glucose regulation in young adults with

very low birth weight. N Engl J Med. 2007 May 17;356(20):2053-63.

58. Horst D, Sedenquist M, Stoll B, et al. Chronic Parenteral Nutrition Reduces Lean Tissue Growth and Induces Insulin Resistance and
Hepatic Steatosis in Neonatal Piglets. Journal of parenteral and enteral nutrition. 2007;31(2):1.
59. Sangild PT, Petersen YM, Schmidt M, et al. Preterm birth affects the intestinal response to parenteral and enteral nutrition in newborn pigs. J Nutr. 2002 Dec;132(12):3786-94.
60. Oste M, Van Ginneken CJ, Van Haver ER, et al. The intestinal trophic response to enteral food is reduced in parenterally fed preterm pigs and is associated with more nitrergic neurons. J Nutr. 2005 Nov;135(11):2657-63.
61. Ergun O, Ergun G, Oktem G, et al. Enteral resveratrol supplementation attenuates intestinal epithelial inducible nitric oxide synthase activity and mucosal damage in experimental necrotizing enterocolitis. J Pediatr Surg. 2007 Oct;42(10):1687-94.
62. Halpern MD, Holubec H, Saunders TA, et al. Bile acids induce ileal damage during experimental necrotizing enterocolitis. Gastroenterology. 2006 Feb;130(2):359-72.
63. Kelly N, Friend K, Boyle P, et al. The role of the glutathione antioxidant system in gut barrier failure in a rodent model of experimental necrotizing enterocolitis. Surgery. 2004 Sep;136(3):557-66.
64. Lu J, Jilling T, Li D, Caplan MS. Polyunsaturated fatty acid supplementation alters proinflammatory gene expression and reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Pediatr Res. 2007 Apr;61(4):427-32.
65. Zani A, Eaton S, Leon FF, et al. Captopril reduces the severity of bowel damage in a neonatal rat model of necrotizing enterocolitis. J Pediatr Surg. 2008 Feb;43(2):308-14.
66. Halpern MD, Dvorak B. Does Abnormal Bile Acid Metabolism Contribute to NEC? Semin Perinatol. 2008 Apr;32(2):114-21.
67. Crissinger KD. Animal models of necrotizing enterocolitis. J Pediatr Gastroenterol Nutr. 1995 Jan;20(1):17-22.
68. Jilling T, Simon D, Lu J, et al. The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. J Immunol. 2006 Sep 1;177(5):3273-82.
69. * Halpern MD, Khailova L, Molla-Hosseini D, et al. Decreased development of necrotizing enterocolitis in IL-18-deficient mice. Am J Physiol Gastrointest Liver Physiol. 2008 Jan;294(1):G20-6.
This article shows the use of a genetically modified neonatal mouse model for investigating mechanisms involved in the development of necrotizing enterocilitis.
70. Ewer AK, Al-Salti W, Coney AM, et al. The role of platelet activating factor in a neonatal piglet model of necrotising enterocolitis. Gut. 2004 Feb;53(2):207-13.
71. Gellen B, Kovacs J, Nemeth L, et al. Vascular changes play a role in the pathogenesis of necrotizing enterocolitis in asphyxiated newborn pigs. Pediatr Surg Int. 2003 Jul;19(5):380-4.
72. ** Sangild PT, Siggers RH, Schmidt M, et al. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. Gastroenterology. 2006 May;130(6):1776-92.
This publication shows that the premature piglet is a unique model to study the development of necrotizing enterocolitis and investigates potential underlying mechanisms.

Chapter 3

Dietary influence on colitis-development in Muc2-deficient mice: *diet matters!*

Nanda Burger-van Paassen
Patrycja J. Puiman
Peng Lu
Nicolas Le Polles
Janneke Bouma
Anita M. Korteland-van Male
Günther Boehm
Johannes B. van Goudoever
Ingrid B. Renes

Manuscript in preparation

Abstract

Background: Muc2 knockout ($Muc2^{-/-}$) mice do not have a protective mucus layer and spontaneously develop colitis.

Objective: To study the effects of a purified diet and probiotic supplementation on growth and disease severity in $Muc2^{-/-}$ mice.

Methods: $Muc2^{-/-}$ and wildtype (WT) mice were fed a non-purified (NP, i.e., standard chow) diet, a NP-diet with daily administration of *Bifidobacterium* breve and *Bifidobacterium animalis subsp*. *lactis* (NP+PRO), or a purified (P) diet during 5 weeks, starting directly after weaning. The purified diet contains 60% less fibers, and milk-casein proteins instead of plant proteins. Clinical symptoms and colonic morphological changes were monitored. Inflammation was studied by immunohistochemistry and determination of cytokine gene expression.

Results: In $Muc2^{-/-}$ mice, the P-diet significantly increased bodyweight compared to the NP and NP+PRO diet. Bodyweights of $Muc2^{-/-}$ mice fed P-diet were similar to WT mice. Crypt length was increased in $Muc2^{-/-}$ mice compared to WT mice regardless the type of diet. In $Muc2^{-/-}$ mice the P diet limited the increase in crypt length compared to NP or NP+PRO diet. In $Muc2^{-/-}$ mice the NP+PRO diet reduced the crypt lengthening compared to NP diet. $Muc2^{-/-}$ mice that were fed the P-diet showed a limited influx of Cd3ε-positive T cells, increased expression of Ebi3 and Il12p35, which as protein dimer is known as the immune suppressive cytokine Il35, decreased abundance of S100a8 and S100a9-positive cells and increased abundance of Muc4-positive cells compared to $Muc2^{-/-}$ mice fed NP or NP+PRO diet.

Conclusions: Type of diet and, to a lesser extent, probiotic supplementation affect colitis severity in $Muc2^{-/-}$ mice. The type of protein and amount of insoluble fibers modulate disease activity in mice prone to develop colitis. Moreover, probiotics might have beneficial effects in $Muc2^{-/-}$ mice as NP+PRO diet reduced crypt lengthening compared to NP diet. Together, these data imply that feeding strategy in subjects with colitis might have considerable implications for disease severity.

Introduction

Innate defense in the gut consists of several components that together prevent bacteria and other micro-organisms from invasion into the intestinal epithelium. Mucus produced by goblet cells is a key component of the physical barrier that covers the intestinal epithelium. The structural component of this mucus layer is the mucin MUC2. We previously showed that deficiency of Muc2, as in Muc2 knockout (Muc2$^{-/-}$) mice, leads to the development of clinical and histological signs of colitis[1]. As the synthesis of the mucin MUC2 is decreased in human intestinal diseases such as ulcerative colitis (UC)[2-4] and necrotizing enterocolitis (NEC)[5-6], the Muc2$^{-/-}$ mouse model is a powerful tool, enabling us to study the physiologic role of the epithelial barrier in many aspects.
Nutrition plays an important role in human inflammatory bowel disease (IBD), *i.e.* enteral nutrition is an effective therapy for the induction of clinical remission in adult Crohn's disease (CD) and is the primary treatment for pediatric CD[7-8]. The exact mechanisms through which enteral nutrition exert these beneficial effects is unknown, but most likely consists of direct anti-inflammatory effects on enterocytes, a suppressive effect on mucosal inflammatory cytokine levels, promotion of the integrity of the epithelium and modulation of the intestinal microbiota[9-14]. NEC is an acquired intestinal disease that predominantly occurs in premature infants. It is known by a very high morbidity and mortality and currently only supportive therapy is available. Besides prematurity, enteral feeding, more specifically formula feeding versus human milk feeding, is a risk factor for the development of NEC[15-16]. Although NEC probably develops due to a combination of risk factors, dietary interventions, such as provision of human milk or supplementation of probiotics, have been suggested for NEC prevention[16,20] an important role in disease prevention.
One of the big advantages of nutritional therapy is the relatively low cost and minimal risk of side effects. Therefore nutritional therapy forms an interesting target for prevention of NEC and treatment in IBD. Probiotics are described as 'live microbial dietary supplements which beneficially affect the host animal by improving its intestinal microbial balance[17]. Although data concerning the effect of probiotics in IBD are conflicting[18-19], enteral supplementation of probiotics significantly reduced the risk of severe NEC in preterm infants[20]. Moreover, colitis was attenuated or prevented in a variety of experimental colitis models that were treated with probiotics[21-22].
The phenotype of Muc2$^{-/-}$ mice is variable and might depend on the genotypic background, a phenomenon that was also described for IL-10–deficient mice[23]. More specifically, Velcich et al. used Muc2$^{-/-}$ mice on a mixed genotypic background (C57/Bl6-129Sv), which hardly displayed intestinal inflammation, whereas we used Muc2$^{-/-}$ mice on a 129Sv background that showed severe colitis[1, 24]. However, differences in the diet might also be responsible as animals that developed colitis were fed a standard,

non-purified rodent diet, whereas animals that did not show a clear colitis phenotype were fed a purified, AIN-based diet (AIN-76A).
In the present study, we investigated the effect of a purified diet, i.e. semi synthetic AIN based diet, relative to a non-purified diet, i.e. standard rodent chow, and supplementation with probiotics on growth and disease severity in Muc2$^{-/-}$ mice. We hypothesized that a purified diet or supplementation with probiotics would decrease disease severity in Muc2$^{-/-}$ mice. Besides clinical disease markers, we studied colonic inflammation markers, serum cytokine profiles and cytokine gene expression profiles in colonic tissue. Together these studies indicate that disease severity is affected by the type of diet.

Material and methods

Animals

Wild type (WT) and Muc2$^{-/-}$ mice were bred as previously described[1]. All mice were generated from Muc2$^{+/-}$ breedings. Mice were housed in the same specific pathogen–free environment in a 12-hour light/dark cycle with free access to acidified tap water. Animal care and procedures were conducted according to institutional guidelines (Erasmus MC Animal Ethics Committee, Rotterdam, the Netherlands). Mice were maintained in a barrier facility. Wild-type and Muc2$^{-/-}$ mice were tested negative for *Helicobacter hepaticus* and norovirus infection.

Experimental Setup

The experiment was divided into three groups. Group 1 received a non-purified (NP) diet, consisting of standard rodent pellets (Special Diets Services, Witham, Essex, England), group 2 received a purified (P), semi-synthetic diet (AIN93G pellets, Research Diet Services BV, Wijk bij Duurstede, the Netherlands) and group 3 received the NP diet supplemented with probiotics (NP+PRO). Animals were weaned from mother's milk at the age of approximately 21 days. Supplementation of probiotics was started immediately thereafter. The probiotic mixture consisted of two probiotic strains: *Bifidobacterium breve* and *Bifidobacterium animalis subsp. lactis* (Danone Research, Wageningen, the Netherlands) in a final concentration of $1x10^9$CFU/animal/day. The probiotic freeze-dried powder was dissolved in NaCl 0,9%. Finally, the mixture was dissolved in sterilized Dutch custard (Stabilac, Campina, the Netherlands). The control suspension existed of maltodextrin dissolved according to the same method as the probiotic suspension. A daily amount of 200µl per animal was inserted into a custom made spoon. To guarantee the administration of a well-defined amount of probiotics, animals were separated by a cage divider during the consumption of the probiotic suspension. Average consumption time was less than 15 minutes, after which the aforementioned

cage divider was removed. Body weight, dietary intake and clinical symptoms were determined thrice weekly. Animals were sacrificed at the age of 8 weeks. Colonic tissue samples were excised immediately and either fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS), stored in RNAlater (Qiagen, Venlo, The Netherlands), at –20°C, or frozen in liquid nitrogen and stored at –80°C.
The animals in the three experimental groups will be referred to as WT NP and $Muc2^{-/-}$ NP for animals that were fed the NP diet, WT P and $Muc2^{-/-}$ P for animals that were fed the P diet and WT NP+PRO and $Muc2^{-/-}$ NP+PRO for animals that were fed the NP diet supplemented with probiotics.

Histology

Tissue fixed in 4% (wt/vol) paraformaldehyde in PBS was prepared for light microscopy, and 4-μm-thick sections were stained with H&E. To detect differences in crypt length in the colon, 10 well-oriented crypts were chosen per intestinal segment and measured using calibrated Leica Application Suite software, version 3.2.0 (Leica Microsystems BV, Rijswijk, The Netherlands).

Immunohistochemistry

Sections were cut and prepared for immunohistochemistry as described previously[25] using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine as staining reagent. Antigen unmasking was carried out by heating the sections for 20 min in 0.01 M sodium citrate (pH 6.0; Merck, Darmstadt, Germany) at 100°C. CD3ε-positive cells were detected using an anti-human CD3ε antibody (DAKO, Heverlee, Belgium; 1:800 diluted in 1% bovine serum albumin, 0.1% Triton X-100 in PBS). As demonstrated by immunocytochemistry, this antibody cross-reacts with the CD3ε-equivalent protein in mouse[26]. Additionally, nonspecific binding was reduced by blocking with TENG-T (10 mmol/L Tris-HCl, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.25% [wt/vol] gelatin, 0.05% [wt/vol] Tween 20). To detect S100a8 and S100a9, both known as cytosolic granulocyte proteins expressed in neutrophils and macrophages, anti-mouse S100a8 and anti-mouse S100a9 antibodies (R&D Systems Europe Ltd., Abingdon, United Kingdom) were used (1:1000 diluted in PBS). Muc4 was stained using an anti-human-MUC4 rabbit-polyclonal antibody (hHA-1) that recognizes the AGYRPPRPAWTFGD amino acid sequence of the C-terminal peptidic region of MUC4α subunit, which is homologous in humans and mice. The antibody was diluted 1:6000 in 1% BSA, 0.1% Triton X-100 in PBS.

Serum Cytokine Levels

Serum was obtained from coagulated blood collected by heart puncture and stored at -80°C until further analysis. The concentrations of several cytokines (Il-12p70, Tnf-α,

interferon gamma (Ifn-γ), monocyte chemoattractant protein-1 (Mcp-1), Il-10 and Il-6) in serum were determined with a BD Cytometric Bead Array mouse inflammation kit (BD-Pharmingen, San Diego, CA, USA).

Quantitative Real-Time PCR (TaqMan Technology)

Total RNA was prepared using the RNeasy midi-kit (Qiagen, Venlo, the Netherlands) and 1.5 μg was used to prepare cDNA. Cytokine mRNA expression levels as well as the housekeeping gene actin were quantified using real-time PCR (qRT-PCR) analysis (TAQman chemistry) based upon the intercalation of SYBR Green on an ABI prism 7900 HT Fast Real Time PCR system (PE Applied Biosystems) as previously described[1]. All primer combinations were designed using OLIGO 6.22 software (Molecular Biology Insights) and purchased from Invitrogen. An overview of the primer sequences used is given in Table 1.

Table 1: primer sequences for quantitative real time PCR

gene	Forward primer	Reverse primer
Il-1β	CCCCAACTGGTACATCA	AGAATGTGCCATGGTTTC
IL-6	CCCAACAGACCTGTCTAT	GGCAAATTTCCTGATTAT
IL-10	CAA GCC TTA TCG GAA ATG	CAT GGC CTT GTA GAC ACC
Il-12alpha (P35 subunit)	GCC TTG GTA GCA TCT ATG AG	TCG GCA TTA TGA TTC AGA GA
Il-12beta (P40 subunit)	CAC GGC AGC AGA ATA AAT A	GAG GGA GAA GTA GGA ATG G
Il-35beta (Ebi3 subunit)	CCC GGA CAT CTT CTC TCT	GAG GCT CCA GTC ACT TG
Tnf-α	TGGCCTCCCTCTCATC	GGCTGGCACCACTAGTT
Ifn-γ	CGG CAC AGT CAT TGA AA	TGC CAG TTC CTC CAG AT
MCP-1	TGG GTC CAG ACA TAC ATT AAA A	GGG TCA ACT TCA CAT TCA AA
β-actin	GGG ACC TGA CGG ACT AC	TGC CAC AGG ATT CCA TAC

Statistical analysis

All data are expressed as median ± SEM or median values. Statistical significance was assessed using the Mann-Whitney U test. (Prism, version 5.00; GraphPad software, San Diego, CA). The data were considered statistically significant at $P < 0.05$.

Results

Type of diet and probiotic supplementation influence disease severity in Muc2$^{-/-}$ mice.

Weight loss or growth retardation can be considered as one of the major clinical symptoms of colitis. At the age of 8 weeks, there was a significant difference in body weight between Muc2$^{-/-}$ NP and WT NP mice (Fig. 1A). Contrastingly, in mice that were fed the

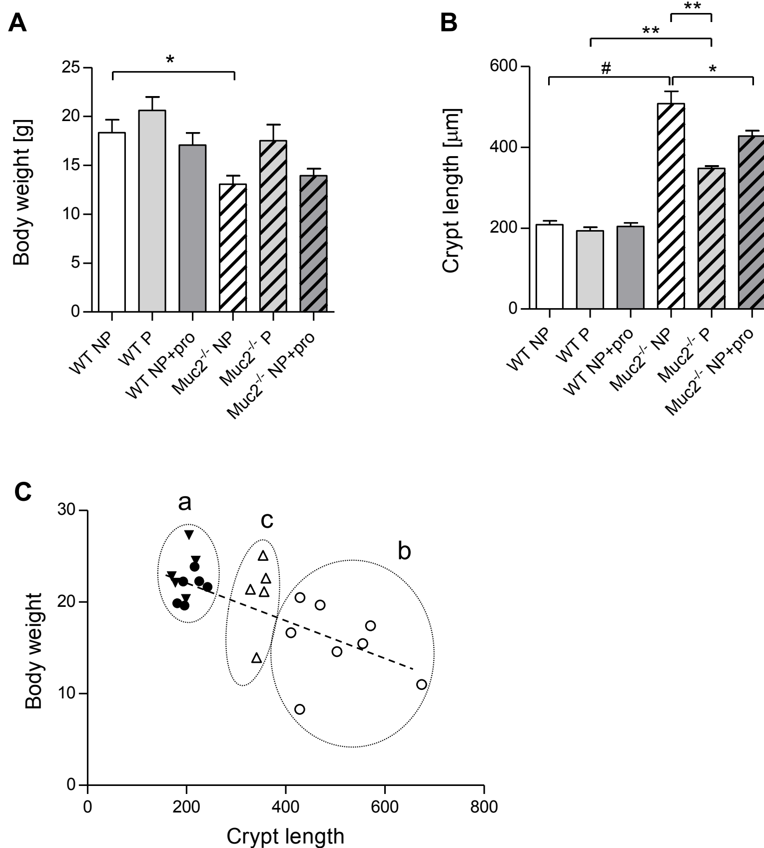


Figure 1. Effect of the type of diet and probiotic supplementation on growth and disease severity. Body weight (A) and crypt length (B) of wild type (WT) and Muc2-/- mice in dietary subgroups are depicted as mean ±SEM. (C) Crypt length (x-axis) significantly correlates with body weight (y-axis) (R2 = 0.4517, P = .0003). Three subgroups can be distinguished: (a) all WT animals in which bodyweight and crypt length are not affected by the type of diet, (b) Muc2-/- NP mice that displayed the highest disease severity reflected in the highest crypt length combined with the lowest bodyweight and (c) Muc2-/- P mice that form an intermediate group displaying increased crypt length but bodyweights comparable with WT mice. Groups are depicted as ▼: WT mice that were fed purified (P) diet, ∆: Muc2-/- mice that were fed the P diet, ●: WT that were fed the non-purified (NP) diet, ○: Muc2-/- mice that were fed the NP diet. (* P < .05, ** P < .01, # P < .001)

P diet, there was no difference between Muc2$^{-/-}$ and WT mice. Probiotic supplementation did not influence body weight in WT mice nor Muc2$^{-/-}$ mice. Total daily consumption of pelleted food did not differ between WT and Muc2$^{-/-}$ mice, nor between mice fed NP diet compared to P diet (data not shown).

As bodyweight is a non-specific, general disease marker, we measured crypt length as a site-specific marker for colitis severity. Similar to body weights, which were different between Muc2$^{-/-}$ NP and WT NP mice, crypt lengths also differed significantly between Muc2$^{-/-}$ NP and WT NP mice (Fig. 1B). Specifically, Muc2$^{-/-}$ NP mice showed increased crypt lengths compared with WT NP mice. Crypt lengths were also significantly increased in Muc2$^{-/-}$ mice fed P diet in comparison with WT mice fed P diet. Furthermore, crypt

length was significantly increased in Muc2$^{-/-}$ NP mice compared to Muc2$^{-/-}$ P mice. Finally, supplementation with probiotics significantly decreased crypt length in Muc2$^{-/-}$ NP+PRO mice compared to Muc2$^{-/-}$ NP mice. Linear regression analysis showed that there is a negative correlation between crypt length and body weight ($P < .0003$) (Fig. 1C). Three subgroups can be distinguished: a) all WT animals in which bodyweight and crypt length are not affected by the type of diet, (b) Muc2$^{-/-}$ NP mice that displayed the highest disease severity reflected in the highest crypt length combined with the lowest bodyweight and (c) Muc2$^{-/-}$ P mice that form an intermediate group as these mice displayed increased crypt length but had bodyweights comparable with WT mice.

Increased Lymphocyte Infiltration and altered cytokine expression in Muc2$^{-/-}$ NP mice

The observed differences between the previously described groups might be explained by an altered inflammatory status of the intestinal mucosa. Therefore, the influx of C3ε-positive T-cells was studied as a marker for inflammation. In mice that were fed the NP diet, the amount of CD3ε-positive T-cells was increased in Muc2$^{-/-}$ mice compared with WT mice (Fig. 2). Interestingly, NP-fed Muc2$^{-/-}$ mice showed an increased influx of CD3ε-positive T cells compared to P-fed Muc2$^{-/-}$ mice.

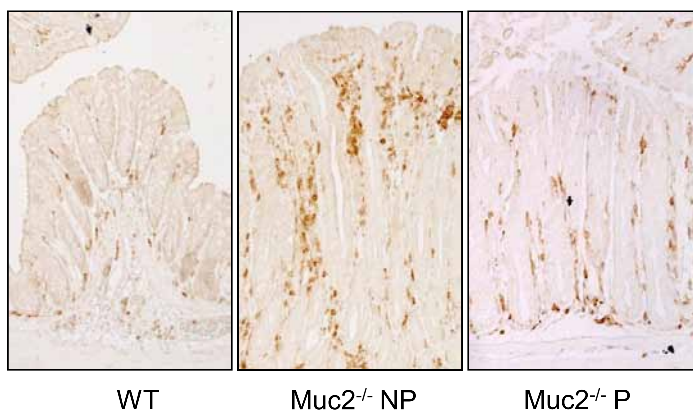


Figure 2. Influx of Cd3ε-positive T-cells
The extent of inflammation in the distal colon was assessed by immunohistochemistry for Cd3ε. Representative stained tissue samples for all groups are shown. Normal WT mice hardly show any Cd3ε-positive T-cells (left panel), whereas Muc2-/- mice that were fed the non-purified (NP) diet show an increased amount of Cd3ε-positive T-cells that are localized along the complete crypt length, but also cluster together at the luminal side of the epithelium (middle panel). Muc2-/- mice that were fed the purified (P) diet showed a decreased amount of CD3ε-positive T-cells compared to Muc2-/- NP mice, that was still increased compared to WT mice (right panel). Crypt lengthening in Muc2-/- mice, as quantified in Fig. 2B, is evident when WT are compared with Muc2-/- NP or Muc2-/- P mice.

No differences in *Cd3ε* mRNA expression were seen upon treatment with probiotics (not shown). To study the increase of CD3ε-positive T-cells into further detail, gene

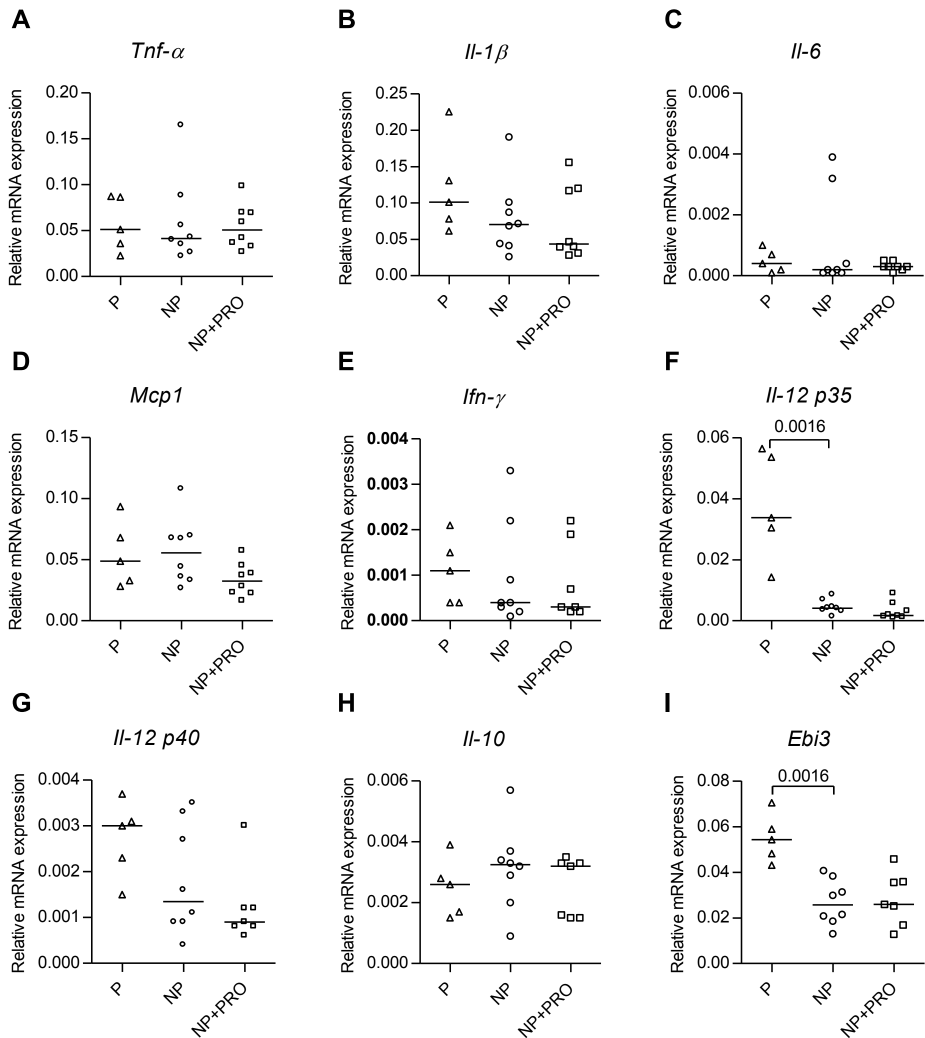


Figure 3. mRNA expression of (anti-)inflammatory cytokines in distal colon of Muc2-/- mice in dietary subgroups

mRNA expression of inflammatory cytokines Tnf-α (A), Il1-β (B), Il-6 (C), Mcp-1 (D), Ifn-γ (E), and Il-12 p35 (F) and p40 (G) and anti-inflammatory cytokines Il-10 (H) and Ebi3 (I) in distal colonic tissue of Muc2-/- mice that were fed a non-purified diet (NP), purified diet (P) or a non-purified diet supplemented with probiotics (NP+PRO). Il-12 p35 and Ebi3 mRNA expression were significantly increased in Muc2-/- that were fed P-diet compared to the NP-diet. Gene expression levels were normalized for β-actin mRNA expression and depicted as median. Groups are depicted as Δ: Muc2-/- mice that were fed the purified (P) diet, ○: Muc2-/- mice that were fed the non-purified (NP) diet, □: Muc2-/- mice that were fed the NP diet substituted with probiotics (NP+PRO)

expression levels of the pro-inflammatory cytokines *Tnf*-α, *Il1*-β, *Il-6*, *Ifn*-γ, and *Il-12* (heterodimer of p35 and p40), chemokine *Mcp-1* and anti-inflammatory cytokines *Il-10* and *Il-35* (heterodimer of p35 and Ebi3), were determined in the distal colon of Muc2$^{-/-}$ mice (Fig. 3). The pro-inflammatory cytokines *Tnf*-α, *Il1*β, *Il-6*, and *Ifn*-γ and

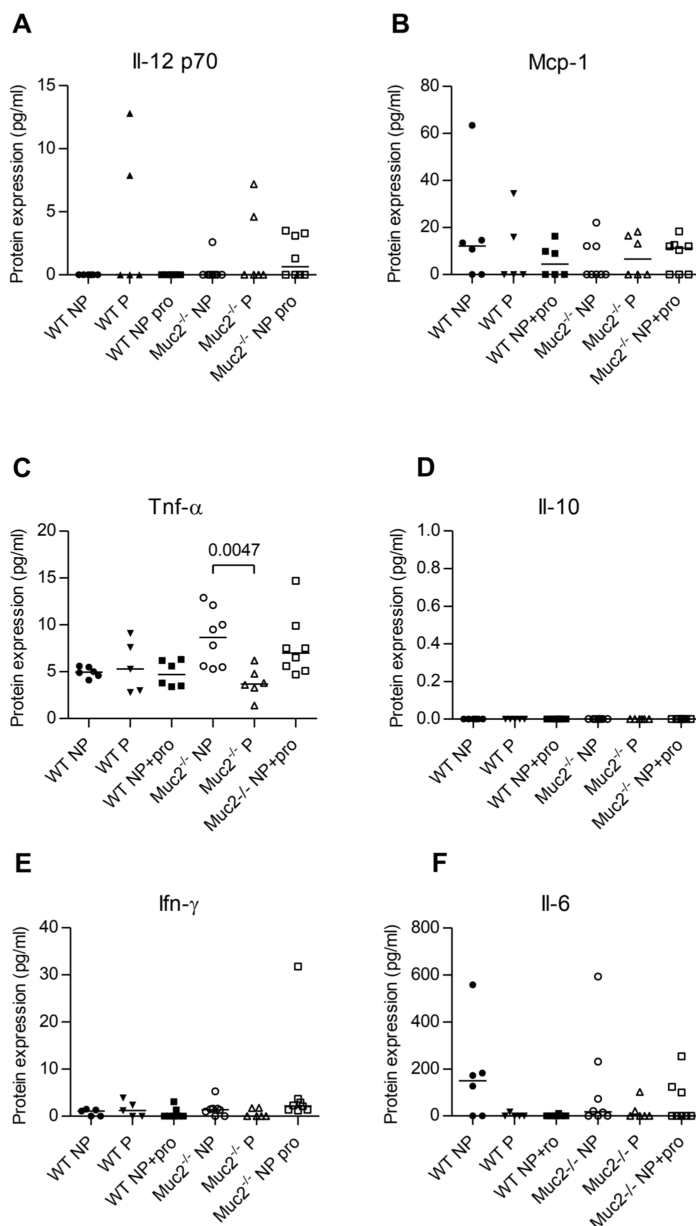


Figure 4. Protein expression of pro- and anti-inflammatory cytokines in serum of wild type and Muc2-/- mice in dietary subgroups

Expression of serum cytokines Il-12 p70 (A), Mcp-1 (B), Tnf-α (C), Il-10 (D), Ifn-γ (E) and Il-6 (F) in ▼: WT mice that were fed purified (P) diet, Δ: Muc2-/- mice that were fed the P diet, ●: WT that were fed the non-purified (NP) diet and ○: Muc2-/- mice that were fed the NP diet, ■ WT mice that were fed the NP diet substituted with probiotics (NP+PRO) and □: Muc2-/- mice that were fed the NP+PRO. Expression of the pro-inflammatory cytokine Tnf-α was significantly increased in serum of Muc2-/- NP mice compared to Muc2-/- P mice. Values are depicted as median.

the chemokine *Mcp-1* were not differentially expressed in Muc2$^{-/-}$ NP compared with Muc2$^{-/-}$ P mice. Expression levels of the *Il-12 p35* subunit and *Ebi3* were significantly increased in Muc2$^{-/-}$ P mice compared to Muc2$^{-/-}$ NP mice. In contrast, expression of the *Il-12 p40* subunit was not significantly different between WT and Muc2$^{-/-}$ mice.
Serum cytokine levels only showed significantly increased expression of Tnf-α protein in Muc2$^{-/-}$ NP mice compared to Muc2$^{-/-}$ P mice. All other serum cytokine levels (Il-12-p70, Mcp-1, Tnf-α, Ifn-γ and Il-6, Il-10) showed variable expression levels that were not significantly different between the different study groups (Fig. 4).

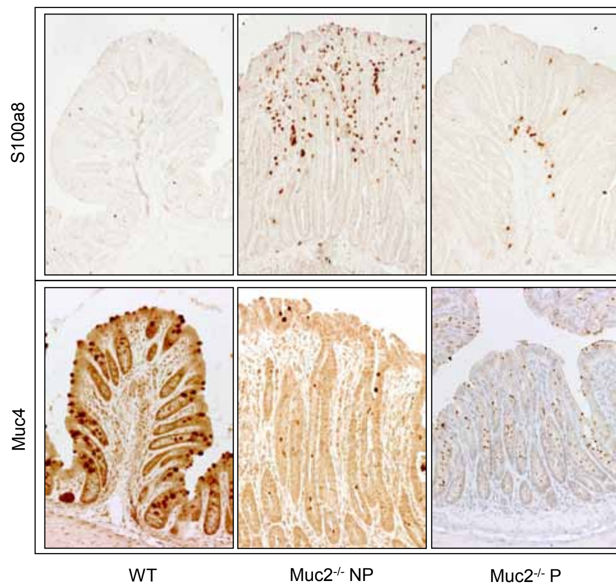


Figure 5. Expression and localization of S100a8 and Muc4 protein in Muc2-/- mice
Expression of S100a8 (upper panels) and Muc4 (lower panel) is depicted in WT (left panel), Muc2-/- mice that were fed the non-purified NP diet (middle panel) and Muc2-/- mice that were fed the purified (P) diet (right panel). The tissue samples are representative for all mice in the studied groups. Note that the expression of S100a8 is increased in Muc2-/- mice compared to WT mice. Moreover, Muc2-/- NP showed increased amounts of S100a8-positive cells compared to Muc2-/- P mice. For Muc4, expression was decreased in Muc2-/- compared to WT mice and with a more pronounced decrease in Muc2-/- NP mice compared to Muc2-/- P mice.

Purified diet reduces expression of S100a8 and S100a9 and increases expression of Muc4 in Muc2$^{-/-}$ mice

To assess the amount of mucosal damage, we performed immunohistochemistry for two S100 proteins, which are produced by neutrophils and macrophages, and are related to inflammation of the intestine, namely S100a8 and S100a9. Moreover, we studied the expression of Muc4 as a marker for epithelial damage. First, the numbers of both S100a8-positive cells (Fig. 5) and S100a9-positive cells (not shown) were increased in Muc2$^{-/-}$ NP mice compared to Muc2$^{-/-}$ P mice. No difference was observed in the number of S100a8-positive

and S100a9-positive cells between $Muc2^{-/-}$ mice that received probiotics and control mice. Contrastingly, the expression of Muc4 was decreased in $Muc2^{-/-}$ NP mice compared to $Muc2^{-/-}$ P mice. Like the S100 proteins, Muc4 expression was not different between $Muc2^{-/-}$ NP+PRO and $Muc2^{-/-}$ NP mice. Increased S100 protein expression and decreased Muc4 expression were seen in the areas where inflammation was the most severe.

Discussion

The present study investigates the effect of two different diets and probiotic supplementation on colitis-severity in $Muc2^{-/-}$ mice. We demonstrate that a purified diet decreases disease severity in $Muc2^{-/-}$ mice and therefore the choice of diet plays a crucial role in this colitis model.

Beneficial effects of the purified diet reflected in increased body weight combined with decreased crypt length in $Muc2^{-/-}$ P mice, indicating that the purified diet reduces disease severity in $Muc2^{-/-}$ mice. As bodyweight and crypt length in WT mice were not affected by the type of diet, the quality of the diet seems to be particularly important in case of disease, more specifically colitis in $Muc2^{-/-}$ mice. Overall, digestibility of the purified diet seems to be more efficient compared to the non-purified diet, resulting in increased availability of nutrients needed for growth, as reflected in increased body weight in $Muc2^{-/-}$ P mice.

Two major differences exist between the two diets used in this study, which might explain the observed difference in disease severity in $Muc2^{-/-}$ mice. First, protein content in the NP diet mainly consists of plant proteins, whereas casein is the main protein source in the purified diet. Although protein composition does not influence the effectiveness of enteral nutrition in the treatment of active adult Crohn's disease[27], a beneficial effect of a casein-based diet in pediatric Crohn's disease has been proposed[9, 28-29]. Second, the amount of insoluble fiber is considerably higher in the NP diet. Bacterial fermentation of insoluble fiber in the proximal colon is less compared to soluble fiber, and thereby limits the amounts of short chain fatty acids (SCFAs) that are produced[30-31]. The protective effect of butyrate, one of the major SCFAs, has been described by several authors, as reviewed by Hamer et al.[32]. Interestingly, butyrate has an anti-inflammatory effect, which is exerted by suppression of nuclear factor kappa B (NF-κB) activation[33]. Colonic contents form a nutrient source for bacteria in the colon and therefore presumably lead to differences in the composition of the microbiota and SCFA production between the two dietary subgroups. Therefore, differences in intestinal inflammation between $Muc2^{-/-}$ NP and $Muc2^{-/-}$ P mice might be related to the amount of SCFAs, more specifically butyrate, that are produced. Finally, insoluble fiber has a more pronounced laxative effect compared to soluble fiber[31], consequently accelerating small bowel transit. This might increase symptoms of diarrhea

that already exist in Muc2$^{-/-}$ mice, regardless of the diet. To further elucidate the effect of specific proteins and the type of fiber on colitis severity in Muc2$^{-/-}$ mice, diets that differ in their protein and fiber content need to be studied into further detail. However, from the current study it is clear that the diet needs to be thoroughly considered in the experimental design as it might significantly influence the disease model. The mechanisms through which probiotics may exert their potential beneficial effects are still largely unknown. We demonstrate that probiotic supplementation leads to a decreased crypt length in Muc2$^{-/-}$ mice. As probiotics can influence cytokine expression[34-37] and we previous showed that *Tnf*-α and *IL-1*β are up-regulated in Muc2$^{-/-}$ mice[1], we hypothesized that probiotic supplementation might restore the disbalanced cytokine profile in Muc2$^{-/-}$ mice and thereby limit disease severity. However, although crypt length was decreased in Muc2$^{-/-}$ NP+PRO mice compared to Muc2$^{-/-}$ NP mice, the abundance of Cd3ε-positive T-cells as well as cytokine expression levels were not different between these mice. Our findings suggest that probiotics might directly influence epithelial proliferation of the intestinal epithelial cells and thereby limit excessive proliferation as seen in Muc2$^{-/-}$ mice that were fed the NP diet without probiotic supplementation. Yet, as probiotics did not restore the disbalance in cytokine expression levels in Muc2$^{-/-}$ NP+PRO mice, disease severity is most likely not limited by the probiotics used in this study. This is in line with the fact that not all probiotic strains have an effect on cytokine expression, let alone have the same effect on the immune system. Interestingly, differing immunological effects have been reported even within the same species of bacteria[38-39].

Several inflammation markers, namely the reduced influx of Cd3ε-positive cells, increased serum Tnf-α levels, and the limited mucosal expression of S100a8 and S100a9 proteins indicate that inflammation is significantly reduced in Muc2$^{-/-}$ mice that were fed P diet compared to Muc2$^{-/-}$ mice that were fed the NP diet. Interestingly, serum cytokine levels only showed significant increased Tnf-α levels in Muc2$^{-/-}$ NP mice compared to Muc2$^{-/-}$ P mice. Tnf-α mRNA levels in the distal colon of Muc2$^{-/-}$ mice were not affected by the type of diet, neither by probiotic supplementation. All other serum cytokines were not affected. Therefore, systemic Tnf-α levels can be regarded as a general marker for colitis in Muc2$^{-/-}$ mice.

Of all cytokines studied in the distal colon, only Il-12 related cytokines showed differences between the studied diets. The interleukin-12 cytokine family includes IL-12, IL-23, IL-27, and the recently identified IL-35[40]. All four are heterodimeric cytokines, composed of an α-chain (p19, p28, or p35) and a β-chain (p40 or Ebi3). IL-12 and IL-23 are highly expressed in the gut of mice and patients with inflammatory bowel diseases[41-42]. Il-12, which consists of a heterodimer of a p35 and a p40 subunit, is known as a pro-inflammatory cytokine, whereas Il-35, the heterodimer of p35 and Ebi3 is linked to regulatory T-cells and possesses immune suppressive capacities. As Il-12 p40 also showed a trend towards increased levels in Muc2$^{-/-}$ mice that were fed the

P-diet, together with significantly increased levels of Il-12 p35, might indicate increased pro-inflammatory Il-12 expression. However, Muc2$^{-/-}$ P mice do not show increased colitis severity. On the contrary, Muc2$^{-/-}$ mice fed the P diet showed a decrease in colitis severity compared to Muc2$^{-/-}$ mice fed the NP diet. Given that Ebi3 is also significantly increased in Muc2$^{-/-}$ mice fed the P-diet, the above described data are in favor of increased Il-35 production (i.e. heterodimer formation of Ebi3 and Il12p35). As Il-35 is known as an immune suppressive cytokine, increased Il-35 levels would also explain the decreased disease severity in Muc2$^{-/-}$ mice that were fed the P-diet compared to Muc2$^{-/-}$ mice that were fed the NP-diet. Yet, further studies are necessary to confirm whether the P diet indeed increases Il35 levels in Muc2$^{-/-}$ mice.

Highest numbers of S100a8- and S100a9-positive cells were observed in Muc2$^{-/-}$ NP mice (Fig. 5). Interestingly these mice also showed the highest disease severity as reflected by lowest body weights and highest increase in crypt lengthening (Fig. 1C). Therefore, the S100 proteins can be regarded as indicators for the degree of colonic inflammation in Muc2$^{-/-}$ mice. In accordance with our findings, fecal calprotectin, the heterodimer of S100a8 and S100a9, is regarded as a marker for inflammation in the gastrointestinal tract, and has been used clinically to follow disease activity in IBD[43-44].

Immunohistochemical staining for Muc4 revealed that abundance of Muc4-positive cells is decreased in Muc2$^{-/-}$ NP mice compared to Muc2$^{-/-}$ P mice. This finding corresponds with previous studies that show decreased numbers of Muc4-positive goblet cells in adult Muc2$^{-/-}$ mice and decreased Muc4 protein expression in patients with CD[45-46]. Interestingly, the areas with the highest abundance of S100a8-positive cells showed the lowest numbers of Muc4-positive cells. We recently showed that Muc4 protein is localized in the intestinal goblet cell[47], implying that Muc4 is not only a membrane bound mucin, but also a secretory mucin in the mouse intestine. Increased secretion of Muc4 in Muc2$^{-/-}$ mice that were fed the NP diet might therefore explain the decreased number of Muc4-positive cells in these mice compared to Muc2$^{-/-}$ mice fed the P diet. Speculating, increased secretion of Muc4 might be caused by a greater need for Muc4 secretion to compensate for Muc2-deficiency in Muc2$^{-/-}$ NP mice. In Muc2$^{-/-}$ P mice the need for this compensatory Muc4 secretion is less as the diet ameliorates colitis-symptoms, and therefore Muc4 protein remains stored in the goblet cell granules of these mice.

In summary, the type of diet significantly influences disease severity, as measured by differences in bodyweight and crypt lengths, in the Muc2$^{-/-}$ colitis model. Secondly, supplementation of the NP diet with probiotics limited crypt lengthening in Muc2$^{-/-}$ mice compared to Muc2$^{-/-}$ mice fed the NP diet only. Compared to Muc2$^{-/-}$ mice fed NP diet, Muc2$^{-/-}$ mice fed the P diet showed a reduced influx of Cd3ε-positive cells that was accompanied by differences in Il-12 related cytokines. These data point to an immune suppressive effect of the P diet, most likely by means of increased Il-35 production. Moreover, a systemic increase of Tnf-α was seen in Muc2$^{-/-}$ mice fed the NP diet, which

was not seen in Muc2$^{-/-}$ mice fed the P diet. Finally, mucosal inflammatory markers S100a8 and S100a9 and the epithelial damage marker Muc4 showed that histological signs of colitis are significantly increased in Muc2$^{-/-}$ mice that were fed the NP diet compared to the P diet. The above described differences, namely decreased growth retardation, reduced crypt lengthening and decreased inflammation markers in Muc2$^{-/-}$ P mice compared to Muc2$^{-/-}$ NP mice might be explained by the type of protein and the type and amount of fibers in the diet. In conjunction, these data imply that feeding strategy in subjects with colitis might have considerable implications for disease severity.

Acknowledgements

This work was supported by Danone Research (Friedrichsdorf, Germany).

References

1. Van der Sluis M, De Koning BA, De Bruijn AC, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006;131:117-29.
2. Pullan RD, Thomas GA, Rhodes M, et al. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. Gut 1994;35:353-9.
3. Tytgat KM, van der Wal JW, Einerhand AW, Buller HA, Dekker J. Quantitative analysis of MUC2 synthesis in ulcerative colitis. Biochem Biophys Res Commun 1996;224:397-405.
4. Jacobs LR, Huber PW. Regional distribution and alterations of lectin binding to colorectal mucin in mucosal biopsies from controls and subjects with inflammatory bowel diseases. J Clin Invest 1985;75:112-8.
5. Burrin DG, Davis TA, Ebner S, et al. Nutrient-independent and nutrient-dependent factors stimulate protein synthesis in colostrum-fed newborn pigs. Pediatr Res 1995;37:593-9.
6. Schaart MW, de Bruijn AC, Bouwman DM, et al. Epithelial Functions of the Residual Bowel After Surgery for Necrotising Enterocolitis in Human Infants. J Pediatr Gastroenterol Nutr 2009.
7. Dziechciarz P, Horvath A, Shamir R, Szajewska H. Meta-analysis: enteral nutrition in active Crohn's disease in children. Aliment Pharmacol Ther 2007;26:795-806.
8. Heuschkel RB, Menache CC, Megerian JT, Baird AE. Enteral nutrition and corticosteroids in the treatment of acute Crohn's disease in children. J Pediatr Gastroenterol Nutr 2000;31:8-15.
9. Fell JM, Paintin M, Arnaud-Battandier F, et al. Mucosal healing and a fall in mucosal pro-inflammatory cytokine mRNA induced by a specific oral polymeric diet in paediatric Crohn's disease. Aliment Pharmacol Ther 2000;14:281-9.
10. Bannerjee K, Camacho-Hubner C, Babinska K, et al. Anti-inflammatory and growth-stimulating effects precede nutritional restitution during enteral feeding in Crohn disease. J Pediatr Gastroenterol Nutr 2004;38:270-5.
11. de Jong NS, Leach ST, Day AS. Polymeric formula has direct anti-inflammatory effects on enterocytes in an in vitro model

of intestinal inflammation. Dig Dis Sci 2007;52:2029-36.
12. Yamamoto T, Nakahigashi M, Saniabadi AR, et al. Impacts of long-term enteral nutrition on clinical and endoscopic disease activities and mucosal cytokines during remission in patients with Crohn's disease: a prospective study. Inflamm Bowel Dis 2007;13:1493-501.
13. Guzy C, Schirbel A, Paclik D, Wiedenmann B, Dignass A, Sturm A. Enteral and parenteral nutrition distinctively modulate intestinal permeability and T cell function in vitro. Eur J Nutr 2009;48:12-21.
14. Leach ST, Mitchell HM, Eng WR, Zhang L, Day AS. Sustained modulation of intestinal bacteria by exclusive enteral nutrition used to treat children with Crohn's disease. Aliment Pharmacol Ther 2008;28:724-33.
15. Noerr B. Current controversies in the understanding of necrotizing enterocolitis. Part 1. Adv Neonatal Care 2003;3:107-20.
16. Schanler RJ, Lau C, Hurst NM, Smith EO. Randomized trial of donor human milk versus preterm formula as substitutes for mothers' own milk in the feeding of extremely premature infants. Pediatrics 2005;116:400-6.
17. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 1995;125:1401-12.
18. Rolfe VE, Fortun PJ, Hawkey CJ, Bath-Hextall F. Probiotics for maintenance of remission in Crohn's disease. Cochrane Database Syst Rev 2006:CD004826.
19. Mallon P, McKay D, Kirk S, Gardiner K. Probiotics for induction of remission in ulcerative colitis. Cochrane Database Syst Rev 2007:CD005573.
20. Deshpande G, Rao S, Patole S, Bulsara M. Updated Meta-analysis of Probiotics for Preventing Necrotizing Enterocolitis in Preterm Neonates. Pediatrics 2010.
21. Roselli M, Finamore A, Nuccitelli S, et al. Prevention of TNBS-induced colitis by different Lactobacillus and Bifidobacterium strains is associated with an expansion of gammadeltaT and regulatory T cells of intestinal intraepithelial lymphocytes. Inflamm Bowel Dis 2009;15:1526-36.
22. Chen CC, Chiu CH, Lin TY, Shi HN, Walker WA. Effect of probiotics Lactobacillus acidophilus on Citrobacter rodentium colitis: the role of dendritic cells. Pediatr Res 2009;65:169-75.
23. Mahler M, Leiter EH. Genetic and environmental context determines the course of colitis developing in IL-10-deficient mice. Inflamm Bowel Dis 2002;8:347-55.
24. Velcich A, Yang W, Heyer J, et al. Colorectal cancer in mice genetically deficient in the mucin Muc2. Science 2002;295:1726-9.
25. Verburg M, Renes IB, Meijer HP, et al. Selective sparing of goblet cells and paneth cells in the intestine of methotrexate-treated rats. Am J Physiol Gastrointest Liver Physiol 2000;279:G1037-47.
26. Jones M, Cordell JL, Beyers AD, Tse AG, Mason DY. Detection of T and B cells in many animal species using cross-reactive anti-peptide antibodies. J Immunol 1993;150:5429-35.
27. Zachos M, Tondeur M, Griffiths AM. Enteral nutritional therapy for inducing remission of Crohn's disease. Cochrane Database Syst Rev 2001:CD000542.
28. Jones S, Shannon H, Srivastava E, Haboubi N. A novel approach to a patient with Crohn disease and a high stoma output: a missed opportunity? Scand J Gastroenterol 2004;39:398-400.
29. Donnet-Hughes A, Duc N, Serrant P, Vidal K, Schiffrin EJ. Bioactive molecules in milk and their role in health and disease: the role of transforming growth factor-beta. Immunol Cell Biol 2000;78:74-9.
30. Floch MH, Narayan R. Diet in the irritable bowel syndrome. J Clin Gastroenterol 2002;35:S45-52.
31. Spiller RC. Pharmacology of dietary fibre. Pharmacol Ther 1994;62:407-27.

32. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther 2008;27:104-19.
33. Andoh A, Fujiyama Y, Hata K, et al. Counter-regulatory effect of sodium butyrate on tumour necrosis factor-alpha (TNF-alpha)-induced complement C3 and factor B biosynthesis in human intestinal epithelial cells. Clin Exp Immunol 1999;118:23-9.
34. Chen CC, Kong MS, Lai MW, et al. Probiotics have clinical, microbiologic, and immunologic efficacy in acute infectious diarrhea. Pediatr Infect Dis J 2010;29:135-8.
35. Pagnini C, Saeed R, Bamias G, Arseneau KO, Pizarro TT, Cominelli F. Probiotics promote gut health through stimulation of epithelial innate immunity. Proc Natl Acad Sci U S A 2010;107:454-9.
36. Foligne B, Nutten S, Grangette C, et al. Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. World J Gastroenterol 2007;13:236-43.
37. He F, Morita H, Hashimoto H, et al. Intestinal Bifidobacterium species induce varying cytokine production. J Allergy Clin Immunol 2002;109:1035-6.
38. Medina M, Izquierdo E, Ennahar S, Sanz Y. Differential immunomodulatory properties of Bifidobacterium logum strains: relevance to probiotic selection and clinical applications. Clin Exp Immunol 2007;150:531-8.
39. Lopez P, Gueimonde M, Margolles A, Suarez A. Distinct Bifidobacterium strains drive different immune responses in vitro. Int J Food Microbiol 2010;138:157-65.
40. Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 2007;450:566-9.
41. Monteleone G, Biancone L, Marasco R, et al. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. Gastroenterology 1997;112:1169-78.
42. Parronchi P, Romagnani P, Annunziato F, et al. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. Am J Pathol 1997;150:823-32.
43. Roseth AG, Aadland E, Jahnsen J, Raknerud N. Assessment of disease activity in ulcerative colitis by faecal calprotectin, a novel granulocyte marker protein. Digestion 1997;58:176-80.
44. Bunn SK, Bisset WM, Main MJ, Golden BE. Fecal calprotectin as a measure of disease activity in childhood inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2001;32:171-7.
45. Buisine MP, Desreumaux P, Debailleul V, et al. Abnormalities in mucin gene expression in Crohn's disease. Inflamm Bowel Dis 1999;5:24-32.
46. Buisine MP, Desreumaux P, Leteurtre E, et al. Mucin gene expression in intestinal epithelial cells in Crohn's disease. Gut 2001;49:544-51.
47. Jonckheere N, Van Der Sluis M, Velghe A, et al. Transcriptional activation of the murine Muc5ac mucin gene in epithelial cancer cells by TGF-beta/Smad4 signalling pathway is potentiated by Sp1. Biochem J 2004;377:797-808.

Chapter 4

Intestinal threonine utilization for protein and mucin MUC2 synthesis is decreased in formula-fed preterm pigs

Patrycja J. Puiman
Mikkel Lykke
Barbara Stoll
Ingrid B. Renes
Adrianus C.J.M. de Bruijn
Kristien Dorst
Henk Schierbeek
Mette Schmidt
Günther Boehm
Douglas G. Burrin
Per T. Sangild
Johannes B. van Goudoever

Submitted

Abstract

Threonine is an essential amino acid necessary for synthesis of intestinal (glyco)proteins such as mucin MUC2 to maintain adequate gut barrier function. In premature infants, reduced barrier function may contribute to the development of necrotizing enterocolitis (NEC). Human milk protects against NEC when compared to infant formula. Therefore, we hypothesized that formula feeding decreases MUC2 synthesis rate concomitant with a decrease in intestinal first-pass threonine utilization, predisposing the preterm neonate to NEC. Preterm pigs were delivered by caesarian section and received enteral feeding with formula (FORM; n=13) or bovine colostrum (COL; n=6) following an initial period of total parenteral nutrition. Pigs received a dual stable isotope tracer infusion of threonine to determine intestinal threonine kinetics. NEC developed in 38% of the FORM pigs, whereas none of the COL pigs were affected. Intestinal fractional first-pass threonine utilization was decreased in FORM pigs compared to COL pigs (49 ± 2% vs. 60 ± 4%, respectively; p=0.02). In FORM pigs compared to COL pigs, protein synthesis (369 ± 31 $mg \cdot kg^{-1} \cdot d^{-1}$ vs. 615 ± 54 $mg \cdot kg^{-1} \cdot d^{-1}$, respectively; p=0.003) and MUC2 synthesis (121 ± 17 %/d vs. 184 ± 15 %/d, respectively p=0.02) were reduced in the distal small intestine. Our results suggest that formula feeding in preterm piglets reduces mucosal growth with a concomitant decrease in first-pass splanchnic threonine utilization, protein synthesis, and MUC2 synthesis in the distal small intestine compared to colostrum feeding. Hence, decreased intestinal threonine metabolism and subsequently impaired gut barrier function may predispose the formula-fed infant to developing NEC.

Introduction

Necrotizing enterocolitis (NEC) is the most common gastrointestinal disorder that affects preterm neonates[1]. Because of the rising incidence of preterm births and improved survival rates of very low birth-weight babies, NEC still remains a challenge in neonatal intensive care. Treatment is limited, and reported mortality rates are as high as 50% for infants requiring surgery[2]. Infants who recover from NEC have an increased risk for complications, such as short bowel syndrome[3] and impaired neurodevelopment[4-5]. Despite extensive research, the pathogenesis of NEC remains poorly understood. Major risk factors identified for the development of NEC are immaturity, enteral (formula) feeding, and bacterial colonization[6]. Therefore, the responses of the immature gut to enteral feeding and bacterial colonization require further investigation.
Feeding preterm infants formula increases NEC incidence compared to their own mother's milk or donor human milk[7-9]. Human milk, especially colostrum, contains various growth factors and immunoglobulins that may reduce the NEC incidence. In preterm piglets, the NEC incidence is greatly increased with formula feeding compared to bovine or porcine colostrum feeding[10]. Formula feeding in preterm pigs decreases digestive capacity, induce mucosal atrophy and disruption, causes microbial overgrowth, and increases gut permeability[10-12]. This negatively affects the gut barrier function that is necessary for epithelial protection.
An important feature for gut barrier function is the mucus layer that overlies the gut epithelium. Goblet cells synthesize and secrete large gel-forming glycoproteins, called mucins. MUC2 is the predominant secretory mucin in the human intestinal tract[13-14]. The mucus layer provides protection against luminal pathogens and toxic substances, and disruption of the mucus layer causes intestinal inflammation and mucosal eruption[15-16]. Decreased gut barrier function caused by a diminished mucus layer may facilitate bacterial translocation and, in combination with an immature immune system, render the preterm infant at risk for the development of intestinal inflammation, sepsis, and NEC[17]. The peptide backbone of MUC2 is particularly rich in the essential amino acid threonine, which constitutes ~30% of the total amino acids in this protein[18-22]. Threonine availability impacts protein mucosal synthesis and mucin synthesis in pigs and rats[23-26]. Using a dual stable isotope tracer method, which allows the determination of dietary first-pass threonine utilization, we showed that in preterm infants, the splanchnic tissues extract 70-82% of dietary threonine, which indicates a high need for threonine[27]. Because very little of the sequestered threonine in the gut is oxidized, a majority is used for gut protein and glycoprotein synthesis[28-29]. However, the effect of colostrum and formula feeding on dietary threonine utilization, protein, and mucin synthesis has not yet been investigated.

We hypothesize that formula feeding predisposes the preterm neonate to developing NEC by a mechanism of a decreased MUC2 synthesis rate, which is accompanied by a decrease in the first-pass intestinal threonine utilization that is necessary for protein and MUC2 synthesis. Thus, the aim of the present study was to determine differences in NEC incidence and first-pass threonine utilization, measured by dual stable isotope tracer technique, in preterm piglets that were fed either formula or colostrum. Furthermore, we aimed to determine differences in gut barrier function in preterm piglets that were fed formula or colostrum by measuring intestinal proteins and MUC2 synthesis.

Materials and methods

Experimental design

Nineteen preterm pigs (Danish landrace X Yorkshire) from three sows were delivered via cesarean section at day 105-107 of gestation, as described in detail previously[10]. Animal protocols and procedures were approved by the Danish National Committee on Animal Experimentation.

Diets

Total parenteral nutrition (TPN) was administered for the first 2 days to mimic the clinical setting in a neonatal intensive care unit, where most preterm infants initially receive TPN. In addition, TPN administration in preterm piglets predisposes them to develop NEC when enteral nutrition is commenced[10]. The parenteral nutrition solution was prepared aseptically and was based on the infusion product Nutriflex Lipid Plus (B. Braun, Melsungen, Germany). The nutrient composition of the TPN solution (glucose, 72 g/L; lipid, 31.1 g/L; amino acids, 45 g/L; solution was provided at a rate of 4-6 $ml \cdot kg^{-1} \cdot h^{-1}$) was identical to that described in detail previously[10]. After 48 h, TPN was discontinued, and the pigs received either human milk formula (FORM, n=13) or bovine colostrum feeding (COL, n=6) via an orogastric tube (at a rate of 15 $ml \cdot kg^{-1} \cdot 3\ h^{-1}$) for 2 days. The milk formula consisted of a mix of three different commercial formulas for human infants (80 g/L pepdite; 70 g/L maxipro; 75 mL/L Liquigen-MCT, all products kindly donated by SHS International, Liverpool, UK) to meet protein and energy requirements. The nutrient composition of the formula mix was calculated from the specifications of the commercial formulas. Bovine colostrum was obtained from the first milking of Holstein-Friesian cows and irradiated (1 x kGy) before use. To make the diets isocaloric, the colostrum was diluted 2:1 with water. An aliquot of the diluted colostrum was assayed for protein content using the Pierce assay (BCA, Protein Assay, Thermo Scientific, Rockford, USA). The macronutrient compositions of the formula and colostrum administered are shown in Table 1. The threonine concentration of both formula and colostrum were determined

using gas chromatography-mass spectrometry. An aliquot of colostrum and formula was hydrolyzed for 24 h at 110°C in 6 N HCl and dried (Speedvac Savant, Thermofisher, Breda, the Netherlands). Samples were then esterified, derivatized, and analyzed using the same method used for plasma threonine concentrations.

Table 1. Macronutrient composition of formula and colostrum (per L)

	COLOSTRUM*	FORMULA†
Energy, kJ	4,000	4,151
Protein, g	86	64
Carbohydrate, g	16-20	47
Lactose, g	16-20	5.3
Fat, g	30-44	61

* Protein content was measured by the Pierce assay. Energy, lactose, and fat contents of colostrum were adapted from (39-42) and adjusted for a 2:1 dilution with water.
† Data were calculated from the specifications of the commercially available products used to prepare the formula: 80 g Pepdite, 70 g Super Soluble Maxipro, 75 mL MCT Liquigen per 1 L formula

Isotope infusion protocol

FORM pigs (n=7, randomly assigned) and COL piglets (n=6) were both subjected to the dual stable isotope tracer infusion protocol. The stable isotope infusion was started 9 h prior to euthanasia to measure intestinal threonine utilization, protein synthesis, and Muc2 synthesis. A primed (25 $\mu mol \cdot kg^{-1}$), continuous (25 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) infusion of [U-^{13}C] threonine (99.47 atom%, Cambridge Isotope Laboratories, Endover, Massachusetts) was administered through an arterial catheter. Simultaneously, a primed (25 $\mu mol \cdot kg^{-1}$) hourly bolus (25 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of [^{15}N]threonine (98 atom%, Cambridge Isotope Laboratories) was administered via an orogastric tube. During the infusion protocol, piglets were switched from 3-h feeding intervals to 1-h feeding intervals. Blood samples were taken at 0 h, 6 h, 8.5 h, and 9 h after the start of the tracer infusion for mass spectrometry analyses. Blood samples were centrifuged immediately after collection to separate plasma and cells. The plasma was stored at -80°C until further analysis. After the 9 h-infusion protocol, piglets were euthanized with an overdose of pentobarbital (200 mg/kg iv; LIFE Faculty Pharmacy, University of Copenhagen, Denmark).

Tissue collection

Immediately after the animal was euthanized, the entire small intestine (SI) and colon were removed, weighed, and sampled for protein analysis and histology as previously described in detail[10]. From each SI segment, the ratio of mucosa to total intestine (in %) was determined after drying both the mucosa and the underlying tissues. Mucosal scraping of the last 10 cm of the distal SI was frozen in liquid nitrogen for mucin analysis (below). The lungs, liver, spleen, heart, kidneys, and stomach were removed, and wet weights recorded.

NEC evaluation & histology

The piglets were evaluated for clinical symptoms of NEC every 3 h as previously described[10]. If any suffering was observed from NEC prior to the end of the study protocol, euthanasia and tissue collection were immediately performed. Upon removal of the gut, the proximal, middle, and distal SI segments and colon were evaluated for NEC lesions and scored from 1 (no/minimal inflammation) to 6 (severe extensive hemorrhage and necrosis), as described previously[10]. A score > 3 was indicative of NEC.

Distal small intestinal and colonic tissue sections (5 µm) were stained with Alcian blue – periodic acid Schiff (AB-PAS) to study morphological changes of the mucosa and identify the presence of neutral and acidic mucins in goblet cells. Goblet cell numbers were analyzed using Visiopharm integrator system in a blinded manner (Visiopharm, Hoersholm, Denmark). The number of goblet cells and the total amount epithelial cells were counted using a specially constructed counting grid, which moved around randomly in the tissue section and analyzed ~25% of the section. In total, five tissue sections per animal were analyzed. The number of goblet cells was expressed as a percentage of total epithelial cells per crypt or villous.

Protein content

Intestinal tissue samples were pulverized in liquid nitrogen and homogenized in ice-cold HIS buffer (50 mM Tris/HCl (pH 7.5), 5 mM EDTA (pH 8), 1% triton, 10 mM iodacetamide, SBTI (100 µg/mL), pepstatin A (10 µg/mL), leupeptin (10 µg/mL, aprotinin (1%), and 1 mM PMSF) at a concentration of 100 mg/mL. Samples were assayed for protein concentration using the Pierce assay.

MUC2 isolation

In the distal SI and colon, MUC2 was isolated using a cesium chloride (CsCl) density gradient ultracentrifugation method combined with gravity gel filtration chromatography, as previously described for human MUC2 in detail[14,30-31].

Mass Spectrometry

Threonine plasma concentration and enrichment analyses

Plasma samples were prepared to determine threonine concentration and enrichment by GC-MS, as described previously[28,31] with minor modifications. [2,3,4,4,4-D_5,^{15}N] threonine was used as internal standard, and an additional derivatization step was performed to block the free hydroxyl group of threonine by adding 20 µL of pyridine and 50 µL of acetic anhydride to the dried ethyl chloroformate derivatives. The samples were briefly vortexed and incubated for 60 min at 60°C. After cooling, the samples were dried under a gentle nitrogen flow at room temperature and resuspended in 50 µL of ethyl acetate. Standard curves were prepared by mixing aqueous solutions of natural

and labeled threonine for both enrichment and concentration determination. GC-MS analyses were performed in selective ion-monitoring mode (SIM) after electron impact ionization (EI) with a MSD 5975C Agilent GCMS (Agilent Technologies, Amstelveen, Netherlands). SIM was carried out at m/z of 146.1, 147.1, 149.1, and 152.1. Separation was achieved on a VF17MS (30 m x 0.25 mm i.d., 0.25 μm film thickness) fused-silica capillary column (Varian, Middelburg, the Netherlands). Helium was used as a carrier gas at a constant flow of 1.2 mL/min. The column was held at 55°C for 1 min, after which the program was altered to 160°C at 30°C/min, then to 200°C at 5°C/min, and finally to 300°C at 10°C/min, with an 8-min hold time. Threonine enrichment was expressed in mole percent excess (MPE). Threonine concentration was expressed in μmol/L.

Intestinal free amino acids and protein-bound amino acids.

Intestinal tissues from the middle SI, distal SI, and colon were homogenized with ice-cold water to achieve a 100 mg/mL concentration. The protein fraction was isolated as described previously[28]. Isotopic enrichment and concentrations of L-[U^{13}C]threonine in the amino-acid-free tissue pool was determined by GC-MS analysis of the acetyl-ethoxycarbonyl-ethylester using EI with an MSD 5975C Agilent GCMS, as described above with the plasma samples. The washed pellets were hydrolyzed by adding 1 mL of 6 N HCl and incubated at 110°C for 20 h. An aliquot was dried at room temperature in a speedvac, and the residue was dissolved in 0.2 mL MQ. Amino acids were isolated by cation exchange separation, as described above for the plasma amino acid fraction. To measure the enrichment of [U^{13}C]threonine in the protein-bound tissue pool, hydrolyzed samples were derivatized to form acetyl-ethoxycarbonylethyl esters. The [^{13}C/^{12}C] ratio of threonine in protein isolates was measured using GC-IRMS according to the method used in our previous work[28,31]. Enrichment was expressed in MPE.

Mucin MUC2 synthesis

Dried MUC2 samples were hydrolyzed and derivatized, and the [^{13}C/^{12}C] ratio of threonine analyzed, as described above for the protein hydrolysates.

Calculations

Plasma enrichments of threonine were used to calculate the rate of threonine turnover or flux. The rate of threonine flux obtained with the enteral [^{15}N]threonine or the intravenous [U^{13}C]threonine and the determination of first-pass uptake of dietary threonine was calculated, as previously described[27]. The fractional protein synthesis rate (FSR in %·d^{-1}) of the middle SI, distal SI, and colon is expressed as a percentage of the total protein pool synthesized per day, and these values were calculated as previously described[32]. The FSR of MUC2 was calculated similarly; the threonine enrichment of the intracellular free amino acid pool in the ileum or colon was used as a precursor.

FSR MUC2 was expressed as a percentage of the total MUC2-pool that was newly synthesized per day. The absolute protein synthesis rate (ASR) of the middle SI, distal SI, and colon, reflecting the total amount of protein that was newly synthesized per day (in $g \cdot d^{-1}$), was measured as the FSR multiplied by the protein mass of the organ in g/kg of body wt, measured by Pierce assay[32].

Statistics

The primary endpoint of the study was the MUC2 FSR. Based on our previous studies on threonine kinetics in piglets and preterm infants[27-28] and Muc2 FSR measurements in human preterm infants[31], we estimated that six piglets per group would detect a difference of 20% (80% power, type 1 error of 0.05) on MUC2 FSR. Furthermore, based on our previous studies, we anticipated a mortality rate of up to 50% in the FORM group, and hence, we doubled the number of piglets studied in this group[10,12]. Minitab statistical software (Minitab, State College, PA) was used for statistical analysis. Data were analyzed by one-way ANOVA - General Linear Model. The difference in NEC incidence between COL and FORM was analyzed using the Fisher's exact test. Data were analyzed for a correlation with NEC score using the Pearson test. Data are presented as the mean ± SEM, and $P < 0.05$ was considered statistically significant.

Results

NEC development and intestinal evaluation

Before the completion of the study, four pigs were euthanized due to suffering from severe clinical symptoms of NEC. The incidence of NEC, defined as a score >3 and a severity of NEC based on the intestinal scoring, was not significantly different between groups (Table 2).

There was no difference in birth weight between FORM and COL piglets. Weight gain was lower in FORM pigs compared to COL pigs (Table 2). A negative correlation between weight gain and NEC score was found (r -0.69, $p = 0.001$).

No difference was observed between FORM and COL pigs with respect to the wet weights of the small intestine and colon. However, the proportion of mucosa and dry matter (mucosa percentage) was lower in FORM pigs than in COL pigs (Table 2). Wet weights of the heart, lungs, liver, stomach, kidneys, and spleen were not different among groups (data not shown).

Histology was performed and showed mucosal damage in the distal small intestinal and colonic tissue in FORM pigs, as described previously[33]. When extensive damage of the mucosa in FORM pigs was observed, samples were excluded from further analysis (distal SI, n=5; colon, n=1). Intestinal epithelial cells that were stained with AB-PAS had

Table 2. NEC incidence and organ weights in preterm pigs fed formula or colostrum*

	COLOSTRUM	FORMULA	p
NEC incidence†	0% (0/6)	38% (5/13)	0.13
NEC score	1.5 ± 0.2	2.8 ± 0.5	0.08
Birth weight, g	872 ± 47	940 ± 52	0.43
Final body weight, g	941± 50	965 ± 56	0.79
Weight gain, $g{\cdot}kg^{-1}{\cdot}d^{-1}$	20.0 ± 1.7	5.9 ± 3.8	0.02
Small intestinal length, cm/kg body wt	305 ± 13	323 ± 18	0.60
Small intestinal weight, g/kg body wt	29.8 ± 0.8	32.7 ± 2.2	0.38
Colon weight, g/kg body wt	8.7 ± 0.5	10.3 ± 0.8	0.20
Mucosa dry weight, %	72 ± 1.2	65 ± 1.9	0.046

*Values are presented as the means ± SEM. COLOSTRUM n=6, FORMULA n=13.
†NEC incidence was defined as a NEC severity score of >3.

the typical morphology of goblet cells in both FORM- and COL-fed piglets. In the distal small intestinal crypts, blue-stained goblet cells, representing the presence of acidic sialylated mucins, were more abundant than in the villi. Cells showing only PAS stain, i.e., pink goblet cells containing neutral mucins, occasionally occurred at all levels of the crypt or villous. The majority of goblet cells were shades of purple, contained both acidic and neutral mucins, and occurred mainly between the tops of the crypts to the tops of the villi (Figure 1A-B). A similar pattern was found in colon samples; more blue-stained goblet cells were present in the lower crypt, whereas pink- and purple-stained cells were found in the upper crypt and surface (Figure 1C-D). Quantitative analysis of goblet cells expressed as a percentage of total epithelial cells in the distal SI (FORM 5.1 ± 0.6 %; COL 6.6 ± 1.2 %) and colon (FORM 23.8 ± 1.7 %; COL 27.6 ± 1.9 %) showed no difference between FORM and COL piglets. In the colon, lower goblet cell counts correlated with a higher NEC score (r -0.74; p = 0.000).

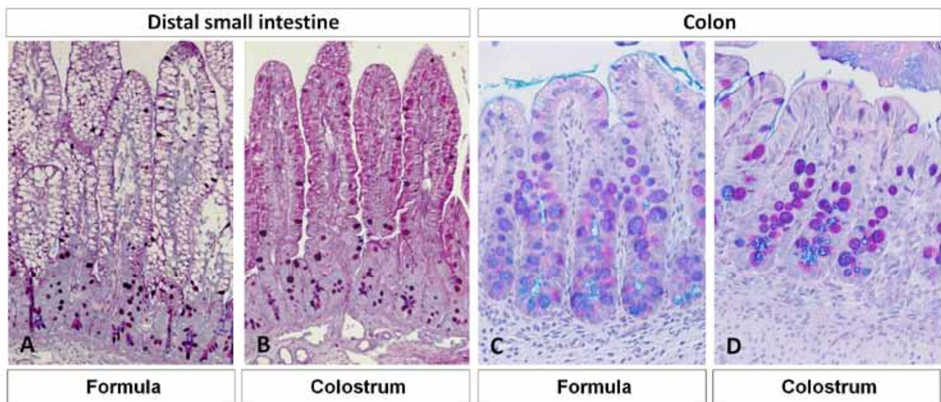


Figure 1. Histology of the distal ileum (A-B) and colon (C-D).
A and C are representative of tissue samples from FORM piglets; B and D are representative of tissue samples from COL piglets.

Threonine kinetics

One pig in the FORM group was excluded from isotopic analyses because of infusion failure of the tracer. All remaining threonine infused pigs (FORM n=6; COL n=6) had a NEC score below 3. Threonine kinetics are presented in Table 3. Plasma threonine concentrations were higher in FORM pigs compared to COL pigs, although their intake was much lower. Plasma threonine flux, based on the iv-infused [$U^{13}C$]threonine tracer, was higher in FORM pigs compared to COL pigs. Plasma threonine flux, based on the intragastric infused [^{15}N]threonine tracer, was not different between FORM and COL pigs. Fractional first-pass utilization of threonine was lower in FORM pigs compared to COL pigs. When corrected for enteral threonine intake, the absolute first-pass utilization of threonine was much lower in FORM pigs compared to COL pigs, which corresponds to the difference in plasma threonine concentrations found.

Table 3. Threonine kinetics in preterm pigs fed colostrum or formula*

	COLOSTRUM	FORMULA	p
Concentration in diet, g/L	4.8	4.52	
Intake, $mg \cdot kg^{-1} \cdot d^{-1}$	576	534	0.000
Plasma concentration, μmol/L	304 ± 50	672 ± 82	0.003
Flux [$U^{13}C$]-threonine (iv) tracer, $\mu mol \cdot kg^{-1} \cdot h^{-1}$	160 ± 14	218 ± 24	0.08
Flux [$1\text{-}^{15}N$]-threonine (ig) tracer, $\mu mol \cdot kg^{-1} \cdot h^{-1}$	404 ± 19	422 ± 39	0.68
First-pass splanchnic utilization, % of intake	60 ± 4	49 ± 2	0.02
First-pass splanchnic utilization, $mg \cdot kg^{-1} \cdot d^{-1}$	343 ± 20	260 ± 11	0.004

* n=6 piglets / group.
The measured threonine concentration of formula was in the same range as the calculated threonine content from the formula specifications (4.0 g/L).

Intestinal protein

The protein contents of the middle SI, distal SI, and colon were determined, and FORM piglets had lower protein contents in the distal SI and colon compared to COL piglets (Table 4). Protein FSR in the middle SI and colon tended to be higher in FORM pigs vs. COL pigs, whereas no difference was observed between the groups in protein FSR in the distal SI (Table 4). Protein ASR in both groups was the highest in the middle SI, decreased towards the distal SI, and declined even further towards the colon (Table 4). However, protein ASR in the distal small intestine was lower in FORM pigs compared to COL piglets, whereas no difference in protein ASR was found in the middle SI or colon between the groups (Table 4).

Mucin MUC2 synthesis

Purified MUC2 isolates from distal SI and colon tissue were further analyzed to determine MUC2 FSR, which is an indication of the percentage of newly synthesized MUC2 per day. In the distal SI, the MUC2 FSR was high, and it was decreased in FORM pigs

Table 4. Protein content, fractional synthesis rate (FSR), and absolute synthesis rate (ASR) in the small intestine and colon of colostrum- and formula-fed piglets

	COLOSTRUM	FORMULA	p
Middle small intestine			
Protein content, g/kg BW	876 ± 69	786 ± 138	0.57
FSR, %/d	55 ± 4.0	68 ± 5.0	0.06
ASR, mg·kg^{-1}·d^{-1}	487 ± 72	523 ± 78	0.75
Distal small intestine			
Protein content, g/kg BW	945 ± 42	678 ± 64	0.006
FSR, %/d	65 ± 6.2	57 ± 6.8	0.36
ASR, mg·kg^{-1}·d^{-1}	615 ± 54	369 ± 31	0.003
Colon			
Protein content, g/kg BW	615 ± 44	448 ± 32	0.012
FSR, %/d	30 ± 3.6	38 ± 1.9	0.08
ASR, mg·kg^{-1}·d^{-1}	191 ± 31	171 ± 14	0.58

Values are presented as the means ± SEM; n=6 per group.

compared to COL pigs (121 ± 17 %/d vs. 184 ± 15 %/d, $p = 0.02$; Figure 2). The FSR of colon MUC2 was approximately half of the MUC2 FSR in the distal SI; however, no difference was detected between the FORM and COL group (73 ± 8 % vs. 69 ± 5 %; Figure 2).

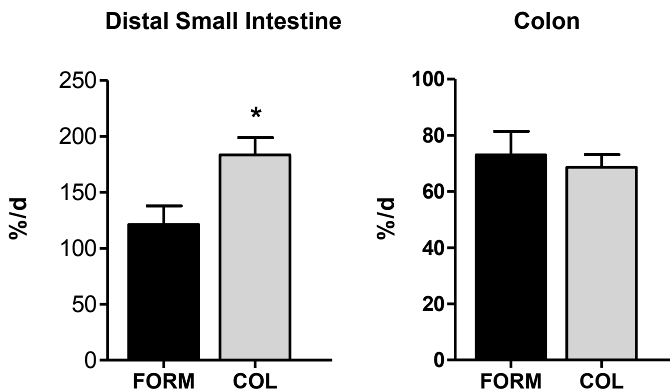


Figure 2. Fractional synthesis rate of MUC2 in the distal SI (A) and colon (B).
Mean ± SEM; n=6 per group.

Discussion

Preterm infants who are fed formula have an increased risk of developing NEC compared to infants who are fed donor or own mother's milk[7,9]. Similarly, formula feeding in preterm pigs increases NEC incidence and induces mucosal atrophy and intestinal

dysfunction when compared to colostrum feeding[10,12]. Colostrum, containing growth factors, immunoglobulins, and other immunostimulatory products may directly stimulate gut barrier function by inducing proliferation and/or differentiation of intestinal epithelial cells and increasing nutrient absorption[10,33]. However, colostrum may indirectly stimulate gut barrier function by activating different metabolic pathways or via enhanced colonization with beneficial bacteria releasing products, such as short-chain fatty acids that stimulate MUC2 synthesis[10,12,34]. We investigated the effect of formula vs. colostrum feeding on intestinal threonine metabolism, which is important for gut growth and barrier function. Our results showed that formula feeding reduced mucosal growth, first-pass threonine utilization, protein synthesis, and mucin synthesis compared to colostrum feeding, whereas NEC incidence was increased.

Fractional and absolute first-pass splanchnic utilization of threonine was markedly decreased in FORM pigs compared to COL pigs. The lower first-pass intestinal threonine utilization in FORM pigs corresponded well with the results obtained for protein and MUC2 synthesis. Both were lower in FORM piglets compared to COL piglets. The fractional first-pass threonine utilization in preterm pigs was lower than that found in 4-wk-old pigs and preterm infants[27-28]. However, the preterm pigs in our study were only enterally fed for 72 hours and still had a low gut-mass/kg of body weight that likely accounted for the lower threonine utilization that we found.

In FORM pigs, lower [U^{13}C]-threonine enrichment and a higher threonine flux or turnover was found compared to COL pigs. Because similar amounts of intravenous threonine were infused, the threonine tracer must have been diluted from either increased dietary threonine amounts passed on to the circulation and / or from endogenous threonine release from protein breakdown. Splanchnic utilization was lower in FORM pigs resulting in increased transport of threonine to the systemic circulation. However, proteolysis might have contributed to the increased threonine turnover and the high threonine plasma concentrations found in FORM pigs as well. Studies in rats and humans have shown that during sepsis, catabolism of muscle protein allowed the mobilization of amino acids required for increased synthesis of defensive proteins in the liver and intestine[35-37]. Because first-pass threonine utilization was lower in FORM pigs, increased threonine supply for protein synthesis in the liver and gut may have been demanded from the systemic pool, most likely at the expense of muscle protein.

Adequate gut barrier function involves multiple intestinal mechanisms for the defense against NEC, such as synthesis of immune cells, defensins, tight junctions, and mucins, such as MUC2[17]. In FORM pigs, protein synthesis was decreased in the distal SI compared to COL pigs. Decreased protein synthesis may reflect decreased synthesis of defensive proteins, which was observed for MUC2. The importance of threonine availability on protein and mucin synthesis has been confirmed in neonatal pigs and rats[24-26]. In mini-pigs with induced ileal colitis, intestinal threonine utilization for mucin synthesis

was increased[38]. Interestingly, feeding increased amounts of mucin precursors, i.e., threonine, cysteine, and proline, enhanced mucin synthesis in a rat model of colitis, which emphasizes the importance of adequate nutrition during inflammation[23]. Future studies will have to elucidate whether increasing amounts of protein and/or threonine in the formula may counteract the negative effect of formula feeding on intestinal threonine metabolism and gut barrier function in preterm piglets.

In the present study we did not investigate the effects of formula vs. colostrum feeding on whole body protein metabolism. Using a whole body protein kinetics model, additional data may be elucidated regarding whole body protein synthesis and proteolysis. Furthermore, because four piglets developed NEC and had to be euthanized, we were unable to study those animals. Therefore, threonine kinetics in an earlier stage of formula feeding may illuminate whether piglets developing NEC have a more affected intestinal threonine metabolism compared to formula fed piglets that were not developing NEC. Additionally, we previously showed that TPN-administration prior to colostrum feeding in preterm pigs diminished intestinal functions and increased NEC, although not to the same extent as formula feeding[10]. Therefore, the differences between the COL- and FORM-fed piglets in intestinal threonine metabolism and protein and mucin MUC2 synthesis found in the present study may be even more pronounced with colostrum vs. formula feeding starting from birth.

In conclusion, our results suggest that feeding preterm piglets formula decreases mucosal mass and first-pass splanchnic threonine utilization when compared to colostrum feeding. This decrease in intestinal threonine metabolism is concomitant with a decrease in both protein and mucin MUC2 synthesis in the distal small intestine. Hence, decreased intestinal threonine metabolism and the subsequently impaired gut barrier function may explain why the formula-fed infant is more prone to develop NEC.

References

1. Lin PW, Stoll BJ. Necrotising enterocolitis. Lancet 2006;368:1271-83.
2. Blakely ML, Lally KP, McDonald S, et al. Postoperative outcomes of extremely low birth-weight infants with necrotizing enterocolitis or isolated intestinal perforation: a prospective cohort study by the NICHD Neonatal Research Network. Ann Surg 2005;241:984-9; discussion 9-94.
3. Petty JK, Ziegler MM. Operative strategies for necrotizing enterocolitis: The prevention and treatment of short-bowel syndrome. Semin Pediatr Surg 2005;14:191-8.
4. Salhab WA, Perlman JM, Silver L, Sue Broyles R. Necrotizing enterocolitis and neurodevelopmental outcome in extremely low birth weight infants <1000 g. J Perinatol 2004;24:534-40.
5. Stoll BJ, Hansen NI, Adams-Chapman I, et al. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. JAMA 2004;292:2357-65.

6. Hsueh W, Caplan MS, Qu XW, Tan XD, De Plaen IG, Gonzalez-Crussi F. Neonatal necrotizing enterocolitis: clinical considerations and pathogenetic concepts. Pediatr Dev Pathol 2003;6:6-23.
7. Lucas A, Cole TJ. Breast milk and neonatal necrotising enterocolitis. Lancet 1990;336:1519-23.
8. McGuire W, Anthony MY. Donor human milk versus formula for preventing necrotising enterocolitis in preterm infants: systematic review. Arch Dis Child Fetal Neonatal Ed 2003;88:F11-4.
9. Sullivan S, Schanler RJ, Kim JH, et al. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. J Pediatr 2010;156:562-7 e1.
10. Bjornvad CR, Thymann T, Deutz NE, et al. Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. Am J Physiol Gastrointest Liver Physiol 2008;295:G1092-103.
11. Thymann T, Moller HK, Stoll B, et al. Carbohydrate maldigestion induces necrotizing enterocolitis in preterm pigs. Am J Physiol Gastrointest Liver Physiol 2009;297:G1115-25.
12. Sangild PT, Siggers RH, Schmidt M, et al. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. Gastroenterology 2006;130:1776-92.
13. Van Klinken BJ, Tytgat KM, Buller HA, Einerhand AW, Dekker J. Biosynthesis of intestinal mucins: MUC1, MUC2, MUC3 and more. Biochem Soc Trans 1995;23:814-8.
14. Tytgat KM, Buller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J. Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology 1994;107:1352-63.
15. Van der Sluis M, De Koning BA, De Bruijn AC, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006;131:117-29.
16. McAuley JL, Linden SK, Png CW, et al. MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. J Clin Invest 2007;117:2313-24.
17. Anand RJ, Leaphart CL, Mollen KP, Hackam DJ. The role of the intestinal barrier in the pathogenesis of necrotizing enterocolitis. Shock 2007;27:124-33.
18. Gum JR, Jr., Hicks JW, Toribara NW, Rothe EM, Lagace RE, Kim YS. The human MUC2 intestinal mucin has cysteine-rich subdomains located both upstream and downstream of its central repetitive region. J Biol Chem 1992;267:21375-83.
19. Lien KA, Sauer WC, Fenton M. Mucin output in ileal digesta of pigs fed a protein-free diet. Z Ernahrungswiss 1997;36:182-90.
20. Mantle M, Allen A. Isolation and characterization of the native glycoprotein from pig small-intestinal mucus. Biochem J 1981;195:267-75.
21. Allen A, Hutton DA, Pearson JP. The MUC2 gene product: a human intestinal mucin. Int J Biochem Cell Biol 1998;30:797-801.
22. Toribara NW, Gum JR, Jr., Culhane PJ, et al. MUC-2 human small intestinal mucin gene structure. Repeated arrays and polymorphism. J Clin Invest 1991;88:1005-13.
23. Faure M, Mettraux C, Moennoz D, et al. Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. J Nutr 2006;136:1558-64.
24. Nichols NL, Bertolo RF. Luminal threonine concentration acutely affects intestinal mucosal protein and mucin synthesis in piglets. J Nutr 2008;138:1298-303.
25. Law GK, Bertolo RF, Adjiri-Awere A, Pencharz PB, Ball RO. Adequate oral threonine is critical for mucin production

and gut function in neonatal piglets. Am J Physiol Gastrointest Liver Physiol 2007;292:G1293-301.
26. Faure M, Moennoz D, Montigon F, Mettraux C, Breuille D, Ballevre O. Dietary threonine restriction specifically reduces intestinal mucin synthesis in rats. J Nutr 2005;135:486-91.
27. van der Schoor SR, Wattimena DL, Huijmans J, Vermes A, van Goudoever JB. The gut takes nearly all: threonine kinetics in infants. Am J Clin Nutr 2007;86:1132-8.
28. Schaart MW, Schierbeek H, van der Schoor SR, et al. Threonine utilization is high in the intestine of piglets. J Nutr 2005;135:765-70.
29. van der Schoor SR, van Goudoever JB, Stoll B, et al. The pattern of intestinal substrate oxidation is altered by protein restriction in pigs. Gastroenterology 2001;121:1167-75.
30. Schaart MW, Schierbeek H, de Bruijn AC, Tibboel D, van Goudoever JB, Renes IB. A novel method to determine small intestinal barrier function in human neonates in vivo. Gut 2006;55:1366-7.
31. Schaart MW, de Bruijn AC, Schierbeek H, Tibboel D, Renes IB, van Goudoever JB. Small intestinal MUC2 synthesis in human preterm infants. Am J Physiol Gastrointest Liver Physiol 2009;296:G1085-90.
32. Stoll B, Chang X, Fan MZ, Reeds PJ, Burrin DG. Enteral nutrient intake level determines intestinal protein synthesis and accretion rates in neonatal pigs. Am J Physiol Gastrointest Liver Physiol 2000;279:G288-94.
33. Oste M, Van Haver E, Thymann T, Sangild P, Weyns A, Van Ginneken CJ. Formula induces intestinal apoptosis in preterm pigs within a few hours of feeding. JPEN J Parenter Enteral Nutr 2010;34:271-9.
34. Burger-van Paassen N, Vincent A, Puiman PJ, et al. The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection. Biochem J 2009;420:211-9.
35. Mansoor O, Cayol M, Gachon P, et al. Albumin and fibrinogen syntheses increase while muscle protein synthesis decreases in head-injured patients. Am J Physiol 1997;273:E898-902.
36. Reeds PJ, Jahoor F. The amino acid requirements of disease. Clinical nutrition (Edinburgh, Scotland) 2001;20:15-22.
37. Faure M, Chone F, Mettraux C, et al. Threonine utilization for synthesis of acute phase proteins, intestinal proteins, and mucins is increased during sepsis in rats. J Nutr 2007;137:1802-7.
38. Remond D, Buffiere C, Godin JP, et al. Intestinal inflammation increases gastrointestinal threonine uptake and mucin synthesis in enterally fed minipigs. J Nutr 2009;139:720-6.
39. Elfstrand L, Lindmark-Månsson H, Paulsson M, Nyberg L, Åkesson B. Immunoglobulins, growth factors and growth hormone in bovine colostrum and the effects of processing. International Dairy Journal 2002;12:879-87.
40. Foley JA, Otterby DE. Availability, Storage, Treatment, Composition, and Feeding Value of Surplus Colostrum: A Review. J Dairy Sci 1978;61:1033-60.
41. Kehoe SI, Jayarao BM, Heinrichs AJ. A survey of bovine colostrum composition and colostrum management practices on Pennsylvania dairy farms. J Dairy Sci 2007;90:4108-16.
42. Rauprich AB, Hammon HM, Blum JW. Influence of feeding different amounts of first colostrum on metabolic, endocrine, and health status and on growth performance in neonatal calves. J Anim Sci 2000;78:896-908.

Chapter 5

Intestinal threonine uptake routes for mucin MUC2 synthesis in preterm pigs and infants

Patrycja J. Puiman
Nanda Burger-van Paassen
Barbara Stoll
Adrianus C.J.M. de Bruijn
Kristien Dorst
Henk Schierbeek
Per T. Sangild
Günther Boehm
Ingrid B. Renes
Johannes B. van Goudoever

Submitted

Abstract

Mucin MUC2 is the major secretory mucin synthesized by goblet cells. Threonine given enterally as well as systemically is rapidly incorporated into small intestinal MUC2 of preterm infants. However, it remains unknown whether there is preferential uptake of enteral or systemic threonine for MUC2 synthesis. Underlining the importance of enteral nutrition in the preterm neonate, we hypothesized that enteral threonine would be the preferred source. We determined the preferential site of threonine absorption for MUC2 synthesis in preterm pigs (n=12) and preterm infants with ileostomies (n=5). We conducted a dual-isotope tracer infusion, allowing incorporation of both enteral and systemic threonine isotope tracers into collected MUC2. Threonine from both the basolateral and luminal side was used for MUC2 synthesis in preterm infants and preterm pigs. Preterm pigs showed higher MUC2 synthesis rates than preterm infants recovering from intestinal disease and surgery.

In preterm pigs, colostrum feeding stimulated threonine uptake for MUC2 synthesis from the luminal vs. the basolateral side, and increased MUC2 fractional synthesis rate when compared to formula feeding (177 ± 17 vs. 121 ± 17%/d respectively). Firstly, we concluded that goblet cells use both luminal and basolateral threonine for synthesis of MUC2. Secondly, colostrum feeding stimulated MUC2 synthesis while increasing threonine absorption from the luminal side. Collectively, colostrum feeding may enhance gut barrier function via stimulation of luminal threonine uptake for MUC2 synthesis.

Introduction

The neonatal gut is in need of threonine to synthesize mucins necessary for epithelial protection. The mucin MUC2 is the most predominant secretory mucin in the human intestinal tract synthesized by goblet cells[1-3]. The peptide backbone of MUC2 is particularly rich in threonine constituting ~30% of the total amino acids[4-8]. Hence, it is not surprising that threonine availability impacts mucosal protein and mucin synthesis in pigs and rats[9-12].

A diminished or disrupted mucus layer causes intestinal inflammation and mucosal eruption, facilitating bacterial translocation and, in combination with an immature immune system, renders the preterm infant at particular risk for the development of sepsis and necrotizing enterocolitis (NEC)[13]. Therefore, adequate threonine uptake by the mucin producing cells is pivotal for prevention of NEC. Intestinal mucosal cells are unique among body cells in that they are presented with substantial quantities of threonine from both the diet and the mesenteric arterial circulation. However, it is unknown whether goblet cells, known to be secretory cells, are able to use enteral substrates like enterocytes do, or whether they are dependent on basolateral absorption as site of precursor uptake.

In preterm neonates, the splanchnic tissues extract 70-82% of dietary threonine[14]. In infants pigs, the intestine is responsible for >70% of splanchnic first-pass metabolism and there is continuous removal of arterial amino acids by the portal-drained viscera which appear to be channeled towards mucosal constitutive protein synthesis[15-16]. Arterial threonine is incorporated rapidly into small intestinal MUC2 of partially enteral fed preterm infants following bowel resection for NEC[17]. However, this study could not determine whether there is a preference for luminal or arterial threonine for MUC2 synthesis. This is of importance for the preterm neonate at risk for NEC when enteral feeding is withheld or delayed. The lack of luminal nutrients, in particular threonine, may decrease MUC2 synthesis and hence gut barrier function.

We hypothesized that enteral threonine would preferentially be used over arterial threonine for MUC2 synthesis by goblet cells, underlining the importance of enteral nutrition in the preterm neonate. We conducted a dual-isotope tracer infusion in preterm pigs and preterm infants, allowing incorporation of both enteral and systemic threonine isotope tracers into MUC2. To be able to compare porcine and human MUC2, our first aim was to isolate and identify porcine MUC2 and determine its homology to human MUC2. Our second aim was to determine the preferential site of threonine absorption for MUC2 synthesis in preterm pigs fed formula or colostrum, and in preterm infants with ileostomies.

Materials and Methods

Materials

Stable isotopes of L-threonine (thr) [^{15}N]thr (98 atom%) and [U-^{13}C]thr (99.5 atom%) were purchased from Cambridge Isotope Laboratories, Andover, Massachusetts. All isotopes were tested and found sterile and pyrogen free.

Preterm pigs

Preterm pigs (Danish landrace X Yorkshire) were delivered via cesarean section at day 105 -107 of gestation, as described in detail previously[18]. Animal protocols and procedures were approved by the Danish National Committee on Animal Experimentation. Total parenteral nutrition (TPN) was administered for the first 2 days as described previously[18]. After 48 h, TPN was discontinued and the pigs were assigned to receive either infant formula (FORM, n=6) or bovine colostrum feeding (COL, n=6) via an orogastric tube (15 $ml \cdot kg^{-1} \cdot 3\ h^{-1}$) for 2 days. The formula consisted of a mix of three different commercial formula's for human infants as described previously to meet protein and energy requirements[18]. Bovine colostrum was derived from the first milking of Holstein-Friesian cows and eradiated (1 x kGy) before use. To make the diets isocaloric, the colostrum was diluted with water in a 2:1 ratio. An aliquot of the diluted colostrum was assayed for protein content using Pierce assay (BCA, Protein Assay, Thermo scientific, Rockford, USA). The threonine concentration of both formula and colostrum were determined using gas chromatography - mass spectrometry. An aliquot of colostrum and formula was hydrolyzed for 24 h at 110°C in 6 mol/L HCl and dried (Speedvac Savant, Thermofisher, Breda, the Netherlands). Samples were then derivatized and analyzed using the same method as that for tissue samples (below).

Isotope infusion protocol

Piglets received a dual- isotope tracer infusion for 9 h before euthanasia. A primed (25 $\mu mol \cdot kg^{-1}$), continuous (25 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) infusion of [U-$^{13}C$]thr was administered through the arterial catheter. Simultaneously, a primed (25 $\mu mol \cdot kg^{-1}$), hourly bolus (25 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) of [$^{15}N$]thr was administered via the orogastric tube. During this protocol, piglets were switched to 1-h feeding intervals. Blood samples were taken at 0, 6, 8.5, and 9 h after start of the tracer infusion for mass spectrometry analyses. Blood samples were centrifuged immediately and the plasma fraction was stored at -80 °C until further analysis. After the 9h-infusion protocol, piglets were euthanized with an overdose of pentobarbital (200 mg/kg iv; LIFE Faculty pharmacy, University of Copenhagen, Denmark).

Tissue collection

Immediately after animals were killed, the entire small intestine (SI) and colon were removed as described in detail previously[18]. The small intestine length was divided into three segments, which were designated proximal, middle, and distal SI. Tissue samples were frozen in liquid nitrogen and stored at -80 °C. Mucosal scraping of the last 10 cm of the distal SI was frozen in liquid nitrogen for MUC2 analysis (below).

Preterm infants

The study was conducted at Erasmus MC - Sophia Children's Hospital (Rotterdam, the Netherlands) after approval from the Erasmus MC Institutional Review Board. Written informed consent was obtained from the parents. Infants who had a bowel resection in the neonatal period and received a temporary ileostomy during surgery were eligible for this study. Exclusion criterion for this study was cystic fibrosis. We included five infants in the study who underwent surgery for NEC (n=2), milk curd obstruction (n=1), midgut volvulus (n=1), and meconium ileus (n=1). Table 1 lists their main clinical characteristics.

Table 1. Demographics and enteral threonine intake of preterm infants

Patient	Sex	GA (wk)	BW (g)	AS (d)	AI (d)	Type of enteral feeding	Enteral intake (ml·kg^{-1}·h^{-1})	Enteral threonine intake (μmol·kg^{-1}·h^{-1})	Parenteral intake (ml· kg^{-1}·h^{-1})	Parenteral threonine intake (μmol· kg^{-1}·h^{-1})
1	F	34.6	2345	1	31	MM + NP1	2.2	10.1	1.0	31.5
2	M	27.9	1130	15	34	Nen 15.4%	3.8	31.9	0.5	16.0
3	M	25.6	865	22	40	Nen 16.5%	4.3	45.3	0.8	25.1
4	M	25.6	755	22	59	MM + BMF	4.4	33.6	0.5	15.8
5	F	33.0	1460	10	28	MM + BMF	4.6	35.3	0.4	12.4

GA gestational age; BW birth weight; AS age at surgery; AI age at isotope infusion; MM mother's milk; NP1 nutrilon premium 1 13.5% (Nutricia, Zoetermeer, the Netherlands); Nen nenatal (Nutricia); BMF breast milk fortifier 4.2% (Nutricia). Parenteral intake: Primene 10% (Baxter, Utrecht, the Netherlands).

Isotope infusion protocol

The study was performed when the infants were clinically stable, i.e. 24 ± 4 days following bowel resection, and received partial enteral nutrition. Then, a primed (10.5 μmol·kg^{-1}), continuous 12-h infusion (10.5 μmol·kg^{-1}·h^{-1}) of [U-^{13}C]thr was administered intravenously. Simultaneously, a primed (21 μmol·kg^{-1}), hourly bolus (21 μmol·kg^{-1}·h^{-1}) of [^{15}N]thr was administered via the nasogastric tube. At baseline, after 9 h, and 12 h of tracer administration, blood samples were collected by heel stick. Blood was centrifuged immediately and the plasma was stored at -80°C. Beginning at the start of the isotope infusion, ileostomy outflow fluid samples were collected at 3-h intervals for 36 h for MUC2 isolation and stored at -80°C.

Mucin MUC2 isolation

MUC2 isolation was performed using a cesium chloride (CsCl) density gradient ultracentrifugation method combined with gravity gel filtration chromatography, as described in detail previously[3, 17, 19]. Briefly, samples were homogenized and, after chemical reduction of the mucin disulfide bonds and carboxymethylation of the sulfhydryl groups, mucins were purified by equilibrium ultracentrifugation (Beckmann Coulter, 50.2 Ti rotor at 250,000 *g* for 72–88 h at 4°C) on a CsCl (Roche, Uppsala, Sweden) density gradient of 1.40 g/mL. CsCl-gradient fractions were collected, dialyzed, and analyzed for the presence of mucins. MUC2 containing fractions, i.e. fractions 3-6 identified by periodic acid schiff[20] positive bands after SDS-PAGE, had a buoyant density between 1.45 and 1.53 mg/mL. This corresponded to relatively high hexose levels in the MUC2 fractions as determined by hexose assay according to François et al.[21] (Figure 1). MUC2 containing fractions were pooled, and further purified by gravity gel filtration chromatography using a Sepharose CL-2B column. MUC2 samples were then extensively dialyzed, analyzed for presence of MUC2 by Western blot, and stored freeze-dried at -20°C.

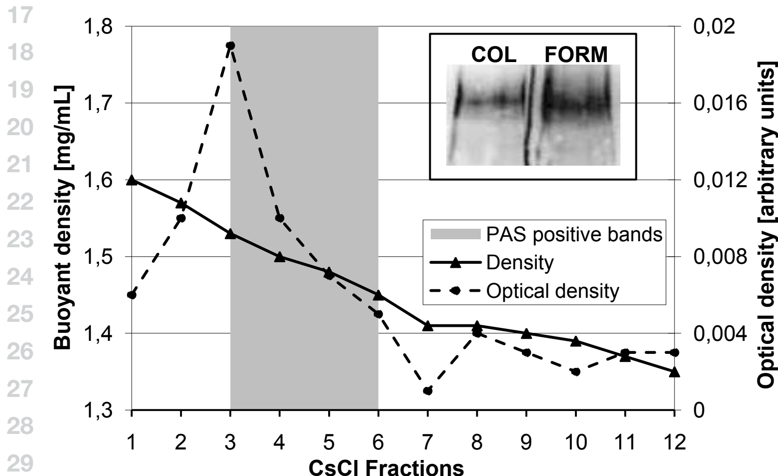


Figure 1. Isolation and determination of porcine Muc2 in intestinal tissue.
Gray area represents periodic acid schiff 20 positive fractions 3-6 after ultracentrifugation. Closed line represents the buoyant density of the fractions. Staggered line represents the hexose content of the fractions. Western blot analysis shows positive bands for porcine MUC2 in colostrum (COL) and formula (FORM) fed piglets.

Porcine MUC2 DNA sequence analysis

To identify porcine MUC2 and further investigate its homology to human MUC2, we used reverse transcriptase-polymerase chain reaction (RT-PCR) and sequence analysis according to the method described previously[22]. Total RNA was isolated from colon samples according to the manufacturer's protocol (RNeasy midi RNA-isolation, Qiagen) and transcribed into cDNA using reverse transcriptase. The final reaction condition was:

2.5 µM pdN6/20 nM oligodT (mix of random hexamers), 200 µM dNTP's, 1 U/µL RNAsin, 8 U/µL mmLV. The entire RT reaction was performed as follows: 45 min at 37°C, then 15 min at 42°C, and was stopped at 94°C (5 min). This was followed by a PCR-reaction in a total volume of 50 µL using 5 µLcDNA as template in combination with human MUC2 primers (Table 2). In one PCR- reaction primer P133-149 was used in combination with P697-713 and P607-623 was used with P1144-1160. Final PCR-reaction conditions were: 4mM $MgCl_2$, 200 µM dNTP's, Taq DNA polymerase, and 0.3 pmol of each primer (forward and reverse). The PCR-reaction was carried out as follows: 5 min 95°C, and 30 cycles of 1 min 95 °C, 1 min 55°C and 1 min 72°C. After the last cycle a 10 min extension step at 72°C followed. The resulting PCR-product was isolated after analysis on a 1.2% agarose gel using Wizard SV Gel and PCR Clean-up System (Promega). Then, BigDye terminator cycle sequencing was performed in a 3130xl Genetic analyzer (Applied Biosystems). Sequencing reactions were prepared as follows: 3-5 ng of PCR-product template, 3 pmol/µL primer, Ready Reaction Primix and BigDye Sequencing buffer. Amplification was over 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. The amplification products were ethanol precipitated for 10-15 minutes at room temperature, centrifuged at maximum speed for 20 minutes, washed with 70% ethanol, and dried by opening the tubes to remove unincorporated dyes. Then pellets were dissolved in HiDi-formamide and run on the sequence analyzer.

Table 2. Human MUC2 primer sequences used for RT-PCR on mRNA isolated from pig colonic tissue.

Primers	Primer sequences
P133 - 149	5′- GTCTGCAGCACCTGGGG -3′
P697 - 713	5′- GAGTGTGAGAGGCTGCT -3′
P607 - 623	5′- GGGAACATGCAGAAGAT -3′
P1144 - 1160	5′- TGTGTCTGTAACGCTGG -3′

Mass spectrometry

Threonine enrichment analyses

Plasma samples were prepared to determine threonine enrichment by GC-MS as described previously[17, 23] with some modifications. As internal standard [2,3,4,4,4-D_5,^{15}N] thr was used and an additional derivatization step was performed to block the free hydroxyl group of threonine by adding 20 µL of pyridine and 50 µL of acetic anhydride to the dried ethyl chloroformate derivatives. The samples were briefly shaken on a vortex mixer and left for 60 min at 60 °C. After cooling down, the samples were dried under a gentle nitrogen flow at room temperature and resuspended in 50 µL of ethyl acetate. Standard curves were prepared by mixing aqueous solutions of natural and labeled

threonine for both enrichment and concentration determination. GC-MS analyses were performed in selective ion-monitoring mode (SIM) after electron impact ionization (EI) on a MSD 5975C Agilent GCMS (Agilent Technologies, Amstelveen, Netherlands). SIM was carried out at m/z 146.1, 147.1, 149.1 and 152.1. Separation was achieved on a VF17MS (30m x 0.25mm i.d., 0.25 μm film thickness) fused-silica capillary column (Varian, Middelburg, the Netherlands). Helium was used as a carrier gas at a constant flow of 1.2 mL/min. The column was held at 55°C for 1 min, and then programmed at 30 °C/min to 160 °C, 5 °C/min to 200°C, and at 10°C/min to 300°C, with a 8-min hold. Threonine enrichment was expressed in mole percent excess (MPE).

Determination of intestinal intracellular free threonine

Intestinal tissues from the distal SI were homogenized with ice cold water in a 100 mg/mL concentration. The protein fraction was isolated as described previously[23]. Isotopic enrichment and concentration of [U-^{13}C]thr and [^{15}N]thr in the tissue amino acid free pool was determined by GC-MS analysis of the acetyl-ethoxycarbonyl-ethylester using EI with a MSD 5975C Agilent GCMS as described before for plasma samples.

Mucin MUC2 synthesis

Dried MUC2 samples were hydrolyzed by adding 1 mL of 6 mol/L HCl and incubating at 110°C for 20 h. Dried hydrolysates (Speedvac Savant, Thermofisher, Breda, the Netherlands) were dissolved in 0.2 mL MilliQ water and amino acids isolated by cation exchange separation as described for the blood amino acid fraction. Enrichment of [U-^{13}C]thr in MUC2 isolates was determined using GCMS as described above.

Calculations and interpretation of threonine labeling.

The FSR of MUC2 is expressed as percentage of the total MUC2-pool newly synthesized per day and calculated as follows[17]:

$$FSR = (SL\text{-}E\ [U\text{-}^{13}C]thr_{MUC2} / E\ [U\text{-}^{13}C]thr_{Plasma/TF}) * 24\ h * 100\% \quad (1)$$

where SL-E [U-^{13}C]thr_{MUC2} is the slope of the linear hourly increase of E [U-^{13}C]thr_{MUC2} where E [U-^{13}C]thr_{MUC2} is the isotopic enrichment of [U-^{13}C]thr incorporated into MUC2. In preterm pigs, SL-E [U-^{13}C]thr_{MUC2} was determined from the slope of the increase of E [U-^{13}C]thr_{MUC2} at the time of euthanasia. E [U-^{13}C]thr_{MUC2} at baseline was assumed to be 0 MPE. [U-^{13}C]thr enrichment of the intracellular tissue free (TF) amino acid pool (E [U-^{13}C]thr_{TF}) was used as the precursor to calculate MUC2 FSR. For preterm infants, [U-^{13}C]thr plasma enrichments (E [U-^{13}C]thr_{Plasma}) were used as the precursor pool because of the absence of intestinal samples for obvious ethical reasons. Similarly, in the following equations, plasma and tissue free (TF) precursor pools will be depicted as E $thr_{Plasma/TF}$ for neonates and piglets respectively.

In the fed state, threonine can be derived from the diet, i.e. luminal uptake, or from the systemic supply, i.e. basolateral uptake. The infusion of intravenous [U-^{13}C]thr leads to exclusive uptake from the basolateral side. During an intragastric [^{15}N]thr infusion, the input of the label is from the luminal side but, after transport by the enterocyte into the systemic pool, can also be derived from the basolateral side. Thus, by the end of the dual-tracer infusion, there are two populations of labeled threonine molecules in MUC2: [U-^{13}C]thr directly from the basolateral side, and [^{15}N]thr derived from both the luminal and basolateral side. The enrichment of the [^{15}N]thr in MUC2 absorbed from the basolateral side (E [^{15}N]thr$_{MUC2\text{-}BL}$) can be calculated by using the percentage of the plasma or tissue free [U-^{13}C]thr incorporated into MUC2 as precursor pool:

$$E\,[^{15}N]thr_{MUC2\text{-}BL} = E\,[^{15}N]thr_{Plasma/TF} * (E\,[U\text{-}^{13}C]thr_{MUC2} / E\,[U\text{-}^{13}C]thr_{Plasma/TF}) \quad (2)$$

where E [^{15}N]thr$_{Plasma/TF}$ is the enrichment of [^{15}N]thr in the plasma or tissue free (TF) pool, E [U-^{13}C]thr$_{MUC2}$ is the enrichment of the [U-^{13}C]thr in MUC2, and E [U-^{13}C]thr$_{Plasma/TF}$ is the enrichment of the [U-^{13}C]thr tracer in the plasma or the tissue free (TF) pool.
Then, the proportion of threonine expressed as % absorbed basolaterally into MUC2 can be calculated by:

$$\text{Basolateral THR absorption (\%)} = (E\,[^{15}N]thr_{MUC2\text{-}BL} / E\,[^{15}N]thr_{Plasma/TF\text{-}BL}) * 100\% \quad (3)$$

where E [^{15}N]thr$_{TF\text{-}BL}$ is the enrichment of the [^{15}N]thr tracer in the TF pool taken up from the basolateral side.
Following, the enrichment of the [^{15}N]thr in MUC2 absorbed from the luminal side (E [^{15}N]thr$_{MUC2\text{-}LUM}$) can be calculated by:

$$E\,[^{15}N]thr_{MUC2\text{-}LUM} = E\,[^{15}N]thr_{MUC2} - E\,[^{15}N]thr_{MUC2\text{-}BL} \quad (4)$$

where E [^{15}N]thr$_{MUC2}$ is the total enrichment of the [^{15}N]thr tracer, i.e. from both the luminal and basolateral side, in MUC2.
The proportion of threonine expressed as % absorbed luminally can then be calculated by:

$$\text{Luminal THR absorption (\%)} = (E\,[^{15}N]thr_{MUC2\text{-}LUM} / E\,[^{15}N]thr_{DIET/TF\text{-}LUM}) * 100\% \quad (5)$$

where the enrichment of the [^{15}N]thr tracer in the diet (E[^{15}N]thr$_{DIET}$) is used as a precursor in the infants, and the enrichment of the [^{15}N]thr tracer in the TF pool taken up from the luminal side is used as precursor for the piglets. From the rate of the dietary threonine intake of the patients during the isotope infusion and the rate of the intragastric [^{15}N]thr administration, E[^{15}N]thr$_{DIET}$ was calculated.

Statistics

Minitab statistical software (Minitab, State College, PA) was used for statistical analysis. Data were analyzed by one-way ANOVA – General Linear Model. Data are presented as the mean ± SEM and $P < 0.05$ was considered statistically significant.

Results

Porcine MUC2

Isolated pig MUC2 showed high similarity to MUC2 found in human preterm infants with respect to its buoyant density, molecular weight, PAS stainability, and hexose content[17, 19]. Specifically, pig MUC2 has a buoyant density between 1.45 and 1.53 mg/mL, has a relative high molecular weight, is visible with PAS on SDS-PAGE (not shown), and contained relatively high amounts of hexose (Figure 1). Recognition of the purified porcine MUC2 samples by western blotting with anti-HCM, i.e. an antibody specific for human MUC2[22], suggested high homology between porcine and human MUC2 (Figure 1). Overlapping parts of pig *MUC2* cDNA were cloned by RT-PCR on pig ileal mRNA following the strategy as shown in Figure 2. Pig *MUC2* cDNA sequences were amplified, sequenced and combined to one fragment of 1028 bp, which showed 85% homology to human *MUC2* cDNA. This 1028 bp fragment was found to encode a fragment of 342 amino acids of the N-terminus of pig MUC2, which showed 81% homology to human MUC2 (Figure 3).

hMUC2:	133	GTCTGCAGCA CCTGGGGCAA CTTCCACTAC AAGACCTTCG ACGGGGACGT
pMUC2:		GTCTGCAGCA CCTGGGGC**G**A CTTCCACTAC AAGACCTTCG ACGG**C**GACGT
hMUC2:	183	CTTCCGCTTC CCCGGCCTCT GCGACTACAA CTTCGCCTCC GACTGCCGAG
pMUC2:		CTTCCGCTTC CC**G**GGCCT**G**T GCGACTACAA CTTCGCCTCC GACTGCCG**G**G
hMUC2:	233	GCTCCTACAA GGAATTTGCT GTGCACCTGA AGCGGGGTCC GGGCCAGGCT
pMUC2:		**A**C**G**CCTACAA GGA**G**TT**C**GCT GTGCACCTGA **GA**CGGGG**C**CC **C**GGC**GGCAG**T
hMUC2:	283	GAGGCCCCCG CCGGGGTGGA GTCCATCCTG CTGACCATCA AGGATGACAC
pMUC2:		G**G**GG**G**CCCC**T** CC**CA**GGT**C**GA GT**A**CATCCTG CTGAC**GG**TCA AGGATGACAC
hMUC2:	333	CATCTACCTC ACCCGCCACC TGGCTGTGCT TAACGGGGCC GTGGTCAGCA
pMUC2:		CATCTACCTC AC**T**C**AG**CA**G**C TGG**TC**GTG**G**T **G**AACGGGGCC **A**TGGTCAGCA
hMUC2:	383	CCCCGCACTA CAGCCCCGGG CTGCTCATTG AGAAGAGCGA TGCCTACACC
pMUC2:		CCCCGCACTA **T**AGCCC**G**GGG CTGCTCATTG AGA**G**GAG**T**G**C** **C**G**T**CTACACC

hMUC2: 433 AAAGTCTACT CCCGCGCCGG CCTCACCCTC ATGTGGAACC GGGAGGATGC
pMUC2: AA**G**GTCTA**T**T CCCG**A**GC**T**GG CCT**TG**C**T**CTC **G**TGTGGAAC**A** G**A**GAGGA**CT**C

hMUC2: 483 ACTCATGCTG GAGCTGGACA CTAAGTTCCG GAACCACACC TGTGGCCTCT
pMUC2: **GG**TCATGCTG GAGCTGGACA **G**TAAGTTCC**A** GAACCACAC**G** TGTGGCCTCT

hMUC2: 533 GCGGGGACTA CAACGGCCTG CAGAGCTATT CAGAATTCCT CTCTGACGGC
pMUC2: GCGG**A**GACTA CAACGGCCTG CAGA**C**CTA**C**T CAGA**G**TTCCT CTC**G**GA**G**GGC

hMUC2: 583 GTGCTCTTCA GTCCCCTGGA GTTTGGGAAC ATGCAGAAGA TCAACCAGCC
pMUC2: **A**T**C**C**C**CTTCA G**C**CCC**T**TGGA GTT**C**GGGAAC ATGCAGAAGA TCAAC**A**AGCC

hMUC2: 633 CGATGTGGTG TGTGAGGATC CCGAGGAGGA GGTGGCCCCC GCATCCTGCT
pMUC2: CGA**G**G**A**G**AA**G TGTGA**C**GA**C**C CCGAGGAGG**C** **ACA**GGCC**AAG** **CTG**TCCTGCT

hMUC2: 683 CCGAGCACCG CGCCGAGTGT GAGAGGCTGC TGACCGCCGA GGCCTTCGCG
pMUC2: C**T**GAGCACCG CGCCGAGTG**C** GAGAGGCTGC TGAC**G**G**A**CG**T** GGCCTTCG**A**G

hMUC2: 733 GACTGTCAGG ACCTGGTGCC GCTGGAGCCG TATCTGCGCG CCTGCCAGCA
pMUC2: GACTG**C**CAGG **GG**CTGGTGCC **A**CTGGAGC**T**G TA**CG**TGC**AG**G CCTGC**GT**GCA

hMUC2: 783 GGACCGCTGC CGGTGCCCGG GCGGTGACAC CTGCGTCTGC AGCACCGTGG
pMUC2: GGACCGCTG**T** C**A**GTGCCCG**C** **AG**GG**CAC**C**T**C CTGCGTCTGC AGCAC**GA**T**C**G

hMUC2: 833 CCGAGTTCTC CCGCCAGTGC TCCCACGCCG GCGGCCGGCC CGGGAACTGG
pMUC2: CCGAGTTCTC CCGCCAGTGC TCCCACGCCG G**T**GG**G**CGGCC **T**GGGAACTGG

hMUC2: 883 AGGACCGCCA CGCTCTGCCC CAAGACCTGC CCCGGGAACC TGGTGTACCT
pMUC2: AGGACCGCCA **A**GCTCTGCCC **T**AAGA**G**CTGC CC**T**GGGAAC**A** TGGT**T**TACCT

hMUC2: 933 GGAGAGCGGC TCGCCCTGCA TGGACACCTG CTCACACCTG GAGGTGAGCA
pMUC2: GGAGAGC**A**GC TCGCCCTGC**G** TGGACACCTG CTC**G**CACCTG GAGGT**C**AGCA

hMUC2: 983 GCCTGTGCGA GGAGCACCGC ATGGACGGCT GTTTCTGCCC AGAAGGCACC
pMUC2: GCCTGTGCGA GGA**A**CACCGC ATGGA**T**GGCT GTTTCTGCCC AGAAGGCAC**T**

hMUC2: 1033 GTATATGACG ACATCGGGGA CAGTGGCTGC GTTCCTGTGA GCCAGTGCCA
pMUC2: GT**G**TATGA**T**G ACATCG**C**GG**G** CAG**A**GGCTGC **A**T**C**CC**C**GTGA GCCAGTG**T**CA

hMUC2: 1083 CTGCAGGCTG CACGGACACC TGTACACACC GGGCCAGGAG ATCACCAATG
pMUC2: CTGCA**A**GCTG CACGG**G**CACC **A**GTA**TG**C**G**CC **C**GGCCAG**C**AG **G**TCACCAA**CA**

hMUC2: 1133 ACTGCGAGCA GTGTGTCTGT AACGCTGG
pMUC2: ACTGCGAGCA **A**TGTGTCTGT AACGCTGG

Figure 2. Part of the 5′ cDNA sequence of porcine MUC2, and sequence comparison with human MUC2.

The sequence of the 1028 bp-long 5′ part of porcine MUC2 (pMUC2) is aligned to the 5′ sequence of human MUC2 (hMUC2). The pMUC2 sequence is for 85% identical to hMUC2. Mismatches are indicated in bold script in the pMUC2 sequence. Underlined sequences correspond with sequences of primers designed on human MUC2 cDNA sequences that were used to amplify porcine MUC2 cDNA.

hMUC2:	36	VCSTWGNFHYKTFDGDVFRFPGLCDYNFASDCRGSYKEFAVHLKRGPGQA
pMUC2:		VCSTWG**D**FHYKTFDGDVFRFPGLCDYNFASDCR**DG**YKEFAVHL**R**RGPG**GS**
hMUC2:	86	EAPAGVESILLTIKDDTIYLTRHLAVLNGAVVSTPHYSPGLLIEKSDAYT
pMUC2:		**GG**P**SQ**VE**Y**ILLT**V**KDDTIYLT**QQ**L**V**V**V**NGA**M**VSTPHYSPGLLIE**R**S**AV**YT
hMUC2:	136	KVYSRAGLTLMWNREDALMLELDTKFRNHTCGLCGDYNGLQSYSEFLSDG
pMUC2:		KVYSRAGL**A**L**V**WNRED**SV**MLELDTKF**Q**NHTCGLCGDYNGLQ**T**YSEFLS**E**G
hMUC2:	186	VLFSPLEFGNMQKINQPDVVCEDPEEEVAPASCSEHRAECERLLTAEAFA
pMUC2:		**IP**FSPLEFGNMQKIN**K**P**EEK**C**D**DPEE**AQ**A**KL**SCSEHRAECERLLT**DV**AF**E**
hMUC2:	236	DCQDLVPLEPYLRACQQDRCRCPGGDTCVCSTVAEFSRQCSHAGGRPGNW
pMUC2:		DCQ**G**LVPLE**LYVQ**AC**V**QDRC**Q**CP**Q**G**TS**CVCST**I**AEFSRQCSHAGGRPGNW
hMUC2:	286	RTATLCPKTCPGNLVYLESGSPCMDTCSHLEVSSLCEEHRMDGCFCPEGT
pMUC2:		RTA**K**LCPK**S**CPGN**M**VYLES**S**SPC**V**DTCSHLEVSSLCEEHRMDGCFCPEGT
hMUC2:	336	VYDDIGDSGCVVSQCHCRLHGHLYTPGQEITNDCEQCVCNAG
pMUC2:		VYDDI**AGR**GC**I**VSQCHC**K**LHGH**Q**Y**A**PGQ**QV**TN**N**CEQCVCNAG

Figure 3. Part of the N-terminal amino acid sequence of porcine MUC2, and sequence comparison with human MUC2.
The sequence of the 342 amino acids-long N-terminal part of the porcine MUC2 (pMUC2) polypeptide fragment is aligned to the N-terminal sequence of human MUC2 (hMUC2). The pMUC2 sequence is for 81% identical to hMUC2. Mismatches are indicated in bold script in the pMUC2 sequence.

Preterm pigs

Body and organ weights

As shown in Table 3 there was no difference in birth weight between FORM and COL piglets. Weight gain was lower in FORM pigs compared to COL pigs. No difference was observed between FORM and COL pigs with respect to the length or wet mass weights of the small intestine. The percentage of mucosa weight to intestinal weight was also not different between groups.

Table 3. Body and organ weights in preterm pigs fed formula or colostrum[*]

	COLOSTRUM	FORMULA	P
Birth weight, g	871 ± 47.3	805 ± 46.7	0.2
Final body weight, g	941 ± 49.7	832 ± 54.4	0.1
Weight gain, $g \cdot kg^{-1} \cdot d^{-1}$	20.0 ± 1.7	8.4 ± 6.2	0.2
Small intestinal length, cm/kg body wt	305 ± 12.7	362 ± 17.4	0.05
Small intestinal weight, g/kg body wt	29.8 ± 0.8	32.5 ± 1.8	0.3
Mucosa dry weight, %	69.5 ± 2.8	67.5 ± 4.7	0.4

[*]Values are presented as means ± SEM; n=6/group

Threonine tracer kinetics

One pig in the FORM group was excluded from isotopic analyses because of infusion failure of the tracer. Enrichments of both [^{15}N]thr and [U-^{13}C]thr were measured in the

plasma (steady state), in the intracellular free pool of the distal SI, and in purified MUC2 samples derived from the distal SI (Table 4). In FORM piglets, 60 ± 6.8 % of threonine in MUC2 was absorbed from the basolateral side and 40 ± 6.8 % was absorbed from the luminal side (Figure 4). In COL piglets opposite results were obtained; 41 ± 4.3 % of dietary threonine in MUC2 was absorbed from the basolateral side and 59 ± 4.3 % was absorbed from the luminal side (Figure 4). MUC2 FSR, representing the percentage of newly synthesized MUC2 per day in the distal SI, was relatively high, and was lower in FORM pigs (121 ± 17 %/d) compared to COL pigs (177 ± 17 %/d) (Figure 5).

Table 4. Threonine kinetics in piglets fed colostrum or formula*

	COLOSTRUM	FORMULA
E [^{15}N]thrDIET (MPE)	11.039	11.804
E [^{15}N]thrPlasma (MPE)	5.9 ± 0.30	5.7 ± 0.53
E [U^{13}C]thrPlasma (MPE)	13 ± 0.85	9.8 ± 0.97
E [^{15}N]thrTF (MPE)	1.5 ± 0.10	5.2 ± 1.5
E [U^{13}C]thrTF (MPE)	1.7 ± 0.07	3.9 ± 0.25
E [^{15}N]thrMuc2 (MPE)	1.2 ± 0.10	1.7 ± 0.46
E [U^{13}C]thrMuc2 (MPE)	1.1 ± 0.09	1.7 ± 0.24
Muc2 FSR† (%/d)	177 ± 17.1	121 ± 16.7

*Values are presented as means ± SEM; COLOSTRUM n=5; FORMULA n=6.
† Muc2 FSR COLOSTRUM vs. FORMULA P = 0.047.
THR threonine; MPE mole percent excess; FSR fractional synthesis rate.

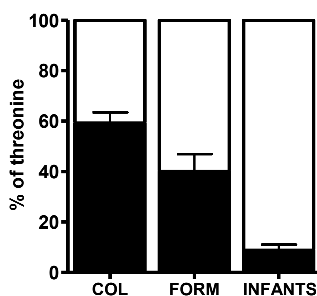


Figure 4. Percentage of luminal and basolateral threonine absorption for MUC2 synthesis in preterm pigs fed colostrum (COL) or formula (FORM) and preterm infants.
Black bars represents luminal absorption of threonine and white bars remaining basolateral absorption of threonine incorporated into MUC2.

Preterm infants

Enrichments of both [^{15}N]thr and [U-^{13}C]thr were measured in the plasma at steady state and in purified MUC2 samples from the ileal outflow fluid (Table 5). In preterm infants, 91 % of the dietary threonine in MUC2 was absorbed from the basolateral side, whereas 9 % was absorbed from the luminal side (Figure 4). The mean MUC2 FSR in the ileum was 108 ± 17 %/d (Figure 5, Table 5).

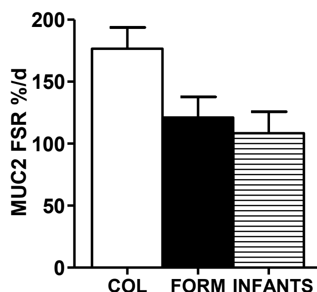


Figure 5. MUC2 FSR expressed as percentage of newly synthesized MUC2 per day in preterm pigs fed either colostrum (COL) or formula (FORM), and in preterm infants.

Table 5. Threonine kinetics in preterm infants.

Patients	1	2	3	4	5
Enteral threonine intake (μmol·kg^{-1}·h^{-1})	10.1	31.9	45.3	33.6	35.3
E [^{15}N]thrDIET (MPE)	40.0	56.7	45.6	57.1	62.8
E [^{15}N]thrPlasma (MPE)	9.0	7.9	7.2	7.6	5.5
E [U^{13}C]thrPlasma (MPE)	3.8	4.2	3.1	3.3	3.1
E [^{15}N]thrMuc2 (MPE)	7.9	5.1	4.1	3.3	4.2
E [U^{13}C]thrMuc2 (MPE)	1.9	1.4	1.6	0.9	1.1
Muc2 FSR (%/d)	147	76	144	61	114

THR threonine; MPE mole percent excess; FSR fractional synthesis rate

Discussion

Threonine is important for mucosal protein and mucin synthesis to provide epithelial protection. The preferential site of threonine uptake, i.e. basolateral or luminal, for mucin synthesis has implications for nutritional therapy in the preterm infant since disruption of the mucus layer increases the risk of NEC. We studied the preferential site of threonine absorption for MUC2 synthesis in partially enterally fed preterm infants and in colostrum or formula-fed preterm pigs. First, we showed by two independent techniques that porcine MUC2 is expressed in the ileum, and that it is 81% homologous to human MUC2. Second, our results show that threonine from both the basolateral and luminal side is used for MUC2 synthesis in preterm pigs and infants. Under these conditions, luminal threonine uptake for MUC2 synthesis and MUC2 synthesis rate was higher in preterm pigs than in partially enterally fed preterm infants. Furthermore, colostrum feeding in preterm pigs stimulated threonine uptake from the luminal side, and increased MUC2 fractional synthesis rate compared to formula feeding.

In both preterm pigs and preterm infants, threonine was taken up for MUC2 synthesis from both the arterial and luminal side. Uptake of both systemically and enterally derived nutrients for synthesis of peptides such as glutathione has been described

before[24]. However, mucin producing goblet cells are secretory cells and according to present day knowledge not designed for nutrient uptake. Amino acids in the small intestinal lumen are actively absorbed across the apical enterocyte membranes[25]. The major apical neutral amino acid transporter is B^0AT1 and transports all neutral amino acids including threonine, albeit to a varying extent[26]. The apical ASCT2 transporter has a higher affinity for small neutral amino acids such as threonine[26]. However, it is unknown whether goblet cells exhibit these transporters on their apical membrane. Studies performed on human and murine tissues have shown that goblet cells do not exhibit the PEPT1 transporter responsible for peptide transport on their apical membrane[27-28]. Based on all these data, it is not likely that goblet cells transport amino acids via the apical membrane. Hence, the uptake of threonine coming directly from the luminal side might come from inter- or paracellular trafficking between enterocytes and goblet cells. It is argued that paracellular transport becomes highly significant at high substrate concentration[29]. At the mucosal surface high amino acid concentrations are present due to local peptidases. However, future studies are warranted to elucidate the detailed mechanisms regulating uptake of threonine from the luminal side. Transporters 4F2/LAT2 and SNAT2 are responsible for basolateral uptake of neutral amino acids[26]. Interestingly, the transporter is upregulated by amino acid depletion and is likely to provide amino acids to intestinal epithelial cells, when recruiting few amino acids from the lumen[30]. Whether this takes place at the basolateral membrane of the goblet cells remains to be investigated as well.

Luminal threonine uptake, as a percentage of the enteral intake, and MUC2 synthesis in preterm infants was lower compared to that observed in preterm pigs. Preterm infants were only partially enterally fed, after receiving full TPN for some time after intestinal surgery, which might have impacted luminal threonine uptake. In piglets receiving TPN, the first pass splanchnic uptake is bypassed, and gut atrophy occurs that is likely to result in diminished gastro-intestinal requirements[31-32]. This is consistent with the finding that whole body threonine requirements of neonatal piglets receiving TPN is considerably lower than that of piglets receiving an identical diet intragastrically[33]. Interestingly, decreased luminal threonine concentrations negatively affected mucin synthesis in piglets[10]. Therefore, in preterm infants the lower luminal threonine uptake might have resulted in the reduced MUC2 synthesis rates observed. Consequently, it is possible that parenteral nutrition, by restricting the supply of enteral threonine, might severely restrict MUC2 synthesis, causing deterioration of gut barrier function. If so, it is possible that, under circumstances of TPN, the provision of additional threonine via the intestine as a factor in trophic feeding, might provide significant functional benefit to the preterm infant at risk for NEC.

Our finding that porcine MUC2 is highly homologous to human MUC2, provides the opportunity to study human MUC2 synthesis and regulation by nutritional factors in an animal model. Our preterm pig model shows that, besides the route of nutrition, type of enteral nutrition, i.e. colostrum or formula, impacts luminal threonine uptake for MUC2 synthesis. MUC2 synthesis rate was higher in colostrum fed piglets, and was accompanied by a higher luminal threonine uptake. Alternatively, luminal threonine concentrations are known to stimulate mucin synthesis but were in the same range for colostrum and formula fed piglets[10]. Therefore, the mechanism for these effects might be indirectly regulated by colostral growth factors and immunoglobulins stimulating mucosal growth, or directly by stimulation of intestinal metabolic pathways. Colostrum feeding in preterm pigs has shown to decrease NEC incidence by stimulation of gut maturation, nutrient absorption, and protection against colonization, in which adequate threonine supply and hence stimulation of MUC2 synthesis might play an important role[18, 34].

The difference in luminal threonine uptake and MUC2 synthesis between colostrum and formula fed piglets, could not be observed between the preterm infants fed formula or their own mothers' milk. However, the sample size was very limited and therefore there was a lack of power to detect any effect. Furthermore, patients were still recovering from intestinal disease and surgery, and were not on full enteral feeds, and hence might not resemble physiological MUC2 synthesis. However, the isotope infusion protocol had to be performed before removal of the (central) venous catheters required for the isotope infusion, according to our NICU guidelines when close to full enteral intake was reached.

The isotopic data from our experiments suggest that there is threonine uptake from both the luminal and basolateral side for MUC2 synthesis. Furthermore, both luminal threonine uptake and MUC2 synthesis seem to be influenced by the amount and type of enteral nutrition. Firstly, luminal threonine uptake and MUC2 synthesis was low in partially enterally fed preterm infants. Therefore, restriction of enteral threonine might diminish MUC2 synthesis, and might cause the deterioration of gut barrier function that occurs when nutrients are supplied predominantly by the parenteral route. Hence, provision of threonine via the intestine during TPN administration might provide significant functional benefit to the preterm infant at risk for NEC. Secondly, type of enteral feeding, i.e. colostrum feeding, enhances luminal threonine uptake and MUC2 synthesis when compared to formula feeding. Thus, the beneficial effect of human or own mother's milk on prevention of NEC in the preterm infant might not only reside in the direct effect of immune- and growth factors, but also indirectly by stimulation of nutrient uptake, and in this case luminal threonine uptake to enhance the MUC2 synthesis necessary for adequate gut barrier function.

References

1. Specian RD, Oliver MG. Functional biology of intestinal goblet cells. Am J Physiol 1991;260:C183-93.
2. Van Klinken BJ, Tytgat KM, Buller HA, Einerhand AW, Dekker J. Biosynthesis of intestinal mucins: MUC1, MUC2, MUC3 and more. Biochem Soc Trans 1995;23:814-8.
3. Tytgat KM, Buller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J. Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology 1994;107:1352-63.
4. Gum JR, Jr., Hicks JW, Toribara NW, Rothe EM, Lagace RE, Kim YS. The human MUC2 intestinal mucin has cysteine-rich subdomains located both upstream and downstream of its central repetitive region. J Biol Chem 1992;267:21375-83.
5. Lien KA, Sauer WC, Fenton M. Mucin output in ileal digesta of pigs fed a protein-free diet. Z Ernahrungswiss 1997;36:182-90.
6. Mantle M, Allen A. Isolation and characterization of the native glycoprotein from pig small-intestinal mucus. Biochem J 1981;195:267-75.
7. Allen A, Hutton DA, Pearson JP. The MUC2 gene product: a human intestinal mucin. Int J Biochem Cell Biol 1998;30:797-801.
8. Toribara NW, Gum JR, Jr., Culhane PJ, et al. MUC-2 human small intestinal mucin gene structure. Repeated arrays and polymorphism. J Clin Invest 1991;88:1005-13.
9. Faure M, Mettraux C, Moennoz D, et al. Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. J Nutr 2006;136:1558-64.
10. Nichols NL, Bertolo RF. Luminal threonine concentration acutely affects intestinal mucosal protein and mucin synthesis in piglets. J Nutr 2008;138:1298-303.
11. Law GK, Bertolo RF, Adjiri-Awere A, Pencharz PB, Ball RO. Adequate oral threonine is critical for mucin production and gut function in neonatal piglets. Am J Physiol Gastrointest Liver Physiol 2007;292:G1293-301.
12. Faure M, Moennoz D, Montigon F, Mettraux C, Breuille D, Ballevre O. Dietary threonine restriction specifically reduces intestinal mucin synthesis in rats. J Nutr 2005;135:486-91.
13. Anand RJ, Leaphart CL, Mollen KP, Hackam DJ. The role of the intestinal barrier in the pathogenesis of necrotizing enterocolitis. Shock 2007;27:124-33.
14. van der Schoor SR, Wattimena DL, Huijmans J, Vermes A, van Goudoever JB. The gut takes nearly all: threonine kinetics in infants. Am J Clin Nutr 2007;86:1132-8.
15. Stoll B, Burrin DG, Henry J, Jahoor F, Reeds PJ. Phenylalanine utilization by the gut and liver measured with intravenous and intragastric tracers in pigs. Am J Physiol 1997;273:G1208-17.
16. Stoll B, Burrin DG, Henry JF, Jahoor F, Reeds PJ. Dietary and systemic phenylalanine utilization for mucosal and hepatic constitutive protein synthesis in pigs. Am J Physiol 1999;276:G49-57.
17. Schaart MW, de Bruijn AC, Schierbeek H, Tibboel D, Renes IB, van Goudoever JB. Small intestinal MUC2 synthesis in human preterm infants. Am J Physiol Gastrointest Liver Physiol 2009;296:G1085-90.
18. Bjornvad CR, Thymann T, Deutz NE, et al. Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. Am J Physiol Gastrointest Liver Physiol 2008;295:G1092-103.
19. Schaart MW, Schierbeek H, de Bruijn AC, Tibboel D, van Goudoever JB, Renes IB. A novel method to determine small intestinal barrier function in human neonates in vivo. Gut 2006;55:1366-7.

20. Sharma R, Tepas JJ, 3rd, Hudak ML, et al. Portal venous gas and surgical outcome of neonatal necrotizing enterocolitis. J Pediatr Surg 2005;40:371-6.
21. Francois C, Marshall RD, Neuberger A. Carbohydrates in protein. 4. The determination of mannose in hen's-egg albumin by radioisotope dilution. Biochem J 1962;83:335-41.
22. van Klinken BJ, Einerhand AW, Duits LA, et al. Gastrointestinal expression and partial cDNA cloning of murine Muc2. Am J Physiol 1999;276:G115-24.
23. Schaart MW, Schierbeek H, van der Schoor SR, et al. Threonine utilization is high in the intestine of piglets. J Nutr 2005;135:765-70.
24. Reeds PJ, Burrin DG, Stoll B, et al. Enteral glutamate is the preferential source for mucosal glutathione synthesis in fed piglets. Am J Physiol 1997;273:E408-15.
25. Silk DB, Grimble GK, Rees RG. Protein digestion and amino acid and peptide absorption. Proc Nutr Soc 1985;44:63-72.
26. Broer S. Amino acid transport across mammalian intestinal and renal epithelia. Physiol Rev 2008;88:249-86.
27. Hussain I, Kellett L, Affleck J, Shepherd J, Boyd R. Expression and cellular distribution during development of the peptide transporter (PepT1) in the small intestinal epithelium of the rat. Cell Tissue Res 2002;307:139-42.
28. Groneberg DA, Doring F, Eynott PR, Fischer A, Daniel H. Intestinal peptide transport: ex vivo uptake studies and localization of peptide carrier PEPT1. Am J Physiol Gastrointest Liver Physiol 2001;281:G697-704.
29. Pappenheimer JR. On the coupling of membrane digestion with intestinal absorption of sugars and amino acids. Am J Physiol 1993;265:G409-17.
30. Franchi-Gazzola R, Visigalli R, Bussolati O, Dall'Asta V, Gazzola GC. Adaptive increase of amino acid transport system A requires ERK1/2 activation. J Biol Chem 1999;274:28922-8.
31. Shulman RJ. Effect of different total parenteral nutrition fuel mixes on small intestinal growth and differentiation in the infant miniature pig. Gastroenterology 1988;95:85-92.
32. Niinikoski H, Stoll B, Guan X, et al. Onset of small intestinal atrophy is associated with reduced intestinal blood flow in TPN-fed neonatal piglets. J Nutr 2004;134:1467-74.
33. Bertolo RF, Chen CZ, Law G, Pencharz PB, Ball RO. Threonine requirement of neonatal piglets receiving total parenteral nutrition is considerably lower than that of piglets receiving an identical diet intragastrically. J Nutr 1998;128:1752-9.
34. Sangild PT, Siggers RH, Schmidt M, et al. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. Gastroenterology 2006;130:1776-92.

Chapter 6

The regulation of the intestinal mucin MUC2 expression by short chain fatty acids: implications for epithelial protection.

Nanda Burger-van Paassen
Audrey Vincent
Patrycja J. Puiman
Maria van der Sluis
Janneke Bouma
Günther Boehm
Johannes B. van Goudoever
Isabelle Van Seuningen
Ingrid B. Renes

Biochem J. 2009 May 13;420(2):211-9.

Abstract

SCFAs (short-chain fatty acids), fermentation products of bacteria, influence epithelial-specific gene expression. We hypothesize that SCFAs affect goblet-cell-specific mucin *MUC2* expression and thereby alter epithelial protection. In the present study, our aim was to investigate the mechanisms that regulate butyrate-mediated effects on *MUC2* synthesis. Human goblet cell-like LS174T cells were treated with SCFAs, after which *MUC2* mRNA levels and stability, and *MUC2* protein expression were analysed. SCFA-responsive regions and *cis*-elements within the *MUC2* promoter were identified by transfection and gel-shift assays. The effects of butyrate on histone H3/H4 status at the *MUC2* promoter were established by chromatin immune precipitation. Butyrate (at 1 mM), as well as propionate, induced an increase in *MUC2* mRNA levels. *MUC2* mRNA levels returned to basal levels after incubation with 5–15 mM butyrate. Interestingly, this decrease was not due to loss of RNA stability. In contrast, at concentrations of 5–15 mM propionate, *MUC2* mRNA levels remained increased. Promoter-regulation studies revealed an active butyrate-responsive region at –947/–371 within the *MUC2* promoter. In this region we identified an active AP1 (c-Fos/c-Jun) *cis* - element at –818/–808 that mediates butyrate induced activation of the promoter. Finally, *MUC2* regulation by butyrate at 10–15 mM was associated with increased acetylation of histone H3 and H4 and methylation of H3 at the *MUC2* promoter. In conclusion, 1 mM butyrate and 1–15 mM propionate increase *MUC2* expression. The effects of butyrate on *MUC2* mRNA are mediated via AP-1 and acetylation/methylation of histones at the *MUC2* promoter.

Introduction

Short chain fatty acids (SCFAs) are produced by fermentation of undigested carbohydrates. SCFAs, and more specifically acetate, propionate and butyrate, are the major anions in the lumen of the large intestine. Several functions of SCFAs have been described, i.e. lowering intestinal pH, energy-source for colonocytes, stimulation of colonic blood flow, smooth muscle contraction, transepithelial chloride secretion and exertion of proliferative stimuli of colonic epithelial cells[1]. It is known that dietary fibers and SCFA have beneficial effects in inflammatory bowel disease (IBD), e.g. by inhibition of proinflammatory cytokine-induced NFκB activation and absorption of sodium and water[2-4]. In addition, SCFAs and especially butyrate are known to influence intestinal specific gene expression, thereby influencing immune responses and oxidative and metabolic stress[5-9].
The composition of SCFAs in the intestine is determined by the composition of the microbiota, which in its turn is influenced by the diet. For example, prebiotics selectively stimulate the growth, and or, activity of bifidobacteria and thereby influence the SCFA composition[10].
Moreover, in human milk-fed infants the large bowel is generally dominated by bifidobacteria and lactic acid bacteria. The flora of formula-fed infants on the other hand, is more diverse, less stable and often contains more *Bacteroides*, *Clostridium* and *Enterobactericeae*[11-14]. This difference in the composition of the microbiota results in a different SCFA composition between human milk-fed and formula-fed infants. It is well known that the ratio between the SCFAs butyrate, propionate, and acetate differ in breast-fed infants compared to formula-fed infants (i.e. 2:6:90 in human milk fed infants versus 5:20:70 in formula fed infants)[15]. Based on the fact that more than 90% of the infants who develop necrotizing enterocolitis (NEC), which is the most common gastrointestinal emergency in premature infants, have received formula feeding, as opposed to human milk solely, one could suggest that the production of SCFAs by bacteria and the composition of SCFAs in the intestine might play a role in the pathogenesis of NEC. Altered fecal concentrations of butyrate have also been reported in patients with ulcerative colitis (UC). In addition, a diminished capacity of the intestinal mucosa to oxidize butyrate has been reported in patients with active UC[16-18].
Both UC and NEC share the feature of an impaired intestinal barrier function. Mucins are required for the maintenance of an adequate mucus layer that covers the intestinal epithelium and thereby forms a physical barrier that protects the intestinal epithelium against toxic agents. The mucin MUC2 is the predominant mucin in the colon and MUC2 synthesis is diminished in UC[19, 20] and presumably also NEC.
It has been shown in cell line studies, experimental animal models and fresh human intestinal tissue specimens, that butyrate alters *MUC2* expression in a dose dependent

manner[21-25]. However, the mechanisms that are responsible for these alterations have not been studied in detail so far.
In the present study, we investigated the role of increasing concentrations of butyrate, as well as acetate and propionate on *MUC2* expression in LS174T cells, a human goblet cell-like cell line. Furthermore, The effects of butyrate on MUC2 expression were respectively studied at the promoter, mRNA and protein levels. We identified butyrate-responsive regions and *cis*-elements within the *MUC2* promoter and determined the effects of butyrate treatment on histone H3 and H4 status at the *MUC2* promoter.

Experimental

Cell culture

The LS174T colonic cancer cell line was cultured in a 37 °C incubator with 10% CO2 in Dulbecco's modified Eagle's minimal essential medium supplemented with non-essential amino acids and 10% foetal calf serum (Boehringer Mannheim) as before[26].

Cell proliferation assay and morphological alterations

LS174T cells (2 × 105) were pre-cultured in 24-well plates overnight to allow them to adhere. Subsequently cells were stimulated with physiological concentrations of butyrate (0, 1, 5, or 10 mM) (sodium butyrate, Sigma-Aldrich, Steinheim, Germany) diluted in cell culture medium for 24 h. After removal of the culture medium, cells were treated with trypsin–EDTA solution and counted. All experiments were performed in triplicate in at least three separate experiments. In addition, cell proliferation and cell death were determined using the WST-I (WST-I proliferation agent, Roche Molecular Biochemicals, Germany) and trypan blue exclusion assays, respectively. Further, butyrate-induced morphological changes were studied microscopically.

Quantitative real- time PCR

LS174T cells were seeded in 6-well plates at 0.5x106 cells/well. Cells were incubated 16 hours after seeding with either a low (1mM), moderate (5 mM) or high (10 and 15 mM) concentration of butyrate, acetate or propionate (Sigma-Aldrich, Steinheim, Germany). After 24 hours of stimulation, cells were lysed and harvested. Total RNA was prepared using the Nucleospin RNA II-kit from Macherey-Nagel. 1.5 μg of total RNA was used to prepare cDNA. The mRNA expression levels of MUC2 as well as the housekeeping gene GAPDH were quantified using real-time PCR (qRT-PCR) analysis (TAQman chemistry) based upon the intercalation of SYBR Green on an ABI prism 7900 HT Fast Real Time PCR system (PE Applied Biosystems) as previously described[27]. The primer combinations for MUC2 (5'-CTC CGC ATG AGT GTG AGT -3', and 5'-TAG CAG CCA CAC TTG

TCT G -3’) and GAPDH (5’-GTC GGA GTC AAC GGA TT -3’, and 5’-AAG CTT CCC GTT CTC AG -3’) were designed using OLIGO 6.22 software (Molecular Biology Insights) and purchased from Invitrogen. The effect of butyrate on MUC2 transcription and *de novo* protein synthesis was respectively studied by co-incubating cells with butyrate and either actinomycin D (0.5 μg/ml) or cycloheximide (10 μg/ml) (Sigma-Aldrich, Steinheim, Germany). qRT-PCR was performed as described above.

RNA stability assay

LS174T cells were seeded at 1x106 cells/well in a 6-well cluster 24h prior to the experiment. At t=0, the cells were treated with either 0 mM or 5 mM butyrate in combination with 4 μg/ml actinomycin D (Sigma-Aldrich, Steinheim, Germany). Cells were harvested after 0, 4, 6, 8, 10 and 24 h of butyrate/actinomycin D treatment. RNA isolation, cDNA synthesis and qRT-PCR were performed as mentioned above. To verify the amplification efficiency as well as the amount of mRNA present in the treated cells, a serial dilution of cDNA derived from non-treated LS174T cells was amplified in duplicate on each plate.

Cell transfections

pGL3-*MUC2* promoter constructs covering the -371/+27, -947/-1, -2096/+27 and -2627/-1 regions of *MUC2* promoter were previously described27. The AP-1-Luc reporter construct was a kind gift from Dr Avery (Pennsylvania State University, USA). LS174T cells were seeded at 2.0 x 105/well in 24-well plates. Transfections and co-transfections were performed the next day by adding 0.25 μg of the pGL3 construct of interest and 0.15 μg of phRG-B as an internal control. Transfection and co-transfection experiments were performed using Effectene® reagent (Qiagen) as described previously[26]. Cells were incubated with the transfection mixture for 24 h at 37 °C. Stimulation with variable dosage of butyrate was performed during 24 h. Total cell extracts were prepared using 1x passive lysis buffer (Promega), as described in the manufacturer’s instruction manual. 10 μl of cell extract was used to determine luciferase activity in a Glomax luminescence counter (Promega) using the dual luciferase assay system (Promega). The luciferase activity is expressed as fold induction of the nonstimulated sample compared with that of the SCFA-stimulated samples, after correction for transfection efficiency as measured by the *Renilla* luciferase activity. All experiments were performed in triplicate in at least three separate experiments.

Site-directed mutagenesis

The consensus AP-1 site (ATGAGTCAGA) found in the MUC2 promoter at -817/-808 was mutated using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The sequence of the oligonucleotides used to mutate the AP-1 site are depicted in table 1. The mutation was confirmed by DNA sequencing.

Table 1. Sequences of the sense oligonucleotides used for site-directed mutagenesis and EMSA.

Mutations	Sequence 5′→3′
Site directed mutagenesis	
WT 114 (-830/-795)	CAG GAT CCC CAC C*AT GAG TCA GAG* GTA GTT CTG GGG
Mutated T114	CAG GAT CCC CAC CA**G** GAG **C**CA GAG GTA GTT CTG GGG
EMSA	
T282 (-822/-804)	CCA CCA TGA GTC AGA GGT A
Mutated T282	CCA CCA T**T**A GTG AGA GGT A

AP-1 consensus sequence is italicized. Mutated nucleotides are bold and underlined. Antisense oligonucleotides were also synthesized and annealed to the sense oligonucleotide to produce double-stranded DNA.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from LS174T cells, untreated or treated with butyrate were prepared as described before[28], quantified using the bicinchoninic acid assay (Pierce, Perbio Science, Brebières, France) and stored at -80°C. Oligonucleotides were synthesized by MWG-Biotech (Germany), sequences are shown in Table 1. Annealed oligonucleotides were radiolabeled using T4 polynucleotide kinase (Promega) and [γ32P]-dATP (GE Healthcare) and purified by chromatography on a Bio-Gel P-6 column (Bio-Rad, Marnes-la-Coquette, France). Nuclear protein incubation with radiolabeled probes and competitions with unlabeled probes were as described in Perrais et al.[26]. For super-shift analyses, 2 µl of the antibody of interest [anti-c-fos (K-25, SC-253X) and anti-c-jun (SC-44X), Santa-Cruz laboratories, Tebu-Bio, Le-Perray-en-Yvelines, France, were added to the proteins and left for 1 h at room temperature (22°C) before adding the radiolabeled probe. Electrophoresis conditions and gel processing were as described before[26].

Western Blotting

Nuclear proteins (10 µg) were separated by running a 10% SDS-polyacrylamide gel electrophoresis, followed by electrotransfer onto a 0.45 µm PVDF membrane (Millipore, Saint-Quentin en Yvelines, France). The membranes were incubated either with specific antibodies against c-fos (sc-253, 1:10,000) or c-jun (sc-44, 1:5,000) (Santa Cruz laboratories) for 1 h at room temperature, or with specific antibodies against histone H3 (anti-acetylated lysine, mono-/di-/trimethylated lysine 4) and histone H4 (anti-acetylated lysine) (Upstate #06-599 (1:10,000 dilution), #05-791 (1:10,000 dilution) and #06-598 (1:1,000 dilution), respectively for 2 h at room temperature. Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit IgGs (Pierce). For detection, blots were processed with West® Pico chemiluminescent substrate (Pierce) and the signal was detected by exposing the processed blots to HyperfilmTM ECL® (enhanced chemiluminescence; Amersham Biosciences). For Sp1 detection, the membranes were incubated 1 h at room temperature with anti-Sp1 antibody (sc-59, 1:10,000, Santa Cruz laboratories) and alkaline phosphatase-conjugated anti-goat IgGs (Promega) as secondary antibody. For detection, the membrane was incubated with

Nitro Blue Tetrazolium Chloride and 5-bromo-4-chloro-3-indolyl phosphate substrate (Life Technologies, Cergy-Pontoise, France)[29].

Immunocytochemistry

LS174T cells were grown on poly-L-Lysine coated microscope glass slides 24 h prior to butyrate treatment. Cells were treated with 0, 1, 2, 5 and 10 mM butyrate for 24h. Cells were fixed in ice-cold methanol at –20°C for 10 min and rinsed in phosphate-buffered saline (PBS). The MUC2 mucin expression was determined by immunocytochemistry. For this purpose, cells were incubated for 60 min at room temperature with the monoclonal MUC2 antibody (WE9)[30] diluted in PBS (1:200), rinsed four times with PBS, and incubated at room temperature for 60 min with the biotinylated horse-anti-mouse antibody (Vector) diluted in PBS (1:1000), followed by 1h incubation with ABC-PO complex (Vectastain Elite Kit, Vector laboratories), each component diluted 1:400 in PBS. After incubation, binding was visualized using 0.5mg/ml 3,3'-diaminobenzidine (DAB), 0.02% v/v H2O2 in 30mM Immidazole, 1 mM EDTA (pH7.0). The slides were counterstained with hematoxylin.

Chromatin Immunoprecipitation (ChIP)

Cells untreated or treated with butyrate (10×106) were fixed in 1% (v/v) formaldehyde and chromatin was sonicated and immunoprecipitated as described in Piessen et al.[31] with either 5 µg of specific antibodies against histone H3 (anti-acetylated lysine, mono-/di-/trimethylated lysine 4, methylated lysine 9 and trimethylated lysine 27) and histone H4 (anti-acetylated lysine) (Upstate) or with normal rabbit IgGs (Upstate) at 4°C. Immunoprecipitated chromatin (50 ng) was then used as a template for PCR using the following pairs of primers: forward primer1: 5'-TTGGCATTCAGGCTACAGGG-3' and reverse primer1: 5' GGCTGGCAGGGGCGGTG-3', covering the -236/+24 region of *MUC2* promoter. PCR was performed using AmpliTaq Gold polymerase as described by Piessen et al.[31]. PCR products (15 µl) were separated on a 2% (w/v) agarose gel containing ethidium bromide run in 1X TBE [Tris/borate/EDTA (1×TBE=45 mM Tris/borate and 1 mM EDTA)] buffer.

Statistical analysis

All values in this article are mean values ± standard deviation (SD).

Results

Butyrate affects cell morphology and proliferation

SCFAs are known to affect epithelial proliferation, differentiation, apoptosis, and gene expression. Butyrate is, compared to acetate and propionate, the most effective SCFA in inducing alterations in these processes. Therefore, we first analyzed the effects of various concentrations of butyrate on morphology, proliferation and apoptosis of LS174T cells. Butyrate induced marked changes in morphology, which are characterized by elongation/stretching of the cells (Fig. 1A). Furthermore, butyrate treatment inhibited the proliferation of the LS174T cells in a dose-dependent manner, as reflected by a decrease in cell number upon butyrate treatment (Fig. 1B). These data were confirmed by WST-1 cell proliferation assays (data not shown). Finally, none of the butyrate concentrations (1-10 mM) used in this study induced cell death of LS174T cells, as determined by trypan-blue exclusion assays and analysis of cell morphology.

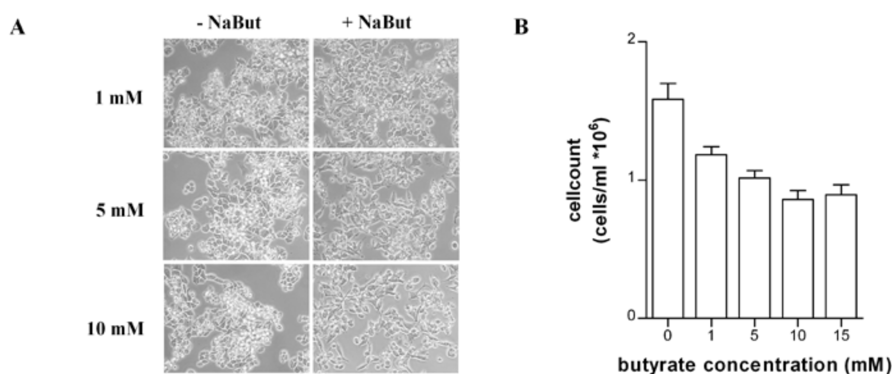


Figure 1. Butyrate affects cell morphology and proliferation
(A) LS174T cells stimulated with 1, 5 or 10 mM butyrate (right panel) demonstrate stretching/ elongation and flattening compared to untreated cells (left panel). (B) Cell counts before and after butyrate stimulation show a dose-dependent decrease in cell number after butyrate stimulation.

SCFAs alter MUC2 mRNA expression

LS174T cells were stimulated with increasing concentrations, from 1 mM to 15 mM, of butyrate, propionate, or acetate to determine the effects of the different SCFAs on *MUC2* mRNA expression (Fig. 2A). One mM butyrate induced a 2.5-fold increase in *MUC2* mRNA levels compared to untreated cells. In contrast, stimulation with higher concentrations, i.e. 5-15 mM butyrate, did not induce an increase in *MUC2* mRNA levels, as at these concentrations *MUC2* mRNA levels were comparable with control levels. Similar to butyrate, 1 mM propionate induced a 2.5 fold increase in *MUC2* mRNA levels. Furthermore, at 5 mM propionate, *MUC2* mRNA levels increased 4.2 fold, whereas at

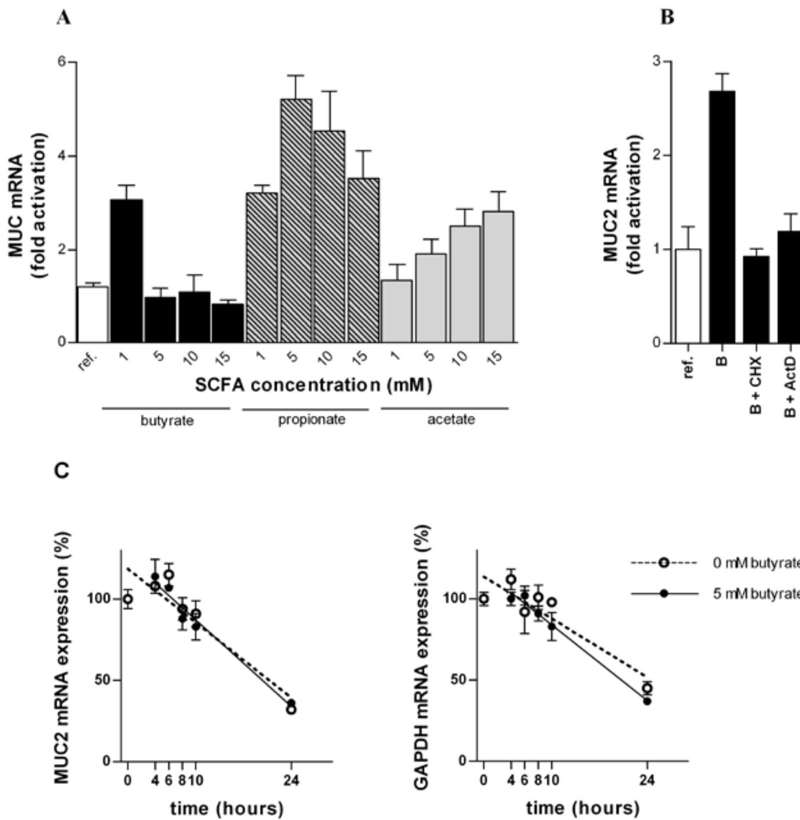


Figure 2. SCFAs alter *MUC2* mRNA expression
(A) MUC2 mRNA fold activation in LS174T cells upon stimulation with SCFAs (butyrate, propionate and acetate) compared to untreated cells (ref.). (B) To determine whether butyrate stimulation influences MUC2 synthesis on transcriptional or translational level, LS174T cells were stimulated with butyrate (B) (1 mM) in the presence of cycloheximide (CHX) or actinomycin D (ActD). (C) Stability of MUC2 mRNA (left panel) and GAPDH mRNA (right panel) over time was measured by RT-PCR. LS174T cells were stimulated with butyrate (5 mM) after which mRNA synthesis was ceased through addition of actinomycin D. Relative mRNA expression was determined at the given time points (0, 4, 6, 8, 10 and 24 hours after addition of actinomycin D). All results represent means ± SD obtained in triplicate in three separate experiments

higher concentrations *MUC2* mRNA levels decreased again. Acetate treatment resulted in a dose-dependent increase in *MUC2* mRNA levels as of 5 mM reaching a 2.2 fold induction at 15 mM. To determine whether the activating effect of SCFAs on MUC2 expression occurred at the transcriptional level, cells were pretreated with actinomycin D, which inhibits transcription. The results indicate that activation of *MUC2* expression by 1 mM butyrate occurred at the transcriptional level, as *MUC2* mRNA levels returned to basal levels when cells were pretreated with actinomycin D (Fig. 2B). This process also requires de novo protein synthesis as pretreatment of LS174T cells with cycloheximide, an inhibitor of mRNA translation, decreased MUC2 mRNA levels to basal levels as well (Fig. 2B). Similar results were obtained when cells were stimulated with propionate or

acetate instead of butyrate (data not shown). Since butyrate increased *MUC2* mRNA levels at low concentrations (1 mM) in contrast to no effect at moderate (5 mM) and high (10 and 15 mM) concentrations, we studied whether this decrease was due to a decrease in *MUC2* RNA stability. For that we pre-treated cells with actinomycin D over a 24h period of time, and then incubated cells with 5 mM butyrate before measuring *MUC2* mRNA amount by qRT-PCR. The results show no differences in MUC2 mRNA stability between butyrate-stimulated and non-stimulated cells (Fig. 2C).

Effect of butyrate on MUC2 protein expression

To determine whether butyrate also induced an increase in MUC2 protein expression in LS174T cells, immunocytochemistry was performed with an antibody specific for MUC2. In non-stimulated cells MUC2 staining was hardly visible (Fig. 3). Stimulation with 1 mM of butyrate clearly shows an increase in MUC2 staining. This effect was even more pronounced in cells stimulated with 2 mM of butyrate (Fig. 3).

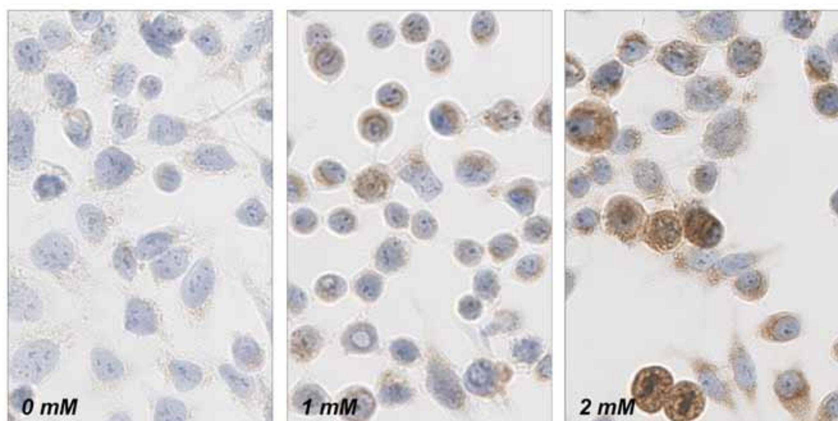


Figure 3. Effect of butyrate on MUC2 protein expression
MUC2 apomucin expression by immunocytochemistry in non-stimulated (0 mM) and butyrate (1 and 2 mM) stimulated LS174T cells. (Magnification x40)

Identification of butyrate responsive regions in the *MUC2* promoter

Transfections with *MUC2* promoter constructs were performed to identify butyrate-responsive regions. The *MUC2* promoter constructs used are indicated in Figure 4A. Stimulation of LS174T cells with low (0.5-2 mM) concentrations of butyrate demonstrated a dose-dependent increase in luciferase-activity after transfection with each of the promoter construct used (Fig. 4B). The highest transactivation was seen using *MUC2* promoter construct -947/-1 indicating a possible butyrate-responsive element within the -947/-372 region. Analysis of the *MUC2* promoter sequence indeed revealed the presence of a consensus putative binding site (ATGAGTCAGA) for the transcription factor AP-1 at –817/-808, a transcription factor known to mediate butyrate-induced

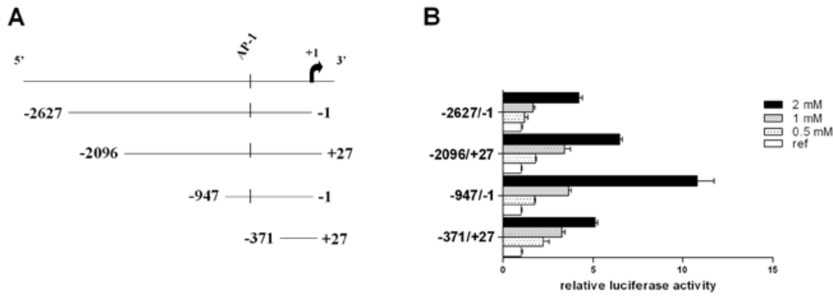


Figure 4. Identification of butyrate responsive regions in the *MUC2* promoter
Schematic representation of MUC2 promoter and the different constructs used to study MUC2 promoter activity (A). Numbering refers to transcription initiation site designated +1. (B) Transfected cells were stimulated with butyrate as described in experimental procedures. Results are expressed as fold activity in the butyrate-stimulated cells compared to non-stimulated cells. Results represent means ± SD obtained in triplicate in three separate experiments.

transcriptional effects. To determine whether this putative AP-1 binding site was responsible for the butyrate-induced *MUC2* promoter transactivation, specific nucleotides within the sequence were mutated (Table 1). The mutation resulted in a 50% reduction of the butyrate-induced *MUC2* transactivation (Fig. 5A). Activation of AP-1 by butyrate in LS174T cells was confirmed by treating AP-1-Luc-transfected cells with butyrate. The stimulation was dose-dependent with a maximal 13.3-fold induction at 2 mM butyrate (Fig. 5B).

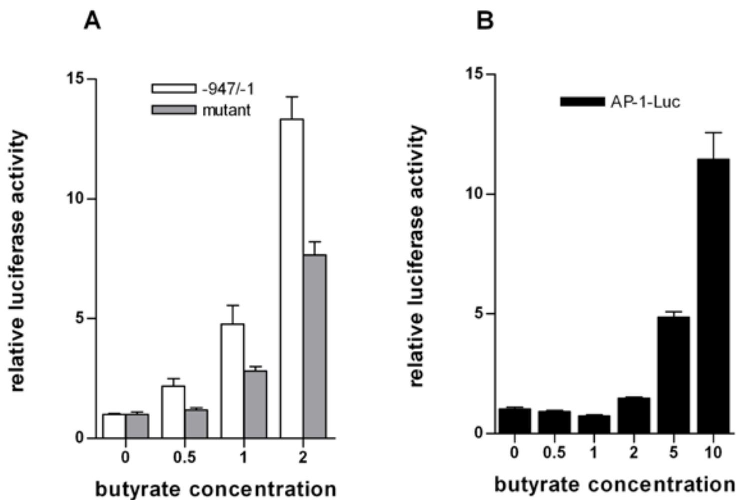


Figure 5. The effect of site directed mutagenesis on *MUC2* promoter activation by butyrate
(A) Relative luciferase activity diagram showing the effect of site-directed mutagenesis on MUC2 promoter activation by butyrate. Cells were transfected either with the wild-type -947/-1 construct (WT, white bars) or with the AP-1 mutated construct (mut, grey bars) before being stimulated with butyrate as described in experimental procedures. (B) Luciferase activity in cells transfected with AP-1-Luc reporter construct and stimulated with butyrate. Results are expressed as fold activity of the butyrate-stimulated compared to non-stimulated cells. Results represent means ± SD obtained in triplicate in three separate experiments.

C-fos and c-jun bind to the AP-1 element in *MUC2* promoter

As the transcription factors c-fos and c-jun are known to bind as a complex to AP-1 binding elements within promoters, EMSAs were carried out to show the binding of these two transcription factors to the AP-1 element found at -817/-808. When incubated with nuclear extract from untreated and butyrate-stimulated (1 mM and 10 mM) LS174T cells, the radiolabeled probe T282 (containing the putative AP-1 binding site, see table I) produced one retarded band (Fig.6, lane 2). Specificity of the protein-DNA complex was confirmed by strong decrease of the shifted band when unlabeled competition was performed with a 50 times excess of unlabeled T282 probe (lane 3), whereas competition with a 50 times excess of unlabeled mutated T282 probe (lane 4) did not affect the shifted band. Involvement of c-jun and c-fos in the complex formation was then proven in supershift experiments carried out with antibodies specific for c-jun (lane 5) and c-fos (lane 6), respectively. Addition of the two antibodies indeed resulted in a supershift that was observed both in untreated cells and butyrate-stimulated cells. This was well-correlated with the amount of c-fos and c-jun found in the cells (Fig. 7). Altogether, this suggests that the decreased *MUC2* mRNA levels after stimulation of cells with 5 mM or 10 mM butyrate compared to 1 mM butyrate stimulation (see Fig. 2), are not caused by a decreased binding capacity of AP-1 (i.e. the c-fos/c-jun complex) to its cis-element within the *MUC2* promoter.

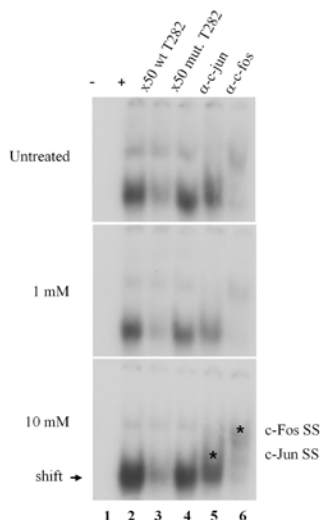


Figure 6. C-fos and c-jun bind to the AP-1 element in *MUC2* promoter
Identification of an AP-1 cis-element in MUC2 promoter by EMSA. Nuclear extracts from untreated cells or cells treated with either 1 or 10 mM butyrate. Radiolabeled probe T282 alone (lane 1), radiolabeled T282 with nuclear extract (lane 2), cold competition with 50x excess of unlabeled wt T282 probe (lane 3), cold competition with 50x excess of unlabeled mutated T282 (lane 4). Supershift analysis was performed by preincubating the nuclear extract with 1 μl of anti-c-jun (lane 5) and anti-c-fos (lane 6) antibodies, respectively. Arrows and stars indicate positions of the shifted and supershifted (ss) protein-DNA complexes.

Butyrate alters histone status at the *MUC2* promoter in a dose-dependent manner

Since butyrate is known to affect histone deacetylase (HDAC) activity, and that *MUC2* promoter is known to be regulated by HDAC[32], we hypothesized that histone status at *MUC2* promoter may be involved in *MUC2* regulation by butyrate. To determine whether alterations in *MUC2* expression correlated to changes in histone acetylation and/or methylation we first examined the effect of butyrate on the levels of acetylated H3 and H4 histones as well as m/d/t methylated H3K4 in LS174T cells by Western blotting (Fig. 7). Acetylated histone H3 and H4 and mono-/di-/tri-methylated H3 on lysine (K) 4, which correlate with activation of transcription, were strongly increased after stimulation with both moderate (5 mM) and high (10 mM) concentrations of butyrate.

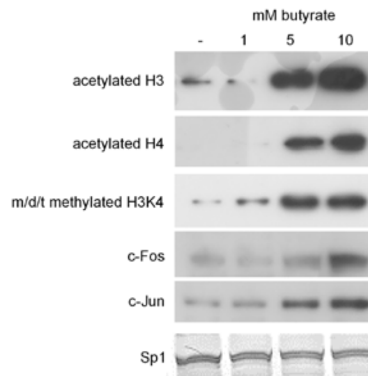


Figure 7. Expression of acetylated histones upon butyrate stimulation
Study of the expression of acetylated histones H3 and H4, m/d/t methylated H3K4, c-Fos, c-Jun and Sp1 in untreated (-) and butyrate-treated (1, 5 and 10 mM) LS174T cells by western-blotting.

To establish the effects of butyrate on histone H3 and H4 status at the *MUC2* promoter, ChIP assays were performed with chromatin from non-stimulated and butyrate-stimulated LS174T cells (Fig. 8). In untreated and 1 mM butyrate-treated cells, *MUC2* promoter covering the -236/+24 region was mainly associated with mono-/di-/tri-methylated K4H3 as well as to a lower extent with acetylated histone H3 and H4, which correlate with activation of transcription. At 5 mM butyrate this status of chromatin activation was confirmed with a stronger association with acetylated H3. At these two concentrations we also observed an increase of 3mK27H3, which is usually indicative of transcription inhibition. At 10 mM butyrate, histone modifications at the *MUC2* promoter were characterized by modifications inducing active chromatin (AcH3, AcH4 and m/d/tK4H3) and usually indicative of inactive chromatin (mK9H3).

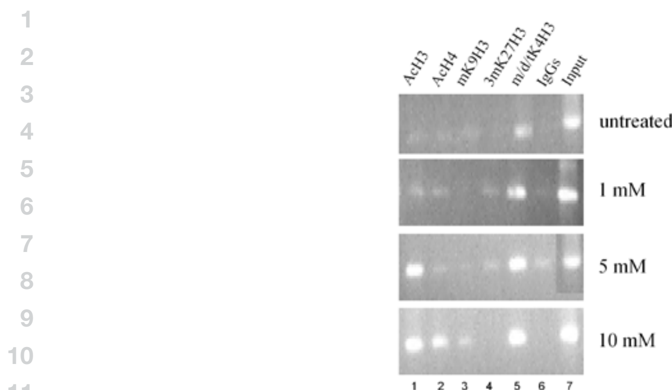


Figure 8. Butyrate alters histone status at the *MUC2* promoter in a dose-dependent manner
Study of the histone status at the MUC2 promoter by ChIP. Acetylated histone H3 (AcH3, lane 1), acetylated histone H4 (AcH4, lane 2), and m/d/tK4H3 (lane 5) are representative of activation of transcription whereas histone H3 methylated on lysine 9 (mK9H3, lane 3) and histone H3 trimethylated on lysine 27 (3mK27H3, lane 4) are representative of inhibition of transcription. Lane 6: negative control with IgGs. Lane 7: Input.

Discussion

In the present study we analyzed the effect of SCFAs on epithelial cell morphology, proliferation, and, as marker for epithelial protection, *MUC2* expression. Moreover, we identified the mechanisms responsible for the butyrate-induced changes in *MUC2* expression. By studying these parameters in conjunction we aimed to gain more insight in the effects of SCFAs on epithelial protection.

The present study revealed that butyrate altered the morphology of LS174T cells by inducing cell elongation/stretching. This suggests that butyrate affects LS174T cell differentiation. Additionally, butyrate caused a dose-dependent decrease in cell number. As this SCFA did not induce apoptosis at the concentrations used in this study, we conclude that butyrate inhibits epithelial proliferation. Several *in vitro* studies support our finding that butyrate inhibits proliferation[33-37]. For example, butyrate inhibited the proliferation of non-confluent and sub-confluent HT-29 cells in a dose-dependent manner (1-8 mM)[36]. Furthermore, Siavoshian et al. demonstrated that the mechanism through which butyrate inhibits proliferation in HT-29 cells is exerted via the induction of cyclin D3, an inhibitor of cell cycle progression and p21, a stimulator of cell differentiation[33].

Next, we studied the effect of SCFAs on MUC2 synthesis in the LS174T cell line. Butyrate, propionate, as well as acetate, were able to increased *MUC2* mRNA synthesis. Specifically, butyrate increased *MUC2* mRNA levels at low concentrations and had no effect at moderate and high concentrations. Both low and moderate propionate concen-

trations increased *MUC2* mRNA levels, whereas at higher concentrations, *MUC2* mRNA levels were still increased but to a lesser extent. Finally, a dose-dependent increase in *MUC2* mRNA levels was seen after stimulation with acetate, with the smallest increase at low concentrations and highest increase at high concentrations.

Of the SCFAs studied in this paper, only the effects of butyrate on mucin expression have been described extensively. Hatayama et al. also showed that concentrations of 1-2 mM butyrate increased MUC2 expression in LS174T cells[22]. Barcelo et al.[23] demonstrated a significant discharge of mucins at concentrations of 5 mM of butyrate, while increasing the concentration to 100 mM decreased this mucus response in mice. Highly relevant, *ex vivo* stimulation of macroscopically normal fresh colon tissue with 0.05-1 mM butyrate stimulates MUC2 synthesis, whereas at stimulation with 10 mM butyrate MUC2 synthesis levels returned to basal levels[24]. These studies correlate with our data with respect to the effects of butyrate on *MUC2* expression (i.e. increase in MUC2 at low concentration and no effect at high concentrations).

Despite previous studies showing induction of MUC2 by butyrate, no precise analysis of the molecular mechanisms has been performed[21-25]. Since butyrate is known to mediate its effects via the AP-1 transcription factor, and because we found a putative consensus binding site (ATGAGTCAGA) for AP-1 at -817/-808 in the *MUC2* promoter, we studied its regulation by AP-1. AP-1 is a multiprotein complex, composed of the products of c-Jun and c-Fos proto-oncogenes. Growth factors, neurotransmitters, polypeptide hormones, bacterial and viral infections as well as a variety of physical and chemical stresses, employ AP-1 to translate external stimuli, both into short-term and long-term changes of gene expression. Interestingly, we found that butyrate was able to activate an AP-1 reporter construct and to induce c-Fos and c-Jun protein expression in the LS174T cell line, indicating that butyrate-induced MUC2 transcription might occur via AP-1 binding to the *MUC2* promoter. That is what we indeed demonstrated by mutating the consensus AP-1 binding site, which abolished both binding of AP-1 and inhibited butyrate-induced *MUC2* activation. As butyrate only increased *MUC2* mRNA and protein levels at low concentrations (1-2 mM), but not at high concentrations (5-10 mM), this suggested that activation of the MUC2 promoter, and up-regulation of MUC2 RNA and protein levels, at low concentration of butyrate was, at least partly, regulated by AP-1.

Since butyrate is a well-known HDAC inhibitor, butyrate-induced alterations in gene expression can also reflect changes in histone modification status and chromatin marks. To assess whether the increase of *MUC2* expression following butyrate treatment was associated with chromatin status, we performed ChIP experiments. As expected, our data indicate that butyrate treatment is associated with dose-dependent increased of both global histone acetylation levels and histone H3 and H4 acetylation at *MUC2* promoter region. Since cross talk between histone post-translational modifications are important

in establishing the histone code, increased mono-/di-/trimethylation of K4H3 observed at *MUC2* promoter after butyrate treatment may be directly linked to increased histone H3 acetylation, as previously shown by Nightingale et al.[38]. For a long time methylation of K9H3 has been considered as a chromatin mark associated with heterochromatin and gene silencing. However, a recent study showed that higher H3K9 monomethylation levels are detected in active promoters surrounding gene transcription start sites, suggesting that this modification may be associated with transcription activation[39]. These data are concordant with our present results, showing that in LS174T cells, the proximal region of *MUC2* promoter is associated with monomethylation of K9H3. However, to our knowledge, this is the first time that a positive effect of high concentrations of butyrate on K9H3 methylation is shown. Strikingly, we found that treatment with low concentration of butyrate induced an increase of H3K27 trimethylation at *MUC2* promoter, which therefore adopted a bivalent chromatin pattern. This bivalent profile has already been described for embryonic stem cell genes as well as DNA-hypermethylated genes which were reexpressed by demethylation[40]. We previously showed that *MUC2* is regulated by a complex combination of DNA (de)methylation and establishment of a (de)repressive histone code[41]. The changes of global epigenetic profile stated at *MUC2* promoter may thus be partly responsible for the increase in *MUC2* expression level induced by low concentration of butyrate.

Surprisingly, treatment with moderate and high concentrations of butyrate, yet associated with active chromatin marks at the *MUC2* promoter, did not induce increased *MUC2* expression. Dual effects of HDAC inhibitors on gene expression have already been shown for numerous genes[42], including mucin genes. In particular, Augenlicht et al.[42] demonstrated that cell treatment with butyrate induce an inhibition of *MUC2* expression, correlated with repression of secretory functions of colonic cells. This repression may be due to changes in histone modification patterns, since trichostatin A, another HDAC inhibitor, has the same inhibiting effect on *MUC2* expression in LS174T cells[41]. However, our results clearly show that *MUC2* promoter is associated with active chromatin marks at high concentrations of butyrate. Therefore, the dual effect observed at high concentrations, is most likely due to dramatic butyrate-induced changes in the global chromatin landscape[43], rather than direct histone modifications at *MUC2* promoter. Numerous studies showing that expression and post-translational modifications of factors known to positively or negatively regulate *MUC2* transcription (including, among others, Sp3 , CDX-2 or p53 transcription factors[44-46]) is dramatically affected by butyrate, support this hypothesis.

Taken together, we have shown that butyrate stimulates *MUC2* expression at low concentrations, but has no effect on *MUC2* expression at moderate and high concentrations. We therefore hypothesize that low concentrations of butyrate could have a protective effect on intestinal barrier function by increasing mucus production, whereas

moderate to high concentrations may decrease intestinal barrier function by decreasing MUC2 production. This effect might partially explain the difference in incidence of NEC between the formula-fed and human milkfed newborn infants. Manipulation of the SCFA profile can be established by influencing the composition of microbiota, for instance by treatment with prebiotics, probiotics and/or human milk. This approach seems to be promising in the treatment of IBD and NEC. In summary, the *in vitro* results in the present study indicate that low concentrations of butyrate stimulate MUC2 mucin expression, which *in vivo* would lead to an increased intestinal epithelial barrier function. In contrast, high concentrations of butyrate decrease MUC2 expression which might diminish intestinal barrier function. Moreover, identification of AP-1 and histone modifications as mechanisms involved in MUC2 regulation by butyrate may represent pathways to target prevention of IBD and NEC by influencing SCFA production by the intestinal microbiota.

Acknowledgements

We thank Dr Avery (Pennsylvania State University, University Park, PA, U.S.A.) for the gift of the AP-1-Luc construct.

Funding

This work was supported by unrestricted grants from The Nutricia Research Foundation and The Association François Aupetit (to I. V. S.). A. V. is the recipient of an Inserm-région Nord-Pas de Calais PhD fellowship.

References

1. Cummings JH RJ, Sakata T Physiological and clinical aspects of short-chain fatty acids. Cambridge UniversityPress, Cambridge 1995.
2. Andoh A, Tsujikawa T, Fujiyama Y. Role of dietary fiber and short-chain fatty acids in the colon. Curr Pharm Des 2003;9(4):347-58.
3. Galvez J, Rodriguez-Cabezas ME, Zarzuelo A. Effects of dietary fiber on inflammatory bowel disease. Mol Nutr Food Res 2005;49(6):601-8.
4. Tedelind S, Westberg F, Kjerrulf M, Vidal A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. World J Gastroenterol 2007;13(20):2826-32.
5. Alvaro A, Sola R, Rosales R, et al. Gene expression analysis of a human enterocyte cell line reveals downregulation of cholesterol biosynthesis in response to short-chain fatty acids. IUBMB Life 2008.
6. Sauer J, Richter KK, Pool-Zobel BL. Physiological concentrations of butyrate favorably modulate genes of oxidative and metabolic stress in primary human colon cells. J Nutr Biochem 2007;18(11):736-45.
7. Welter H, Claus R. Expression of the monocarboxylate transporter 1 (MCT1) in cells of the porcine intestine. Cell Biol Int 2008;32(6):638-45.

8. Kiela PR, Kuscuoglu N, Midura AJ, et al. Molecular mechanism of rat NHE3 gene promoter regulation by sodium butyrate. Am J Physiol Cell Physiol 2007;293(1):C64-74.
9. Daly K, Shirazi-Beechey SP. Microarray analysis of butyrate regulated genes in colonic epithelial cells. DNA Cell Biol 2006;25(1):49-62.
10. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 1995;125(6):1401-12.
11. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr 2000;30(1):61-7.
12. Balmer SE, Wharton BA. Diet and faecal flora in the newborn: breast milk and infant formula. Arch Dis Child 1989;64(12):1672-7.
13. Stark PL, Lee A. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J Med Microbiol 1982;15(2):189-203.
14. Kleessen B, Bunke H, Tovar K, Noack J, Sawatzki G. Influence of two infant formulas and human milk on the development of the faecal flora in newborn infants. Acta Paediatr 1995;84(12):1347-56.
15. Knol J, Scholtens P, Kafka C, et al. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. J Pediatr Gastroenterol Nutr 2005;40(1):36-42.
16. Burrin DG, Davis TA, Ebner S, et al. Nutrient-independent and nutrient-dependent factors stimulate protein synthesis in colostrum-fed newborn pigs. Pediatr Res 1995;37(5):593-9.
17. Den Hond E, Hiele M, Evenepoel P, Peeters M, Ghoos Y, Rutgeerts P. In vivo butyrate metabolism and colonic permeability in extensive ulcerative colitis. Gastroenterology 1998;115(3):584-90.
18. Roediger WE. The colonic epithelium in ulcerative colitis: an energy-deficiency disease? Lancet 1980;2(8197):712-5.
19. Kato K, Ishii Y, Mizuno S, et al. Usefulness of rectally administering [1-(13) C]-butyrate for breath test in patients with active and quiescent ulcerative colitis. Scand J Gastroenterol 2007;42(2):207-14.
20. Pullan RD, Thomas GA, Rhodes M, et al. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. Gut 1994;35(3):353-9.
21. Tytgat KM, van der Wal JW, Einerhand AW, Buller HA, Dekker J. Quantitative analysis of MUC2 synthesis in ulcerative colitis. Biochem Biophys Res Commun 1996;224(2):397-405.
22. Shimotoyodome A, Meguro S, Hase T, Tokimitsu I, Sakata T. Short chain fatty acids but not lactate or succinate stimulate mucus release in the rat colon. Comp Biochem Physiol A Mol Integr Physiol 2000;125(4):525-31.
23. Hatayama H, Iwashita J, Kuwajima A, Abe T. The short chain fatty acid, butyrate, stimulates MUC2 mucin production in the human colon cancer cell line, LS174T. Biochem Biophys Res Commun 2007;356(3):599-603.
24. Barcelo A, Claustre J, Moro F, Chayvialle JA, Cuber JC, Plaisancie P. Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. Gut 2000;46(2):218-24.
25. Finnie IA, Dwarakanath AD, Taylor BA, Rhodes JM. Colonic mucin synthesis is increased by sodium butyrate. Gut 1995;36(1):93-9.
26. Sakata T, Setoyama H. Local stimulatory effect of short-chain fatty acids on the mucus release from the hindgut mucosa of rats (Rattus norvegicus). Comp Biochem Physiol A Physiol 1995;111(3):429-32.
27. Perrais M, Pigny P, Copin MC, Aubert JP, Van Seuningen I. Induction of MUC2 and MUC5AC mucins by factors of the epider-

mal growth factor (EGF) family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. J Biol Chem 2002;277(35):32258-67.
28. Van der Sluis M, De Koning BA, De Bruijn AC, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006;131(1):117-29.
29. Van Seuningen I, Ostrowski J, Bomsztyk K. Description of an IL-1-responsive kinase that phosphorylates the K protein. Enhancement of phosphorylation by selective DNA and RNA motifs. Biochemistry 1995;34(16):5644-50.
30. Jonckheere N, Van Der Sluis M, Velghe A, et al. Transcriptional activation of the murine Muc5ac mucin gene in epithelial cancer cells by TGF-beta/Smad4 signalling pathway is potentiated by Sp1. Biochem J 2004;377(Pt 3):797-808.
31. Tytgat KM, Bovelander FJ, Opdam FJ, Einerhand AW, Buller HA, Dekker J. Biosynthesis of rat MUC2 in colon and its analogy with human MUC2. Biochem J 1995;309 (Pt 1):221-9.
32. Piessen G, Jonckheere N, Vincent A, et al. Regulation of the human mucin MUC4 by taurodeoxycholic and taurochenodeoxycholic bile acids in oesophageal cancer cells is mediated by hepatocyte nuclear factor 1alpha. Biochem J 2007;402(1):81-91.
33. Vincent A, Ducourouble MP, Van Seuningen I. Epigenetic regulation of the human mucin gene MUC4 in epithelial cancer cell lines involves both DNA methylation and histone modifications mediated by DNA methyltransferases and histone deacetylases. Faseb J 2008.
34. Siavoshian S, Segain JP, Kornprobst M, et al. Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression. Gut 2000;46(4):507-14.
35. Whitehead RH, Young GP, Bhathal PS. Effects of short chain fatty acids on a new human colon carcinoma cell line (LIM1215). Gut 1986;27(12):1457-63.
36. Barnard JA, Warwick G. Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. Cell Growth Differ 1993;4(6):495-501.
37. Comalada M, Bailon E, de Haro O, et al. The effects of short-chain fatty acids on colon epithelial proliferation and survival depend on the cellular phenotype. J Cancer Res Clin Oncol 2006;132(8):487-97.
38. Archer S, Meng S, Wu J, Johnson J, Tang R, Hodin R. Butyrate inhibits colon carcinoma cell growth through two distinct pathways. Surgery 1998;124(2):248-53.
39. Nightingale KP, Gendreizig S, White DA, Bradbury C, Hollfelder F, Turner BM. Cross-talk between histone modifications in response to histone deacetylase inhibitors: MLL4 links histone H3 acetylation and histone H3K4 methylation. J Biol Chem 2007;282(7):4408-16.
40. Barski A, Cuddapah S, Cui K, et al. High-resolution profiling of histone methylations in the human genome. Cell 2007;129(4):823-37.
41. McGarvey KM, Van Neste L, Cope L, et al. Defining a chromatin pattern that characterizes DNA-hypermethylated genes in colon cancer cells. Cancer Res 2008;68(14):5753-9.
42. Vincent A, Perrais M, Desseyn JL, Aubert JP, Pigny P, Van Seuningen I. Epigenetic regulation (DNA methylation, histone modifications) of the 11p15 mucin genes (MUC2, MUC5AC, MUC5B, MUC6) in epithelial cancer cells. Oncogene 2007;26(45):6566-76.
43. Augenlicht L, Shi L, Mariadason J, Laboisse C, Velcich A. Repression of MUC2 gene expression by butyrate, a physiological regulator of intestinal cell maturation. Oncogene 2003;22(32):4983-92.

44. Reczek PR, Weissman D, Huvos PE, Fasman GD. Sodium butyrate induced structural changes in HeLa cell chromatin. Biochemistry 1982;21(5):993-1002.
45. White NR, Mulligan P, King PJ, Sanderson IR. Sodium butyrate-mediated Sp3 acetylation represses human insulin-like growth factor binding protein-3 expression in intestinal epithelial cells. J Pediatr Gastroenterol Nutr 2006;42(2):134-41.
46. Domon-Dell C, Wang Q, Kim S, Kedinger M, Evers BM, Freund JN. Stimulation of the intestinal Cdx2 homeobox gene by butyrate in colon cancer cells. Gut 2002;50(4):525-9.
47. Palmer DG, Paraskeva C, Williams AC. Modulation of p53 expression in cultured colonic adenoma cell lines by the naturally occurring lumenal factors butyrate and deoxycholate. Int J Cancer 1997;73(5):702-6.

Chapter 7

Modulation of the gut microbiota with antibiotic or probiotic treatment suppresses body urea production and stimulates mucosal mucin production in neonatal pigs.

Patrycja Puiman

Barbara Stoll

Lars Mølbak

Adrianus de Bruijn

Henk Schierbeek

Mette Boye

Günther Boehm

Ingrid Renes

Johannes van Goudoever

Douglas Burrin

Submitted

Abstract

Production and utilization of amino acids by the intestinal microbiota may impact amino acid requirements in neonates. We hypothesized that modulation of the intestinal microbiota by antibiotics or probiotics would impact amino acid metabolism. We investigated the impact of the gut microbiota whole body nitrogen and amino acid turnover in neonatal pigs receiving no treatment (control), antibiotics, or probiotics. We quantified whole body urea kinetics, threonine fluxes, and threonine disposal into protein, oxidation, and tissue protein synthesis. Compared to controls, antibiotics reduced the amount of bacterial species in the distal small intestine (SI) and colon. Antibiotics decreased plasma urea concentrations via decreased urea synthesis. Antibiotics elevated threonine plasma concentrations and turnover, as well as whole body protein synthesis and proteolysis. Antibiotics decreased protein synthesis rate in the proximal SI and liver but did not affect the distal SI, colon or muscle. Probiotics induced a bifidogenic microbiota. Probiotics decreased plasma urea concentrations, but did not affect whole body threonine or protein metabolism. Probiotics decreased protein synthesis in the proximal SI but not in other tissues. Probiotics tended to increase mucin MUC2 synthesis rate and increased MUC2 expression in the colon. In conclusion, alteration of the gut microbiota by antibiotics or probiotics differentially affected urea and threonine kinetics as well as intestinal protein synthesis and mucin expression. Alteration of the gut microbiota may therefore have implications for protein homeostasis in human neonates treated with antibiotics or probiotics.

Introduction

Optimal nutritional support remains a challenge in clinical care of preterm infants. Most preterm infants, especially those born very preterm with extremely low birth weight, are not fed sufficiently to achieve normal, i.e. fetal, growth rate and as a result end up growth-restricted during their hospital period after birth[1]. Growth restriction is a significant problem, as numerous studies have shown that undernutrition at critical stages of development, especially of protein, produces long-term short stature, organ growth failure, and adverse behavioral and cognitive outcomes[2,3]. Dietary protein and amino acids are essential not only for body growth, but for metabolic signaling, protein synthesis, and protein accretion.

The prominent role of the gut microbiota in host metabolic homeostasis has become an intense topic of research with the advent of rapid advances in the genomic characterization of the microbiome[4-6]. The nitrogenous and carbohydrate substrates derived from dietary input and host secretions are utilized for growth of the intestinal microbiota, but microbial derived substrates can also become available to the host[7]. Recent studies in growing pigs show that amino acids considered to be dietary essential, such as lysine, are synthesized by gut microbiota and absorbed in the small intestine[8,9]. Thus, bacterial amino acid synthesis appears to contribute to the host nitrogen requirements, however, the quantitative importance is not well defined; recent estimates suggest that microbial lysine contributes up to 12-68 mg/kg per day[10,11].

The potential of the intestinal microbiota to support growth and amino acid homeostasis in neonates is unknown. The use of broad-spectrum antibiotics in neonatal care for prophylactic treatment of infections negatively impacts both the number and diversity of commensal bacteria that normally colonize the infant gut, especially lactobacilli and bifidobacteria[12,13]. Decreased bacterial colonization might translate into a reduction in gut bacterial amino acid synthesis and hence limit their availability for host body protein synthesis in neonates. On the other hand, probiotics, most commonly *Lactobacillus* and *Bifidobacteria*, have been used to induce a more beneficial microbiota and there is increasing interest in the potential health benefits of proactive colonization of the gastrointestinal tract of (preterm) infants. Enteral supplementation of some probiotics has shown to decrease the risk of severe necrotizing enterocolitis and mortality in preterm infants[14-16]. There is compelling evidence for beneficial effects of probiotics on intestinal barrier function[17], yet there is a very limited understanding of how probiotics affect intestinal and/or host body protein metabolism.

We hypothesized that suppression of the intestinal microbiota by antibiotic treatment would decrease gut amino acid utilization and whole body urea production and thereby increase the systemic availability of dietary amino acids. We also hypothesized that probiotic supplementation would reduce gut ammonia and thus urea production. Our

aim was to investigate the impact of an altered intestinal microbiota resulting from treatment with either antibiotics or probiotics on nitrogen metabolism in neonatal pigs by stable isotope infusions of urea to determine whole body urea kinetics. Additionally, we used a stable isotope infusion of threonine to determine threonine flux, threonine first-pass splanchnic utilization, threonine disposal into protein, and threonine oxidation. We chose threonine because of its importance in intestinal amino acid metabolism and protein synthesis, specifically that of mucin MUC2.

Materials and methods

Animals and design

The study protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and conducted in accordance with the Guide for the Care and Use of Laboratory Animals [Department of Health and Human Services publication no. (National Institutes of Health; NIH) 85–23, revised 1985, Office of Science and Health Reports, NIH, Bethesda, MD]. Three-day-old, crossbred piglets (n=21) were obtained from the Texas Department of Criminal Justice (Huntsville, TX), transported to the animal facility at the Children's Nutrition Research Center (Houston, TX), and immediately placed in cages in a heated room (30°C). Piglets were weaned to a non-medicated milk-replacer formula (40 $g \cdot kg^{-1} \cdot d^{-1}$; Advance Liqui-Wean; Milk Specialties Company, Dundee, IL), fed 4-5 times per day, and weighed daily to adjust their intake.
At five days of age, surgery was performed after overnight fasting under isoflurane general anesthesia. Silastic catheters were inserted into the jugular vein, carotid artery, and gastric fundus as previously described[18]. Piglets received no prophylactic antibiotic treatment but did receive one dose of analgesic (0.1 $mg^{-1} \cdot kg^{-1}$ butorphenol tartrate; Torbugesic®, Fort Dodge Laboratories, Fort Dodge, IA) post surgery. During the initial 24 hours postoperatively, all pigs received total parenteral nutrition (TPN) at 50% of full intake providing (in $g \cdot kg^{-1} \cdot d^{-1}$) 25 glucose, 13 amino acid, 5 lipid, and 108 $kcal^{-1} \cdot kg^{-1} \cdot d^{-1}$ at a volume of 120 $mL^{-1} \cdot kg^{-1} \cdot d^{-1}$. Thereafter, TPN was stopped and enteral feeding was commenced.
On day eight of life, after complete recovery from surgery and adjustment to full enteral nutrition, piglets were assigned to one of three groups based on equal body weights: 1) Control (CO, n=7), receiving no additional treatment or supplementation; 2) Antibiotic (AB; n=7) receiving intravenous administration of ampicillin (150 $mg \cdot kg^{-1} \cdot d^{-1}$), gentamicin (4 $mg \cdot kg^{-1} \cdot d^{-1}$), and metronidazole (30 $mg \cdot kg^{-1} \cdot d^{-1}$); or 3) Probiotic (PRO; n=7) receiving a mixture of *Bifidobacterium breve* and *Bifidobacterium animalis subspecies lactis* (*BB12*) orally once daily before the first feeding (1:1; $2*10^{10}$ CFU/d; kindly provided by Danone Research, Friedrichsdorf, Germany).

Infusion protocols and sample collection

After eight treatment days, piglets received a primed (150 μmol·kg body wt^{-1}), 6-h continuous, intravenous infusion (15 μmol·kg body $wt^{-1}·h^{-1}$) of [$^{15}N_2$]urea (98 atom%, Cambridge Isotope Laboratories, Andover, Massachusetts, USA) to quantify urea kinetics. Blood samples were collected hourly for [$^{15}N_2$]urea enrichment. After 10 treatment days, piglets received a primed (5 μmol·kg body wt^{-1}), 2-h (0-120 min) continuous intravenous infusion (5 μmol· kg body $wt^{-1}·h^{-1}$) of [^{13}C]bicarbonate (99 atom%, Cambridge Isotope Laboratories) to determine CO_2 production rate. The bicarbonate infusion was followed by a primed (80 μmol·kg body wt^{-1}), 6-h (120-480 min) continuous intragastric infusion (80 μmol·kg body $wt^{-1}·h^{-1}$) of [U-^{13}C,^{15}N]threonine (97-99 atom%, Cambridge Isotope Laboratories) and a primed (40 μmol·kg^{-1}), continuous intravenous infusion (40 μmol·$kg^{-1}·h^{-1}$) of [^{15}N]threonine (98 atom%, Cambridge Isotope Laboratories) administered simultaneously. Blood samples were collected at 0, 90, 105, 120, 360, 420, 450, and 480 min. Measurements of ^{13}C and ^{15}N enrichment were used to determine whole body threonine flux, threonine disposal into protein, threonine oxidation, and tissue protein synthesis rates. On the day of stable isotope infusions, pigs were fed a bolus meal before start of the urea and threonine infusions and maintained in the fed state throughout the infusions by continuous administration of diet through the intragastric catheter. Pigs were fasted during [^{13}C]bicarbonate infusion and given their daily dose of AB or PRO before the start of feeding. Four hours prior to euthanasia, each animal was injected with an intravenous bolus of 5-bromodeoxyuridine (BrdU; 50 mg·kg body wt^{-1}; Sigma Aldrich, St. Louis, MO) to measure the *in vivo* crypt cell proliferation index[18]. After completion of the tracer infusion protocol, pigs were euthanized with a venous injection of pentobarbital sodium (50 mg·kg body wt^{-1}) and sodium phenytoin (5 mg·kg body wt^{-1}, Beuthanasia-D®; Schering-Plough Animal Health, Kenilworth, NJ). The intestine was excised from the ligament of Treitz to the rectum. The SI was divided into 2 segments of equal length, which were designated as proximal and distal SI. Intestinal content from proximal and distal SI, and colon was sampled for analysis of the gut microbiota. The SI and colon were quickly flushed with ice-cold saline and weighed. SI and colon segments were placed in 10% buffered formalin for morphological and BrdU analysis. Stomach, liver, spleen, and kidneys were removed and wet weights recorded. An aliquot of each intestinal segment, liver, and muscle was snap-frozen in liquid nitrogen.

Analysis of intestinal microbiology

Terminal restriction fragment length polymorphism (T-RFLP) analysis was used to analyze the intestinal microbiota. Total DNA was extracted from contents from the proximal and distal SI, and colon by the QIAamp DNA Mini kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions, with the addition of a bead-beating step for 3 min. T-RFLP was profiled and analyzed as described previously[19]. The uni-

versal bacterial primers S-D-Bact-0008-a-S-20 (5'-AGAGTTTGATCMTGGCTCAG-3')[20] and S-D-Bact-0926-a-A-20 (5'-CCGTCAATTCCTTTRAGTTT-3') were used for the PCR[19]. Primer S-D-Bact-0008-a-S-20 was labeled with 5'-FAM (carboxyfluorescein-*N*-hydroxysuccinimide ester dimethylsulfoxide). Similarly, bifido-specific primers Lm26 (5'-GATTCTGGCTCAGGATGAAC-3') and LM3-new (5'-CTRCCCACTTTCATGACT-3') were used for determination of intestinal *Bifidobacterial population*[19]. Primer Lm26 was labeled with 5'JOE (4',5'-dichloro-2',7'-dimethoxyfluorescein). Purified PCR products were digested by restriction enzymes and analyzed by electrophoresis on an automatic sequence analyzer (ABI Prism 373, PE Biosystems, Foster City, CA). ABI traces were imported into BioNumerics version 4.5 (Applied Maths, Belgium) and T-RF's between 35 and 625 basepairs were analyzed.

Plasma urea and amino acids

Plasma amino acids were analyzed by reverse-phase HPLC of their phenyl isothiocyanate derivatives (Pico Tag, Waters, Milford, MA, USA). Urea concentrations were measured with the use of an end-point enzyme assay (Thermo Scientific, Rockford, IL).

Analysis of plasma tracer enrichment

Plasma samples were analyzed for [$^{15}N_2$]urea enrichment and measured by electron ionization mass spectrum of the 2-pyrimidinol-tBDMS derivative of urea as previously described[21]. The cluster of ions at 153 – 155 *m/z* was used for selected ion monitoring of the isotopic enrichment by gas chromatography-mass spectrometry (GC-MS).
Whole blood samples were analyzed for ^{13}C enrichment in CO_2 from [^{13}C]bicarbonate and [U-^{13}C,^{15}N]threonine using gas isotope ratio-mass spectrometry, with monitoring of ions at *m/z* 44 and 45.
Plasma samples were analyzed for [^{15}N]threonine and [U-^{13}C,^{15}N]threonine enrichment. Threonine was isolated by cation exchange chromatography (AG-50W resin, Bio-Rad, Hercules, CA) and analyzed as its*n*-propyl ester n-heptafluorobutyramide derivative using methane-negative chemical ionization and monitoring ions at *m/z* 533 to 539 (5890 series II gas chromatograph linked to a model 5989B quadrupole mass spectrometer, Hewlett Packkard, Palo Alto, CA). In a subset of pigs (n=3 per group) [$^{13}C_2$,^{15}N]glycine enrichments were measured for determination of threonine oxidation through the L-threonine 3-dehydrogenase (TDG) pathway. GC-MS analysis for glycine was performed in the same derivatized samples as for threonine by monitoring ions at *m/z* 293 to 296.

Protein synthesis

Protein and DNA concentrations of tissue samples from jejunum, ileum, colon, liver, and muscle were determined and aliquots were prepared for GC-MS analysis as previously described[22]. The isotopic enrichment of [^{15}N]threonine and [U-^{13}C,^{15}N]threonine in

the tissue amino acid free pool was determined as described for plasma samples. To measure the enrichment of [^{15}N]threonine and [U-^{13}C,^{15}N]threonine in the tissue protein-bound pool, hydrolyzed samples were analyzed as their *N*-pivaloyl-i-propyl esters and measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS, Thermo Finnigan Deltaplus XL GC-C-IRMS; Thermo Electron Corp.,Waltham, MA)[23].

MUC2 isolation and synthesis

MUC2 was isolated from colonic tissue using a cesium chloride (CsCl) density gradient ultracentrifugation method combined with gravity gel filtration chromatography, as previously described for human MUC2 in detail[24-26]. A small aliquot of purified MUC2 was used for confirmation of MUC2 by Western blot and the remaining was hydrolyzed as described above for the tissue protein hydrolysates. To measure the enrichment of [U-^{13}C,^{15}N]threonine, hydrolyzed samples were analyzed as their acetyl-ethoxycarbonylethyl esters. The [^{13}C/^{12}C] ratio of threonine in MUC2 isolates was measured by a Delta-XP isotope ratio MS linked online with a Trace gas chromatograph and a combustion interface type 3 (Thermo Fischer, Bremen, Germany) according to[24,27]. Enrichment was expressed in mole percent excess (MPE).

MUC2 Western blotting

Colon tissue samples were homogenized in HIS buffer containing protease inhibitors and an aliquot was used for Western blotting of MUC2 as described previously[24]. MUC2 expression was normalized to β-actin immunoreactivity.

Histology and Immunohistochemistry

Morphometry analysis was performed on formalin-fixed, paraffin embedded, hematoxylin and eosin-stained intestinal sections (5 μm) as described previously[28]. Villus height, crypt depth, intestinal surface area and muscularis thickness were measured in 15 vertically well-oriented crypt-villus units. *In vivo* crypt cell proliferation was measured by BrdU crypt-cell labeling[28].

Calculations

Plasma enrichments of [$^{15}N_2$]urea turnover or flux (Q_{urea}, μmol · kg^{-1} · h^{-1}) were used to calculate the rate of whole body urea turnover or flux as described previously[21].
Plasma enrichments of [^{15}N]threonine and [U-^{13}C,^{15}N]threonine were used to calculate the rate of whole body threonine turnover or flux as described previously[29].
$^{13}CO_2$ enrichments at steady state during [^{13}C]bicarbonate infusion were used to calculate CO_2 production rate as described before[21,30] and $^{13}CO_2$ enrichments at steady state

during [U-^{13}C,^{15}N]threonine infusion were used to calculate threonine oxidation[21]. Under the assuming that threonine flux (Q_{THR})

$$Q_{THR} = \text{synthesis} + \text{oxidation} = \text{proteolysis} + \text{intake}$$

the non-oxidative threonine disposal (NOTD), an indication of whole body protein synthesis, equals threonine flux (Q_{THR}) minus threonine oxidation (Ox_{THR}). Threonine appearance from protein (endogenous Ra), an indication of whole body proteolysis, equals Q_{THR} minus threonine intake (325 μmol · kg^{-1} · h^{-1}) calculated from the infusion and the diet. Threonine intake from the diet (231 μmol · kg^{-1} · h^{-1}) was determined by analysis of the amino acid composition of the diet as described previously[21]. Ultimately, protein net balance was calculated from protein synthesis minus protein degradation.
Plasma glycine enrichment was measured to determine the contribution of threonine to glycine production. The fractional contribution of threonine to glycine production ($FC_{THR-GLY}$ in %) was calculated as described previously[31].
The fractional protein synthesis rate (FSR, %·d^{-1}) of intestinal, liver, and muscle proteins reflects the percent protein mass synthesized in a day[32]. The FSR of MUC2 is expressed as percentage of the total MUC2-pool newly synthesized per day. Threonine enrichment of the intracellular free amino acid pool in the ileum or colon was used as a precursor.
Absolute protein synthesis rates (ASR) of intestinal and liver proteins (g·kg body wt^{-1}·d^{-1}), reflecting the absolute protein mass synthesized in a day, were calculated according as previously described[32].

Statistics

Minitab statistical software (Minitab 15, State College, PA) was used for statistical analysis. Plasma amino acid data were first analyzed by one-way ANOVA using a general linear model and followed by a Bonferoni post analysis. All other data were first analyzed by one-way ANOVA using a general linear model and followed by a Tukey's means comparison test. For correlations between urea plasma levels, urea enrichment and flux the Pearson correlation test was used. Data are presented as the mean ± SEM and $P < 0.05$ was considered statistically significant. Intestinal bacterial diversity was analyzed using principal component analysis (PCA) generated by BioNumerics on band patterns. Division by the variances over the entries and subtraction of the averages over the characters was included in the PCA. Curve-based pairwise comparisons of T-RFLP profiles were calculated by BioNumerics using the Pearson's correlations.

Results

Weight data and histology

During the course of the study piglets were active and healthy. Data on body weight, organ weights, and intestinal morphometry are summarized in Table 1. Initial body weights, final body weights, and daily weight gain were not different amongst groups. Wet mass weight, protein content, and DNA content of proximal SI were lower in AB and PRO treated animals compared to CO. In the distal SI and colon no differences were observed between groups with respect to organ weights and protein and DNA content. Wet mass weight of liver was not different between groups whereas protein content of the liver was significantly lower in AB pigs compared to PRO pigs, and tended to be lower when compared to CO. DNA content of livers of AB and CO pigs was lower compared to PRO pigs. Wet mass weights of stomach, spleen, kidneys and heart were not different amongst groups (data not shown).

Intestinal microbiology

AB and PRO differentially affected the colonic and small intestinal microbiota. First, the colonic universal bacterial Terminal restriction fragments (T-RFs) from AB pigs digested with *HhaI* revealed a total of 34.0 ± 4.4 different T-RF's in the T-RFLP analysis. The number of T-RF's, representing bacterial species, was significantly lower in AB compared to CO pigs (48.3 ± 2.5; $P = 0.04$), and tended to be lower compared to PRO pigs (47.4 ± 4.9; $P = 0.06$). Principal component analysis (PCA) of the representative T-RF's for each treatment group showed that there was a high diversity in bacterial composition among the AB treated pigs (Figure 1A). Moreover, AB treatment did not only diminish the amount of T-RF's, but also altered the composition of the microbiota compared to CO and PRO. The difference in microbial diversity between CO and PRO could be identified when PCA was performed excluding the AB pigs. T-RF's, and thus bacterial species, within the colon showed great diversity both within the CO and PRO group, as well as between the CO and PRO group. This demonstrates that PRO supplementation altered the composition of the colonic microbiota when compared to CO pigs (Figure 1B). No differences between groups were detected in the amount of T-RF's in the T-RFLP analysis of the ileum-derived samples (CO 45.7 ± 9.1; AB 49.2 ± 8.2; PRO 67.1 ± 10.3) or jejunum-derived samples (CO 8.8 ± 3.7; AB 8.7 ± 2.6; PRO 15.0 ± 5.3) although PRO pigs tended to have higher T-RF counts in both intestinal segments. The small intestinal microbiota is known to be less stable than the colonic microbiota. For this reason, combined with the wide diversity of the bacterial composition found within all treatment groups, differences between groups might not have been detected.

Second, the digested *Bifidobacteria*-specific T-RFs from colon, ileum, and jejunum samples demonstrated the absence of Bifidobacterium species in AB pigs. In CO

pigs, limited bifidobacterial T-RF's were detected in the colon, whereas no T-RF's were detected in the ileum or jejunum (Figure 2). In contrast to AB and CO pigs, PRO supplementation resulted in the detection of *Bifidobacterium breve* and *Bifidobacterium animalis subspecies lactis* in colon, ileum, and jejunum. Interestingly, *Bifidobacterium breve* was more abundant in the jejunum and ileum, whereas *Bifidobacterium animalis subspecies lactis* was abundant in ileum and colon (Figure 2). Moreover, analysis of the bifidogenic microbiota of PRO pigs resulted in more *Bifidobacterium*-specific T-RF's than only those representing the supplemented probiotics, suggesting increased species diversity (Figure 2).

Table 1 Weight gain, organ weights, and tissue analyses in CO, AB and PRO pigs[A].

	CO	AB	PRO	*P*-value
Final body weight, g	4039 ± 204	4240 ± 212	4137 ± 163	NS
Weight gain, $g \cdot kg^{-1} \cdot d^{-1}$	40.7± 1.9	44.3 ± 1.9	42.6 ± 2.0	NS
Proximal small intestine				
Weight, $g \cdot kg^{-1}$	19.4 ± 0.8*†	17.6 ± 0.6*	16.6 ± 0.5†	*0.02 †0.0007
Protein mass, $mg \cdot kg^{-1}$	2007 ± 70*†	1778 ± 79*	1707 ± 81†	*0.038 †0.007
DNA mass, $mg \cdot kg^{-1}$	143 ± 11.7*†	113 ± 9.6*	113 ± 8.6†	*0.006 †0.005
Villus height, μm	855 ± 64	875 ± 42	729 ± 26	NS
Crypt depth, μm	140 ± 3.9	133 ± 4.4	136 ± 2.1	NS
Muscularis thickness, μm	148 ± 6.6	155 ± 10.2	144 ± 11.5	NS
Surface area, mm^2	82 ± 6.5	78 ± 4.0	68 ± 2.4	NS
BrdU positive crypt cells, %	31 ± 1.9	28 ± 1.6	29 ± 2.4	NS
Distal small intestine				
Weight, $g \cdot kg^{-1}$	20.4 ± 1.4	21.6 ± 1.7	18.0 ± 0.7	NS
Protein mass, $mg \cdot kg^{-1}$	1937 ± 161	1947 ± 120	1677 ± 67	NS
DNA mass, $mg \cdot kg^{-1}$	161 ± 13.8	189 ± 15.1	166 ± 9.3	NS
Villus height, μm	485 ± 76	651 ± 92	547 ± 79	NS
Crypt depth, μm	140 ± 4.6	142 ± 6.6	134 ± 6.3	NS
Muscularis thickness, μm	226 ± 9.4	186 ± 14.3	179 ± 18.2	NS
Surface area, mm^2	44 ± 7.0*	68 ± 13.0*	55 ± 10.8	0.04
BrdU positive crypt cells, %	20 ± 2.4	20 ± 2.9	19 ± 3.6	NS
Colon				
Weight, $g \cdot kg^{-1}$	8.8 ± 0.6	8.1 ± 0.7	7.7 ± 0.3	NS
Protein mass, $mg \cdot kg^{-1}$	736 ± 44	695 ± 101	669 ± 43	NS
DNA mass, $mg \cdot kg^{-1}$	51 ± 5.4	51 ± 6.2	45 ± 2.7	NS
Crypt depth, μm	327 ± 18.9*	270 ± 13.5*	279 ± 10.7	0.037
Liver				
Weight, $g \cdot kg^{-1}$	33.2 ± 1.3	30.7 ± 1.1‡	35.0 ± 2.2‡	‡0.046
Protein mass, $mg \cdot kg^{-1}$	4570 ± 206	3972 ± 127‡	4735 ± 249‡	‡0.043
DNA mass, $mg \cdot kg^{-1}$	174 ± 10.7†	172 ± 9.5‡	196 ± 15.2†‡	†0.017 ‡0.01

[A]Mean ± SEM; n=7 pigs per group

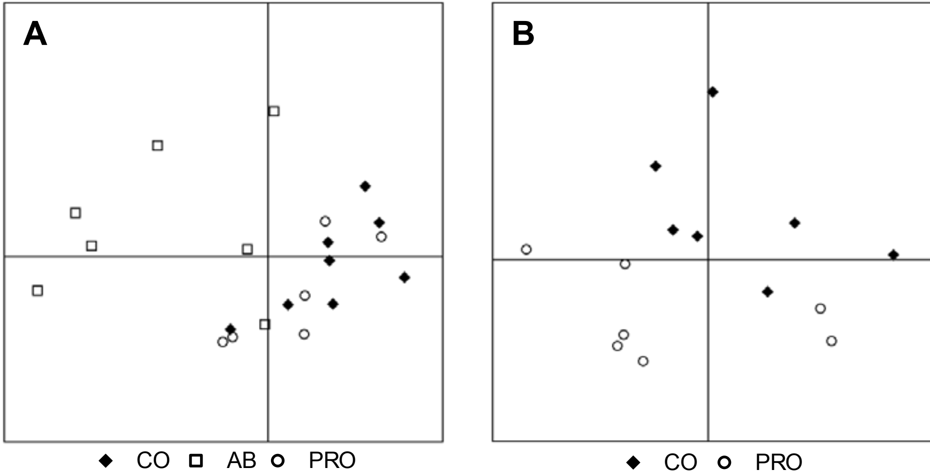


Figure 1. Principal component analysis (PCA) of the treatment effect on bacterial composition in colonic content of CO pigs or pigs treated with AB or PRO.
A. PCA of TRF's of all treatment groups. The PCA plot shows a markedly different bacterial composition in AB compared to CO and PRO (x= 16.9 % Y= 10.6%). Among the AB pigs there was a high variation in the bacterial composition but they clustered in the same area. B: PCA of TRF's of CO and PRO treated pigs. The PCA plot shows a high variation in bacterial composition both between (X= 11.7 %; Y= 8.6%) and within groups.

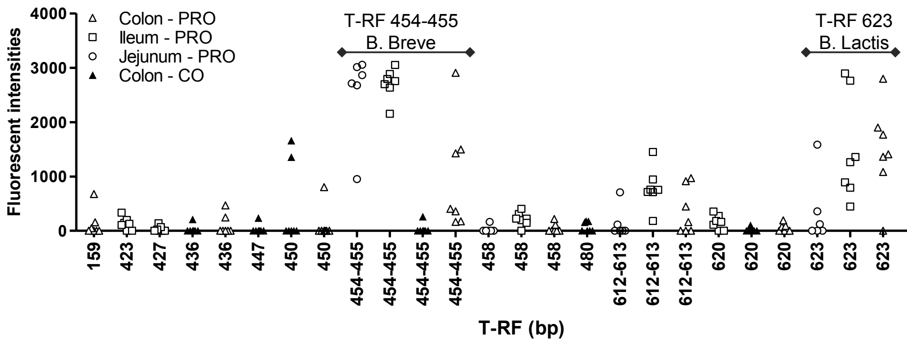


Figure 2. Graph representing Bifidobacterial T-RFs of PRO pigs in proximal SI, and T-RFs of CO and PRO pigs in dist SI and colon.
Bifidobacteria were undetectable in proximal SI in CO pigs. In AB pigs no bifidobacteria were present in all intestinal segments.

Plasma amino acid concentrations

Plasma concentrations of ornithine, cystine, glycine, threonine, and tyrosine were higher in AB piglets compared to CO (Table 2). Except for lower histidine levels in PRO, no differences were observed between PRO and CO pigs. Plasma concentrations of citrulline, glycine, histidine, ornithine, serine, threonine, and tyrosine were higher in AB compared to PRO pigs, whereas glutamine was lower in AB than in PRO pigs (Table 2).

Table 2 Plasma amino acids in CO, AB, and PRO pigs[A].

Amino acids [B]	CO	AB	PRO	P-value
Alanine	452 ± 36	529 ± 36	453 ± 30	NS
Arginine	82 ± 9.1	97 ± 10.6	84 ± 9.2	NS
Asparginine	91 ± 7.7	100 ± 7.3	91 ± 9.4	NS
Aspartate	7.2 ± 1.2	6.4 ± 1.0	7.0 ± 0.8	NS
Citrulline	44 ± 4.8	58 ± 4.5‡	31 ± 3.6‡	‡0.002
Cysteine	263 ± 22	253 ± 17	316 ± 43	NS
Cystine	19 ± 1.7*	28 ± 1.3*	24 ± 1.9	*0.003
Glutamine	347 ± 31	308 ± 23‡	419 ± 31‡	‡0.046
Glutamate	120 ± 21	116 ± 14	128 ± 15	NS
Glycine	553 ± 29*	682 ± 57*‡	515 ± 41‡	*0.036 ‡0.006
Histidine	19 ± 4.4†	24 ± 5.2‡	11 ± 4.4†‡	†0.035 ‡0.002
Isoleucine	146 ± 6.2	155 ± 9.4	162 ± 11.0	NS
Leucine	184 ± 11	211 ± 16	204 ± 15	NS
Lysine	185 ± 16	210 ± 14	201 ± 13	NS
Methionine	136 ± 8.8	156 ± 3.4	144 ± 17.4	NS
Ornithine	21 ± 1.6*	33 ± 1.8*‡	21 ± 1.1‡	*‡0.0001
Phenylalanine	43 ± 6.1	47 ± 7.3	43 ± 3.5	NS
Proline	285 ± 20	332 ± 17	298 ± 17	NS
Serine	164 ± 7.2	202 ± 11‡	160 ± 14‡	‡0.035
Taurine	57 ± 4.9	71 ± 4.6	58 ± 5.3	NS
Threonine	522 ± 22*	794 ± 31*‡	476 ± 40‡	*†0.000
Tryptophan	14 ± 1.1	18 ± 1.5	15 ± 2.0	NS
Tyrosine	89 ± 8.0*	123 ± 7.4*‡	84 ± 8.3‡	*0.015 ‡0.006
Valine	204 ± 8.5	219 ± 14	233 ± 15	NS

[A] Mean ± SEM, n=7 pigs per group.
[B] Plasma amino acids, μmol/L.

Urea kinetics

Plasma levels of urea were lower in AB pigs compared to CO (P = 0.03) and PRO pigs (P = 0.03) (Figure 3A). Plasma [$^{15}N_2$]urea had reached isotopic steady state during the last 4 h of infusion (Figure 3B). Urea enrichment was higher (P = 0.004) (Figure 3B), and consequently urea flux was lower (P = 0.009) (Figure 3C) in AB pigs compared to CO pigs. Urea enrichment and flux in PRO pigs was not significantly different from either CO or AB pigs. Urea plasma levels from all pigs correlated negatively with urea enrichments (P = 0.001) and thus correlated positively with urea flux (P = 0.000).

Whole body threonine kinetics

Threonine kinetics are depicted in Table 3.Threonine flux was significantly higher in AB pigs compared to CO and PRO pigs. CO_2 production and threonine oxidation were not affected by treatment. Protein synthesis (NOTD) was higher in AB pigs vs. CO (P = 0.019) and PRO pigs (P = 0.039) (Figure 4A). Proteolysis (endogenous Ra) was also

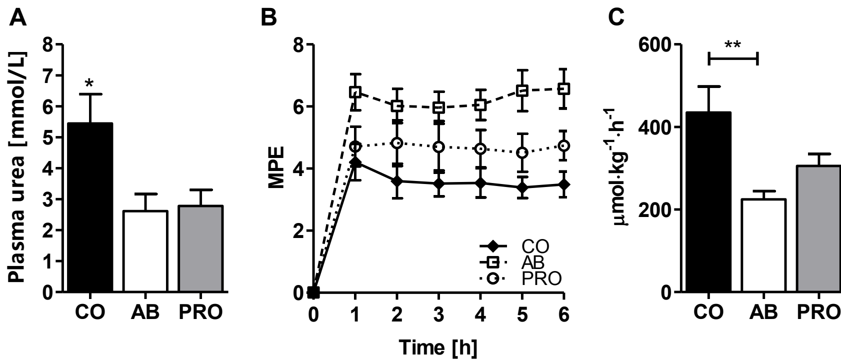


Figure 3. Whole body and plasma urea kinetics in neonatal pigs in control (CO), antibiotic (AB) and probiotic (PRO) treated groups. Statistics based on ANOVA and Tukey's test.
Panel A shows plasma urea concentration measured at the end of the 8-day treatment period; CO vs. AB P = 0.03, CO vs. PRO P = 0.03. Panel B shows the steady-state plasma 15N-urea enrichments during the 6 hr infusion of 15N-urea in control (solid diamond), AB (open square) and PRO (open circle) groups; CO vs. AB P = 0.004. Panel C shows the calculated whole body urea flux in CO (solid bar), AB (open bar) and PRO (shaded bar) groups; CO vs. AB P = 0.009, CO vs. PRO P = 0.09.

increased in AB pigs vs. CO (P = 0.008) and PRO pigs (P = 0.02) (Figure 4B). The net result was that net balance was not different amongst treatment groups (CO, AB, PRO: 273, 270, 267 µmol · kg^{-1} · h^{-1}). The fractional contribution of threonine to glycine production ($FC_{THR-GLY}$) was not different amongst groups, and accounted for 29% in CO, 32% in AB, and 29% in PRO pigs.

Table 3 Whole body threonine kinetics in CO, AB, and PRO pigs[A].

	CO	AB	PRO	P-value
Intake, mg·kg^{-1}·d^{-1}	325	325	325	
Flux [U^{13}C]threonine (ig) tracer, µmol·kg^{-1}·h^{-1}	541 ± 22*	661 ± 27*‡	559 ± 27‡	*.008 ‡.022
Flux [^{15}N]threonine (iv) tracer, µmol·kg^{-1}·h^{-1}	521 ± 20	573 ± 13	531 ± 19	NS
First-pass splanchnic utilization, % of intake	3.5 ± 3.0	12.9 ± 2.5	4.2 ± 3.7	NS
First-pass splanchnic utilization, mg·kg^{-1}·d^{-1}	2.3 ± 2.0	8.5 ± 1.6	2.8 ± 2.5	NS
CO_2 production, mmol·kg^{-1}·h^{-1}	48 ± 5.9	47 ± 5.4	47 ± 4.0	NS
^{13}C THR oxidized, µmol·kg^{-1}·h^{-1}	56 ± 8.4	55 ± 7.7	57 ± 6.2	NS
% of flux oxidized, %	10.6 ± 1.8	8.5 ± 1.4	10.5 ± 1.5	NS
$FC_{THR-GLY}$, %[B]	29 ± 6.5	32 ± 2.2	29 ± 5.6	

[A]Mean ± SEM; n=7 pigs per group.[B] n=3 pigs per group

Tissue protein synthesis

Protein synthesis rates in proximal SI, distal SI, and colon were measured by mass spectrometry analysis of incorporation of the intravenous administered [^{15}N]threonine tracer (Table 4). Protein FSR was lower in AB compared to CO pigs, whereas protein ASR in both AB and PRO were lower compared to CO. Protein FSR and ASR in the distal SI were not different between groups. Protein FSR and ASR in the colon was lower compared to the small intestine but were not affected by treatment. In the liver,

protein FSR was lower in AB compared to CO pigs, whereas protein ASR was lower in AB pigs compared to both CO and PRO pigs. In muscle, protein FSR was not different between treatment groups.

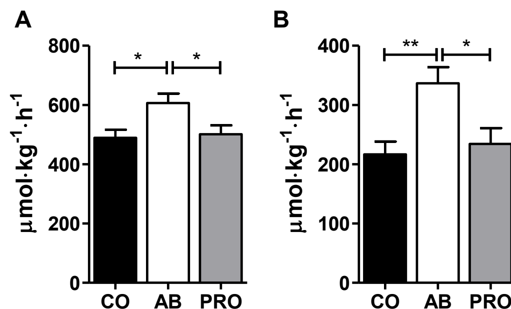


Figure 4. Whole body protein synthesis and proteolysis based on threonine kinetics in neonatal pigs.
Panel A shows whole body protein synthesis rate in CO (solid bar), AB (open bar) and PRO (shaded bar) groups; CO vs. AB P = 0.019, AB vs. PRO P = 0.039. Panel B shows whole body proteolysis CO (solid bar), AB (open bar) and PRO (shaded bar) groups; Proteolysis; CO vs. AB P = 0.008, AB vs. PRO P = 0.02

Table 4 Protein FSR and ASR in the small intestine, colon, liver, and muscle in CO, AB, and PRO pigs[A].

	CO	AB	PRO	*P*-value
Proximal small intestine				
FSR, %/d	71 ± 7.1*	56 ± 4.0*	65 ± 4.1	*.013
ASR, $g \cdot kg^{-1} \cdot d^{-1}$	1.4 ± 0.12*†	1.0 ± 0.05*	1.1 ± 0.04†	*.0013 †.011
Distal small intestine				
FSR, %/d	66 ± 5.2	57 ± 6.2	68 ± 3.6	NS
ASR, $g \cdot kg^{-1} \cdot d^{-1}$	1.3 ± 0.15	1.2 ± 0.18	1.1 ± 0.1	NS
Colon, $g^{-1} \cdot kg^{-1}$				
FSR, %/d	52 ± 3.7	44 ± 3.1	52 ± 4.0	NS
ASR, $g \cdot kg^{-1} \cdot d^{-1}$	0.4 ± 0.03	0.3 ± 0.03	0.4 ± 0.04	NS
Liver,				
FSR, %/d	54 ± 4.1*	46 ± 2.6*	52 ± 3.6	*.047
ASR, $g \cdot kg^{-1} \cdot d^{-1}$	2.5 ± 0.22*	1.8 ± 0.12*‡	2.5 ± 0.20‡	*.022 ‡.024
Muscle				
FSR, %/d	39 ± 1.9	43 ± 2.7	39 ± 1.2	NS

[A]Mean ± SEM; n=7 pigs per group.

MUC2 analyses

Identified MUC2 fractions showed great similarity to that found in human preterm infants[24,25]. Colonic MUC2 FSR representing the percentage of MUC2 newly synthesized per day was 48, 34, and 57 %/d in CO, AB, and PRO pigs respectively (Figure 5A). MUC2 FSR was not significantly different between treatment groups although a trend towards a higher MUC2 FSR was observed in PRO pigs compared to AB pigs (*P* =

0.07). Quantitative Western blotting showed increased colonic MUC2 expression in PRO pigs compared to CO (P = 0.007) and AB (P = 0.016) (Figure 5B).

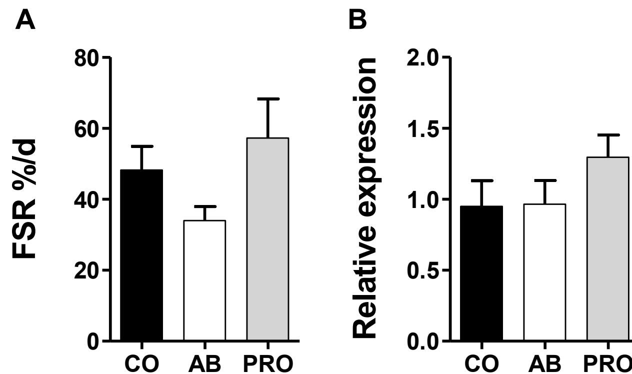


Figure 5. Fractional synthesis rate (FSR) and relative expression of MUC2 kinetics in neonatal pigs in control (CO), antibiotic (AB) and probiotic (PRO) treated groups.
Panel A shows the MUC2 FSR in CO (solid bar), AB (open bar) and PRO (shaded bar) groups; PRO vs. AB p = 0.07. Panel B shows colonic expression of MUC2 performed by quantitative Western blotting; CO vs. PRO P = 0.007, AB vs. PRO P = 0.016

Histology

Histology measurements of villus height, crypt depth, intestinal surface area, and muscularis thickness showed no significant differences between treatment groups for all segments (Table 1). However, villus height and intestinal surface area in the proximal SI tended to be decreased by PRO treatment. Analyses of BrdU positive cells indicating proliferation in proximal and distal SI were not affected by treatment.

Discussion

The intestinal microbiota utilizes and synthesizes amino acids that are available to the host and contributes to amino acids requirements[33]. In the neonate, modulation of the gut microbiota by treatment with antibiotics and probiotics, might therefore impact amino acid and hence protein metabolism. We investigated the impact of antibiotics and probiotics on urea production and both whole body and intestinal threonine kinetics in neonatal pigs. Consistent with our hypothesis, we found that antibiotics lowered urea production and increased threonine turnover, whole body protein synthesis, and whole body proteolysis, but this did not translate into a net increase in body threonine balance. Furthermore, antibiotics decreased SI weight, SI protein synthesis, and liver protein synthesis. Probiotics decreased urea plasma levels, but did not affect whole body threonine or protein metabolism. Probiotics did decrease small intestinal weight

and protein synthesis similar to AB pigs, but increased MUC2 expression suggesting stimulation of the intestinal mucus layer and enhanced gut barrier function.

Antibiotics and probiotics differentially changed urea kinetics in our piglet model. Antibiotic treatment reduced urea production reflected as both lower plasma urea concentration and urea rate of appearance compared to CO. Urea is synthesized in mammals as part of the urea cycle, to dispose of ammonia generated mainly from deamination of amino acids in the gut lumen or liver. In this cycle, amino groups donated by ammonia and aspartate are converted to urea, while ornithine, citrulline, argininosuccinate, and arginine act as intermediates. Decreased urea production from host amino acid oxidation is unlikely to be responsible for the observed effect since threonine oxidation was not different amongst groups. A large proportion of amino acids extracted in first pass by the gut are catabolized by the neonatal pig intestine[34,35]. A more recent report studying isolated intestinal epithelial cells indicated that there was neither production of CO_2 or citric acid cycle intermediates from carbons of various essential amino acids, nor activity of amino acid catabolizing enzymes[36]. The extensive in vivo first pass catabolism of essential amino acids by the pig SI may result from oxidation by luminal microbes[37]. Therefore, in the present study, we suspect that antibiotics resulted in decreased bacterial mass and thus amino acid catabolism and resulting in decreased amino acid deamination, ammonia production and hepatic urea synthesis.

Similar to antibiotics, probiotics reduced plasma urea concentrations and tended to lower urea rate of appearance versus CO pigs. In pigs and human infants about 50% of urea secreted into the gut lumen is hydrolyzed by bacteria and used for recycling[38,39]. Thus, increased urease activity by the intestinal microbiota could explain the reduced plasma urea concentrations observed in probiotic treated pigs. However, earlier reports showed that probiotic supplementation decreased urease activity by diminishing urease-producing bacteria[40]. Nonetheless, different probiotic products with different species and even different strains may exhibit different results. We suspect that probiotic treatment also reduced body urea production by increasing the utilization of free ammonia produced in the gut for bacterial amino acid synthesis.

Threonine is an essential amino acid important for intestinal and whole body protein metabolism, and may therefore be influenced by modulation of the intestinal microbiota. Threonine oxidation was not different amongst treatment groups whereas AB pigs had significantly higher threonine plasma concentrations and threonine flux compared to CO and PRO pigs. Proteolysis resulting in endogenous threonine release was increased in AB pigs and responsible for the higher threonine flux and plasma concentration. Yet, the higher threonine flux with unchanged oxidation of threonine in AB pigs also reflects increased protein synthesis. Despite the finding of higher whole body protein synthesis, this was not explained by increased protein synthesis rates in the gut, liver and muscle tissues. Interestingly, protein synthesis rates in liver and proximal SI were

lowest in AB pigs. A major question that arises from our study is why AB treatment resulted in increased proteolysis and decreased protein synthesis rates in liver and gut. One might expect that AB treatment could translate into a lower need for synthesis of immune defensive and acute phase proteins associated with reduced bacterial-related inflammatory stimulation[41-43]. Another explanation might be that increased proteolysis was caused by AB as a side effect of the treatment. Gentamicin is an aminoglycoside antibiotic that causes mistranslation of mRNA as a consequence of binding to the bacterial 70S ribosomal A-site decoding region of rRNA[44]. Mistranslation of mRNA then interrupts bacterial protein synthesis and hence induces cell death. Although eukaryotic cells mainly comprise 80S ribosomes, clinical use of aminoglycosides has shown to be potentially be toxic to human cells, possibly by mistranslation by the proposed mechanism above. We are not aware of any reports describing changes in tissue proteolysis induced by aminoglycosides or other antibiotics. Further studies are needed to confirm proteolysis by antibiotic treatment in preterm infants and to elucidate the mechanism responsible.

Despite the wide-spread nutritional use and application of probiotics in human nutrition, there are surprising few reports of their impact on protein and amino acid metabolism. We found that probiotic supplementation did not affect threonine oxidation, whole body protein synthesis, or protein synthesis of liver and muscle proteins compared to CO. Recently, a stimulatory effect of probiotics on plasma and liver protein synthesis was reported in a study concerning a piglet colitis model[45]. However, the presence of colitis in that model, as well as difference in probiotics and microbial microbiota, might explain the difference in results. Interestingly, protein content and hence absolute protein synthesis rates in the proximal SI were decreased in PRO compared to CO pigs. This decrease in protein synthesis in the proximal gut in PRO pigs remains open for further investigation. The proximal SI preferentially uses dietary threonine for mucosal protein synthesis[27]. Therefore, the presence of probiotic bacteria in the proximal SI might have resulted in competition for dietary amino acid with the host and hence decreased availability of threonine for protein synthesis. It is also possible that probiotics induced an anti-inflammatory action in the mucosal tissue resulting in lower protein synthesis rates[17,41-43,46].

MUC2, the most predominant intestinal secretory mucin forming the protective mucus layer, is a major component of gut barrier function[26,47]. Probiotics are associated with increased gut barrier function and a decrease in the incidence of necrotizing enterocolitis in preterm infants[15,17]. Thus, we were interested to examine how either probiotics or antibiotic treatment would alter MUC2 synthesis in the neonatal intestine. Our results show that PRO pigs increased colonic MUC2 protein expression and synthesis compared to CO and AB. This provides further evidence that certain probiotic bacteria increase MUC2 synthesis in colonic epithelial cells[48-50]. Moreover, it suggests a specific

action of probiotic bacteria on epithelial innate immune function, since the total protein synthesis rates were not different between groups. We have recently shown that MUC2 synthesis is upregulated by short chain fatty acids, especially butyrate[51]. It was also shown that supplementing infant formula with prebiotics increased bifidogenic microbiota and altered short chain fatty acid composition mimicking that of the breastfed infant[52]. Therefore, increased butyrate levels in PRO treated pigs might have accounted for the increase in MUC2 observed.

In summary, this study is among the first to comprehensively examine the impact of gut microbiota on gut growth and metabolism of both nitrogen and amino acids in a neonatal animal model highly relevant to human infants. Our results show that antibiotics commonly given to premature infants and to a lesser extent probiotic treatment both decreased urea production in neonatal pigs. We suspect that the changes in whole body urea kinetics are due to suppressed gut ammonia production and not host amino acid deamination. Furthermore, while AB pigs exhibited increased threonine turnover, whole body protein synthesis and proteolysis the fractional protein synthesis rates in the proximal SI and liver were decreased. These changes in metabolism were not associated with changes in intestinal growth or mucosal structure despite the significant suppression of gut colonization due to AB treatment. In contrast, probiotics did not markedly alter whole body threonine or protein metabolism despite the modest decrease in protein synthesis in the proximal gut. A major finding was the stimulatory effect of PRO on colonic MUC2 synthesis and MUC2 protein expression indicating increased gut barrier function and specific induction of mucosal innate immunity. Again, this was despite any remarkable changes in intestinal villus structure or crypt cell proliferation in AB-treated pigs. Our results indicate that neither antibiotic nor probiotic result in adverse outcomes associated with nitrogen and amino acid metabolism. Contrary to our expectation, neither antibiotic nor probiotic had a measurable net anabolic effect on whole body protein metabolism. However, clearly more studies are warranted in infants to confirm the observed effects of antibiotics and probiotics. Finally, our results suggest that rather than positive protein anabolic effects related to infant growth, probiotics lead to localized stimulation of intestinal mucosal defense that translates into longer term health and development outcomes.

References

1. Hulst JM, van Goudoever JB, Zimmermann LJ, et al. The effect of cumulative energy and protein deficiency on anthropometric parameters in a pediatric ICU population. Clin Nutr 2004;23(6):1381-9.
2. Hay WW, Jr. Nutrient supplies for optimal health in preterm infants. J Pediatr Gastroenterol Nutr 2007;45 Suppl 3:S163-9.
3. Larroque B, Ancel PY, Marret S, et al. Neurodevelopmental disabilities and special care of 5-year-old children born

before 33 weeks of gestation (the EPIPAGE study): a longitudinal cohort study. Lancet 2008;371(9615):813-20.
4. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science 2005;307(5717):1915-20.
5. Turnbaugh PJ, Gordon JI. The core gut microbiome, energy balance and obesity. J Physiol 2009;587(Pt 17):4153-8.
6. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444(7122):1027-31.
7. Metges CC, Petzke KJ, El-Khoury AE, et al. Incorporation of urea and ammonia nitrogen into ileal and fecal microbial proteins and plasma free amino acids in normal men and ileostomates. Am J Clin Nutr 1999;70(6):1046-58.
8. Torrallardona D, Harris CI, Fuller MF. Lysine synthesized by the gastrointestinal microflora of pigs is absorbed, mostly in the small intestine. Am J Physiol Endocrinol Metab 2003;284(6):E1177-80.
9. Metges CC, Eberhard M, Petzke KJ. Synthesis and absorption of intestinal microbial lysine in humans and non-ruminant animals and impact on human estimated average requirement of dietary lysine. Curr Opin Clin Nutr Metab Care 2006;9(1):37-41.
10. Jackson AA, Gibson NR, Bundy R, Hounslow A, Millward DJ, Wootton SA. Transfer of (15)N from oral lactose-ureide to lysine in normal adults. Int J Food Sci Nutr 2004;55(6):455-62.
11. Metges CC, El-Khoury AE, Henneman L, et al. Availability of intestinal microbial lysine for whole body lysine homeostasis in human subjects. Am J Physiol 1999;277(4 Pt 1):E597-607.
12. Westerbeek EA, van den Berg A, Lafeber HN, Knol J, Fetter WP, van Elburg RM. The intestinal bacterial colonisation in preterm infants: a review of the literature. Clin Nutr 2006;25(3):361-8.
13. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol 2007;5(7):e177.
14. Alfaleh K, Bassler D. Probiotics for prevention of necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev 2008(1):CD005496.
15. Deshpande G, Rao S, Patole S. Probiotics for prevention of necrotising enterocolitis in preterm neonates with very low birthweight: a systematic review of randomised controlled trials. Lancet 2007;369(9573):1614-20.
16. Alfaleh K, Anabrees J, Bassler D. Probiotics reduce the risk of necrotizing enterocolitis in preterm infants: a meta-analysis. Neonatology 2010;97(2):93-9.
17. Madsen K. Probiotics and the immune response. J Clin Gastroenterol 2006;40(3):232-4.
18. Stoll B, Price PT, Reeds PJ, et al. Feeding an elemental diet vs a milk-based formula does not decrease intestinal mucosal growth in infant pigs. JPEN J Parenter Enteral Nutr 2006;30(1):32-9.
19. Molbak L, Thomsen LE, Jensen TK, Bach Knudsen KE, Boye M. Increased amount of Bifidobacterium thermacidophilum and Megasphaera elsdenii in the colonic microbiota of pigs fed a swine dysentery preventive diet containing chicory roots and sweet lupine. J Appl Microbiol 2007;103(5):1853-67.
20. Leser TD, Lindecrona RH, Jensen TK, Jensen BB, Moller K. Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with Brachyspira hyodysenteriae. Appl Environ Microbiol 2000;66(8):3290-6.
21. Vann RC, Nguyen HV, Reeds PJ, et al. Somatotropin increases protein balance by lowering body protein degradation in fed,

growing pigs. Am J Physiol Endocrinol Metab 2000;278(3):E477-83.
22. Niinikoski H, Stoll B, Guan X, et al. Onset of small intestinal atrophy is associated with reduced intestinal blood flow in TPN-fed neonatal piglets. J Nutr 2004;134(6):1467-74.
23. Metges CC, Petzke KJ, Hennig U. Gas chromatography/combustion/isotope ratio mass spectrometric comparison of N-acetyl- and N-pivaloyl amino acid esters to measure 15N isotopic abundances in physiological samples: a pilot study on amino acid synthesis in the upper gastro-intestinal tract of minipigs. J Mass Spectrom 1996;31(4):367-76.
24. Schaart MW, de Bruijn AC, Schierbeek H, Tibboel D, Renes IB, van Goudoever JB. Small intestinal MUC2 synthesis in human preterm infants. Am J Physiol Gastrointest Liver Physiol 2009;296(5):G1085-90.
25. Schaart MW, Schierbeek H, de Bruijn AC, Tibboel D, van Goudoever JB, Renes IB. A novel method to determine small intestinal barrier function in human neonates in vivo. Gut 2006;55(9):1366-7.
26. Tytgat KM, Buller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J. Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology 1994;107(5):1352-63.
27. Schaart MW, Schierbeek H, van der Schoor SR, et al. Threonine utilization is high in the intestine of piglets. J Nutr 2005;135(4):765-70.
28. Burrin DG, Stoll B, Jiang R, et al. GLP-2 stimulates intestinal growth in premature TPN-fed pigs by suppressing proteolysis and apoptosis. Am J Physiol Gastrointest Liver Physiol 2000;279(6):G1249-56.
29. van der Schoor SR, Wattimena DL, Huijmans J, Vermes A, van Goudoever JB. The gut takes nearly all: threonine kinetics in infants. Am J Clin Nutr 2007;86(4):1132-8.
30. Spear ML, Darmaun D, Sager BK, Parsons WR, Haymond MW. Use of [13C] bicarbonate infusion for measurement of CO2 production. Am J Physiol 1995;268(6 Pt 1):E1123-7.
31. Le Floc'h N, Obled C, Seve B. In vivo threonine oxidation rate is dependent on threonine dietary supply in growing pigs fed low to adequate levels. J Nutr 1995;125(10):2550-62.
32. Stoll B, Chang X, Fan MZ, Reeds PJ, Burrin DG. Enteral nutrient intake level determines intestinal protein synthesis and accretion rates in neonatal pigs. Am J Physiol Gastrointest Liver Physiol 2000;279(2):G288-94.
33. Metges CC. Contribution of microbial amino acids to amino acid homeostasis of the host. J Nutr 2000;130(7):1857S-64S.
34. van Goudoever JB, Stoll B, Henry JF, Burrin DG, Reeds PJ. Adaptive regulation of intestinal lysine metabolism. Proc Natl Acad Sci U S A 2000;97(21):11620-5.
35. Stoll B, Burrin DG. Measuring splanchnic amino acid metabolism in vivo using stable isotopic tracers. J Anim Sci 2006;84 Suppl:E60-72.
36. Chen LX, Yin YL, Jobgen WS, et al. In vitro oxidation of essential amino acids by jejunal mucosal cells of growing pigs. Livestock Science 2007(109):19–23.
37. Fuller MF, Reeds PJ. Nitrogen cycling in the gut. Annu Rev Nutr 1998;18:385-411.
38. Millward DJ, Forrester T, Ah-Sing E, et al. The transfer of 15N from urea to lysine in the human infant. Br J Nutr 2000;83(5):505-12.
39. Mosenthin R, Sauer WC, de Lange CF. Tracer studies of urea kinetics in growing pigs: I. The effect of intravenous infusion of urea on urea recycling and the site of urea secretion into the gastrointestinal tract. J Anim Sci 1992;70(11):3458-66.
40. Brigidi P, Vitali B, Swennen E, Bazzocchi G, Matteuzzi D. Effects of probiotic administration upon the composition and

enzymatic activity of human fecal microbiota in patients with irritable bowel syndrome or functional diarrhea. Res Microbiol 2001;152(8):735-41.
41. Cooney RN, Kimball SR, Vary TC. Regulation of skeletal muscle protein turnover during sepsis: mechanisms and mediators. Shock 1997;7(1):1-16.
42. Lang CH, Frost RA, Vary TC. Regulation of muscle protein synthesis during sepsis and inflammation. Am J Physiol Endocrinol Metab 2007;293(2):E453-9.
43. Vary TC, Kimball SR. Regulation of hepatic protein synthesis in chronic inflammation and sepsis. Am J Physiol 1992;262(2 Pt 1):C445-52.
44. Ryu DH, Rando RR. Aminoglycoside binding to human and bacterial A-Site rRNA decoding region constructs. Bioorg Med Chem 2001;9(10):2601-8.
45. Harding SV, Fraser KG, Wykes LJ. Probiotics stimulate liver and plasma protein synthesis in piglets with dextran sulfate-induced colitis and macronutrient restriction. J Nutr 2008;138(11):2129-35.
46. Madsen K, Cornish A, Soper P, et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 2001;121(3):580-91.
47. Anand RJ, Leaphart CL, Mollen KP, Hackam DJ. The role of the intestinal barrier in the pathogenesis of necrotizing enterocolitis. Shock 2007;27(2):124-33.
48. Caballero-Franco C, Keller K, De Simone C, Chadee K. The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 2007;292(1):G315-22.
49. Ohland CL, Macnaughton WK. Probiotic bacteria and intestinal epithelial barrier function. Am J Physiol Gastrointest Liver Physiol 2010;298(6):G807-19.
50. Mack DR, Michail S, Wei S, McDougall L, Hollingsworth MA. Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. Am J Physiol 1999;276(4 Pt 1):G941-50.
51. Burger-van Paassen N, Vincent A, Puiman PJ, et al. The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection. Biochem J 2009;420(2):211-9.
52. Knol J, Scholtens P, Kafka C, et al. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. J Pediatr Gastroenterol Nutr 2005;40(1):36-42.

Chapter 8

Paneth cell hyperplasia and metaplasia in necrotizing enterocolitis

Patrycja J. Puiman
Nanda Burger - van Paassen
Maaike W. Schaart
Adrianus C.J.M. de Bruijn
Ronald R. de Krijger
Dick Tibboel
Johannes B. van Goudoever
Ingrid B. Renes

Accepted Pediatric Research

Abstract

Paneth cell dysfunction has been suggested in necrotizing enterocolitis (NEC). The aim of the present study was to: i) study Paneth cell presence, protein expression, and developmental changes in preterm infants with NEC; ii) determine Paneth cell products and antimicrobial capacity in ileostomy outflow fluid. Intestinal tissue from NEC patients (n=55), preterm control infants (n=22), and term controls (n=7) was obtained during surgical resection and at stoma closure after recovery. Paneth cell abundance and protein expression were analyzed by immunohistochemistry. RNA levels of Paneth cell proteins were determined by quantitative real-time RT-PCR. In ileostomy outflow fluid, Paneth cell products were quantified and antimicrobial activity was measured in vitro. In acute NEC, Paneth cell abundance in small intestinal tissue was not significantly different from preterm controls. After recovery from NEC, Paneth cell hyperplasia was observed in the small intestine concomitant with elevated human alpha-defensin 5 (HD5) mRNA levels. In the colon, metaplastic Paneth cells were observed. Ileostomy fluid contained Paneth cell proteins and inhibited bacterial growth. In conjunction, these data suggest an important role of Paneth cells and their products in various phases of NEC.

Introduction

Paneth cells, named after Joseph Paneth[1], play a significant role in the innate immune response. Localized at the base of the base of the crypts of Lieberkühn, Paneth cells are abundant in the ileum and only occasionally found in the proximal colon[2]. Paneth cells have defensive functions including: 1) protection of stem cells in response to invading microbes[3-4]; 2) eradication of ingested pathogens[5-7]; 3) regulation of the composition, distribution, and number of commensal bacteria in the intestinal lumen[8-9]; 4) induction of cytokine secretion, immune cell recruitment, and chloride secretion to flush the intestinal crypts of pathogens[10-11].

Paneth cells execute their functions by production of antimicrobial proteins/peptides such as lysozyme[12-13], secretory phospholipase-A2 (sPLA2)[14], and human α-defensins (HD5 and -6)[15-16]. In the human fetal intestine, Paneth cells are present from 20 weeks of gestation, however HD5 and -6 mRNAs are expressed from 13.5 weeks onwards[17-18]. Both Paneth cell numbers and HD5 and -6 mRNA expression are lower in premature infants at 24 weeks of gestation compared to term infants, and up to 200-fold lower than in adults[18]. In the premature infant, who is often exposed to nosocomial pathogens and has delayed colonization with beneficial commensals, this phenomenon could result in higher susceptibility to bacterial infection and inflammation.

Although Paneth cell dysfunction in necrotizing enterocolitis (NEC) has been suggested[19], little is known about Paneth cell abundance and function in preterm infants at risk for NEC. NEC is the most common gastrointestinal disease in premature infants with mortality rates up to 50% for infants needing surgery[20-21]. Risk factors for NEC are prematurity, very low birth weight, enteral formula feeding, and bacterial colonization[22-24]. However, the underlying etiology and the impact of the innate immune system on the development of NEC require further investigation.

We hypothesized that preterm infants with acute NEC have fewer Paneth cells compared to control patients, and that Paneth cell numbers are up-regulated during recovery from NEC, thereby enhancing the innate immune response. Our aim was to i) study Paneth cell presence, Paneth cell protein expression, and disease-related changes in Paneth cell numbers over time in premature infants with NEC compared to control patients, and ii) to measure Paneth cell products in ileostomy outflow fluid during NEC recovery and to determine the bactericidal activity of ileal outflow fluid.

Methods

Study population.

Premature infants, who underwent bowel resection for NEC between August 2003 and September 2009, were eligible for the study. NEC was diagnosed and staged according to Bell's criteria[25]. Diagnosis was confirmed during surgery and by histopathological evaluation of resected intestinal tissue. Samples of both ends of the resected intestine represented macroscopically vital tissue and were collected for histology and quantitative real-time RT-PCR (qRT-PCR).

Approximately 3-5 weeks after surgery, when infants received an enteral intake of at least 100 $ml \cdot kg^{-1}.d^{-1}$, enterostomy outflow fluid was collected every 3h for 24h and immediately stored at -20°C. After full recovery, patients underwent a second surgical procedure when eligible for reanastomosis (i.e. stoma closure). To allow proper reanastomosis tissue was resected, which was collected for histology.

Patients who underwent surgery for resection of post-NEC strictures, as a result of obstructive fibrotic intestinal tissue that developed during non-surgical therapy for NEC, were also included. These samples were not subjected to surgical manipulation or exposed to the extra-abdominal environment, and are thus representative for the effects of NEC only. Again, samples were taken from both the proximal and distal part of the resected intestine.

Finally, both preterm and term neonates who underwent resection for developmental defects or diseases other than NEC were included as control patients, and intestinal tissue was collected as described above. Infants diagnosed with cystic fibrosis were excluded.

The study protocol was approved by the 'Central Committee on Research involving Human Subjects' (The Hague, the Netherlands) and written informed consent was obtained from the parents.

Histology and Immunohistochemistry.

Intestinal tissues were fixed in 4% (wt/vol) paraformaldehyde in phosphate buffered saline for 24h at 4°C and processed for light microscopy. Five micrometer-thick sections were cut and deparaffinized through a graded series of xylol-ethanol. For histology, tissue samples were stained with hematoxylin and eosin. To determine Paneth cell-specific expression of lysozyme, trypsin and HD5, immunohistochemistry was performed as described previously[26-27]. Antibodies used were anti-human lysozyme (Dako, Glostrup, Denmark), anti-human trypsin (MAB 1482; Millipore, Billerica, MA, USA) and anti-human HD5 (HyCult Biotechnology, Uden, the Netherlands). Secondary antibodies applied were biotinylated horse anti-mouse IgG diluted (Vector Laboratories, Burlingame, England) and biotinylated goat anti-rabbit IgG (Vector Laboratories). Detection

was performed using the ABC-PO complex (Vectastain Elite Kit, Vector Laboratories). Staining was developed using diaminobenzidine.
Collected tissue samples were assigned to intestinal segments according to their origin: jejunum, ileum, coecum, colon ascendens, colon transversum, colon descendens, and rectosigmoid. When samples originated from the same intestinal segment, only one sample per segment was analyzed. Samples from NEC and control infants were matched and analyzed according to their segment of origin. Tissue morphology was qualitatively assessed by 2 trained observers (PJP&IBR). A semi-quantitative assessment of Paneth cell abundance based on lysozyme and HD5 positive cells was performed. Paneth cells within 10 crypts per tissue sample were scored blinded as follows: 0: no Paneth cells; 1: 0-1 Paneth cells per crypt and at least 1 Paneth cell per 10 crypts; 2: 1-4 Paneth cells per crypt; 3: more than 4 Paneth cells per crypt. Scores were given by one observer and validated by the second observer.

Quantitative real-time RT-PCR.

RNA was isolated from snap-frozen ileal tissue (RNeasy Midi kit; Qiagen Benelux, Venlo, the Netherlands) and used for cDNA synthesis. Expression levels of DEFA5 (HD5), LYZ (lysozyme), TRY2 (anionic trypsin), and PLA2G2A (sPLA2) were quantified using qRT-PCR analysis based upon the intercalation of SYBR Green on an ABI prism 7900HT Fast Real-Time PCR system (PE Applied Biosystems, Foster City, CA, USA) as described previously[28]. Messenger RNA levels were expressed relative to the epithelial-specific housekeeping gene VIL1 (Villin). Primer combinations are given in table 1.

Table 1. Primer sequences used for quantitative real time RT-PCR

Gene	Forward primer	Reverse primer
LYZ	5′- TTT GCT GCA AGA TAA CAT C -3′	5′- GAC GGA CAT CTC TGT TTT G -3′
DEFA5	5′- TGC AGG AAA TGG ACT CTC -3′	5′- GCC ACT GAT TTC ACA CAC -3′
PLA2G2A	5′- TGG CAC CAA ATT TCT GA -3′	5′- GCA GCC TTA TCA CAC TCA -3′
TRY2	5′- GCT CCA AGG AAT TGT CTC -3′	5′- GGG GCT TTA GCT GTT G -3′
VIL1	5′- CTG CCT TCT CTG CTC TG -3′	5′- ATC GGT GAG AAA ATG AGA C -3′

Primers for DEFA5 (HD5), LYZ (lysozyme), TRY2 (anionic trypsin), and PLA2G2A (secretory phospholipase A2) were designed using OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO, USA).

Protein isolation and Western blot analyses.

To isolate proteins from the small intestinal outflow fluid, cesium-chloride density gradient ultracentrifugation was performed as described previously[29-30]. Fractions with a buoyant density >1.35 g/ml, containing high density proteins, were discarded. Fractions with a buoyant density <1.35 g/ml were pooled, dialyzed, and used to quantify lysozyme, trypsin, and sPLA2 by Western-blot. The used dialysis membrane had a cut-off size of 10 kD and therefore did not allow analysis of HD5 as HD5 is smaller than 10 kD. Primary antibodies used were rabbit anti-lysozyme (Dako), rabbit anti-sPLA2 (H-

74) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-α-trypsin (MAB 1482, Chemicon International). Bound antibodies were detected using HRP-conjugated goat anti-rabbit or anti-mouse antibodies and Luminol Enhancer (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA). Western-blots were analyzed using densitometry.

Antimicrobial assay.

Escherichia coli (E. coli), Lactococcus lactis (L. lactis), and Lactobacillus rhamnosus (LGG) were grown overnight in LB medium, GM17 medium, and MRS medium (BD, Franklin Lakes, NJ, USA) respectively, at 37°C. The pooled antimicrobial protein fractions (100 μg) were added to 200 μl of bacterial cultures of $2x10^7$ colony forming units (CFU)/ml and incubated for 1h at 37°C. A 10-4 dilution was prepared and 100 μl of the suspension was plated. After overnight incubation at 37°C, CFUs were counted. Bacterial growth inhibition was analyzed by calculating the number of CFUs in comparison to untreated bacteria.

Statistical analysis.

Comparisons between patient groups were made using ANOVA with a post-hoc Tukey T-test for normally distributed data or the Kruskal-Wallis Test followed by Dunn's Multiple Comparison Test for not-normally distributed data. Protein and mRNA levels of antimicrobial proteins were determined using the Mann Whitney test. Analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Significance was defined at $P < .05$.

Results

Study population.

A total of 84 infants were included in the study. Patient characteristics are shown in table 2. Forty-nine preterm infants underwent bowel resection for stage III acute NEC, further referred to as A-NEC. After resection of necrotic tissue, primary anastomosis was performed in 4 patients, an enterostomy was created in 40 infants, and in 5 patients NEC lesions were too extensive to allow further surgical treatment compatible with life. In total 13 infants (27%) died of complications of NEC. After recovery, 31 out of the 40 enterostomy patients were eligible for reanastomosis (further referred to as NEC-R), allowing repeated tissue collection. Additionally, six premature infants were included, who had received non-surgical treatment for NEC, but developed post-NEC strictures requiring surgery (Post-NEC Stricture).

Twenty-two premature infants were included as control (Preterm CO). These patients were diagnosed with small intestinal atresia (n=8), milk curd obstruction (n=3), perfora-

tion due to herniation (n=2), solitary perforation (n=1), ileus (n=1), Meckel's diverticulum (n=1), volvulus (n=2), gastroschizis (n=3) and cloacal malformation (n=1). After intestinal resection, 6 infants received a primary anastomosis and 16 an enterostomy. Post-surgically 1 patient died of additional clinical complications. After full recovery, 13 infants were eligible for stoma closure (Preterm CO-R), allowing repeated tissue collection.
Seven term infants who were resected for small intestinal atresia (n=3), intestinal perforation (n=3), and volvulus (n=1), were included as term controls (Term CO).
Figure 1 depicts the corrected gestational age at the time of tissue sampling during surgery for all patient groups.

Table 2. Patient Demographics

	NEC	Post-NEC Stricture	Preterm CO	Term CO	P-value
No. of patients	49	6	22	7	
Demographics					
Sex, % male	61	67	50	57	
Gestational age (wk)	29.5 ± 3	31.8 ± 3.7	32.9 ± 3.9	38.2 ± 1.2	a,c,i,k
Birth weight (g)	1239 ± 501.8	1618 ± 507.5	2051 ± 874.8	2946 ± 738.9*	a,c,h,j
Apgar score 1 min	7 (4 - 9)	7.5 (2.9 - 9)	8 (5 - 9)*	9 (8 - 9)†	NS
Apgar score 5 min	9 (8 - 10)	9.5 (8.3 - 10)	9 (8 - 9)*	9 (9 - 10)†	NS
Clinical and surgical outcome					
Postnatal age at 1st surgery (d)	11 (7 - 17.5)	40 (35.8 – 51)	1.5 (0 - 16)	2 (0 - 3)	b,d,e,g,h
Gestational age at 1st surgery (wk)	31.7 (29.2 - 34)	38 (35.8 - 40.5)	34.4 (31 - 36.1)	38 (37.6 – 40.3)	b,c,f
Primary anastomosis, %	8	100	27	42.3	
Jejunostomy, %	12	-	23	14.3	
Ileostomy, %	61	-	45	42.3	
Colostomy, %	8	-	4	-	
Deaths, %	27	-	4	-	
Patients eligible for reanastomosis, %	63	-	59	-	
Postnatal age at reanastomosis (d)	95 (57 - 125)	-	55 (44 - 102)	-	NS
Gestational age at reanastomosis (wk)	43 (40.1 - 46.6)	-	41 (39.5 – 46.4)	-	NS

Data provided are percentages or means ± SD. Medians (interquartile range) are provided for variables that are not normally distributed.
a P < .001 for comparisons between NEC and Preterm controls.
b P < .05 for comparisons between NEC and Preterm controls.
c P < .001 for comparisons between NEC and Term Controls.
d P < .05 for comparisons between NEC and Term Controls.
e P < .05 for comparisons between NEC and Post-NEC Strictures.
f P < .001 for comparisons between NEC and Post-NEC Strictures.
g P < .001 for comparisons between Post-NEC Strictures and Preterm Controls.
h P < .001 for comparisons between Post-NEC Strictures and Term controls.
i P < .01 for comparisons between Post-NEC Strictures and Term controls.
j P < .05 for comparisons between Preterm controls and Term controls.
k P < .01 for comparisons between Preterm controls and Term controls.
NS not significant.
*Data on 1 patient was not recorded. † Data on 2 patients was not recorded.

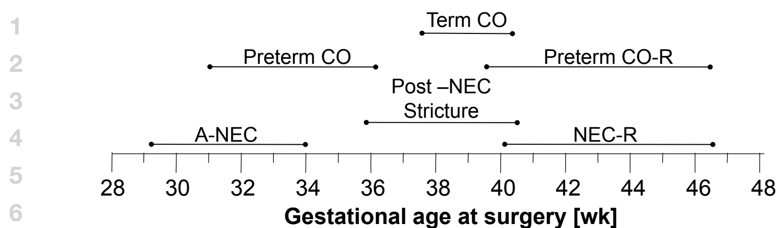


Figure 1. Time-frame representing an overview of intestinal tissue sampling according to gestational age. Bars represent interquartile ranges.
A-NEC patients are significantly younger than Preterm CO (Mann-Whitney $P < .01$), but corrected gestational age is comparable during reanastomosis. Corrected gestational age during reanastomosis is in both groups not different from patients with Post-NEC strictures and Term Controls during surgery.

Sample evaluation.

In most infants the resected intestine covered multiple intestinal segments. Samples were cut from both ends of the resected material and therefore sample numbers do not correlate with the number of patients included. Samples from jejunum, ileum, and colon were used for morphological analysis (n=157). Samples that showed complete mucosal erosion were excluded from further analyses (n=36). The remaining samples were stained for lysozyme, HD5, and trypsin to analyze Paneth cell-specific protein expression. Scoring was based upon both lysozyme and HD5 staining. Table 3 demonstrates the number of samples that were obtained and evaluated for each patient group.

Table 3. Sample evaluation of intestinal tissue obtained during acute surgery (A) and reanastomosis (B)

A. Acute surgery	Small Intestine			Colon		
Patient Groups	Total	Excluded	Evaluated	Total	Excluded	Evaluated
NEC	46	16	30	18	8	10
Post-NEC Stricture	0	0	0	5	0	5
Preterm CO	21	4	17	2	1	1
Term CO	7	1	6	1	0	1
B. Reanastomosis	Small Intestine			Colon		
Patient Groups	Total	Excluded	Evaluated	Total	Excluded	Evaluated
NEC	29	3	26	13	2	11
Preterm CO	12	1	11	3	0	3

Note: Tissue samples were obtained from both ends of resected intestine and might originate from multiple intestinal segments. Therefore patient numbers do not correlate with sample numbers.

Paneth cell hyperplasia during NEC recovery.

Paneth cells were present in small intestinal tissue of NEC patients and Preterm CO, and were positive for lysozyme (Figure 2A-D), HD5 (Figure 2E-H), and trypsin (not shown). Abundance of Paneth cells, determined by lysozyme and HD5 staining, was not significantly different between A-NEC and Preterm CO (Figure 3). In reanastomosis samples, no differences in Paneth cell abundance between NEC-R and Preterm CO-R were observed (Figure 3). However, small intestinal samples in both NEC-R and Preterm

CO-R showed increased numbers of Paneth cells, determined by lysozyme and HD5 staining, when compared to A-NEC and Preterm CO samples, respectively (Figure 3). To determine whether this increase in Paneth cell numbers was age- or disease-related, we compared Term CO samples to NEC-R and Preterm CO-R samples, as the corrected gestational age at time of surgery was not significantly different between these groups (Figure 1). Interestingly, Paneth cell abundance in small intestinal NEC-R and Preterm CO-R tissue was increased compared to Term CO indicating a disease-related effect.

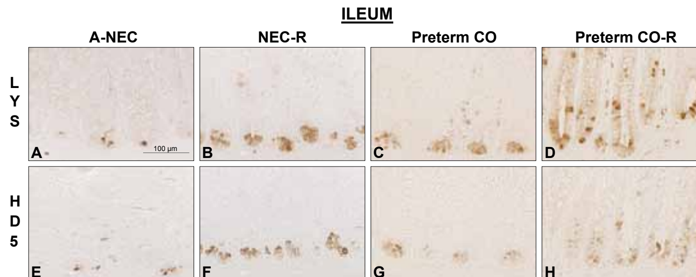


Figure 2. Immunohistochemistry for lysozyme- (LYS) (A-D) and HD5-positive (E-H) Paneth cells in ileal tissue from NEC patients and Preterm CO. (A, E) A-NEC; (B, F) NEC-R; (C, G) Preterm CO; (D, H) Preterm CO-R. Images are representative of 19 A-NEC, 21 NEC-R, 9 Preterm CO, and 6 Preterm CO-R specimens derived from the ileum.

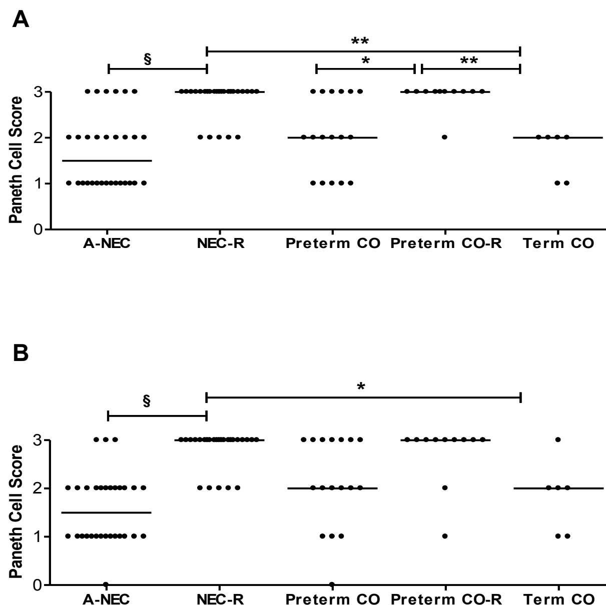


Figure 3. Paneth cell abundance in small intestinal tissue from A-NEC and NEC-R patients, Preterm CO and Preterm CO- R, and Term CO obtained during acute surgery and reanastomosis. Scoring of HD5-positive (A) and lysozyme–positive (B) Paneth cells, based upon the number of stained cells per crypt from an average of 10 crypts (see methods). Lines represent medians. Statistics were performed using the Kruskal-Wallis - Dunn's Multiple Comparison test. * P < .05; ** P < .01; § P < .001.

Paneth cell metaplasia during NEC recovery.

In 9 out of 10 samples, no Paneth cells were found in colonic tissue from A-NEC patients (Figure 4 and 5). Unexpectedly, at the time of reanastomosis nearly all proximal and distal colon samples showed metaplastic Paneth cells, determined by lysozyme and HD5 staining (Figure 4). This finding was confirmed by the Post-NEC Stricture colon samples which all displayed metaplastic Paneth cells (Figure 4). Figure 5 presents the scoring of Paneth cell-abundance in the colon and demonstrates the induction of metaplastic Paneth cells, assessed by staining for HD5 (Figure 5A) and lysozyme (Figure 5B), in NEC-R and Post-NEC Stricture. In Preterm CO-R, only few colon samples (n=3) were obtained, nevertheless all samples showed Paneth cell metaplasia (not shown).

Figure 4. Representative immunohistochemistry for lysozyme (LYS; A-C) and HD5 (D-F) in metaplastic Paneth cells in colonic tissue from NEC patients.
(A, D) A-NEC. (B, E) NEC-R. (C, F) Post-NEC Stricture. Images are representative of 10 A-NEC, 11 NEC-R, and 5 Post-NEC Stricture specimens.

Figure 5. Scoring of Paneth cell abundance in colonic tissue from NEC A-NEC, NEC-R and Post-NEC Stricture patients. Scoring of HD5-positive (A) and lysozyme-positive (B) Paneth cells, based upon the number of stained cells per crypt from an average of 10 crypts (see methods).
Lines represent medians. Statistics were performed using the Kruskal-Wallis - Dunn's Multiple Comparison test. * P < .05;** P < .01.

LYZ, TRY2, PLA2G2A, and DEFA5 mRNA levels in NEC.

Analyses of ileal samples from 21 NEC patients by qRT-PCR showed that LYZ, TRY2, and PLA2G2A mRNA levels were not different in tissue from A-NEC (n=8) vs. NEC-R (n=13) (Figure 6). However, expression of DEFA5 was significantly higher in NEC-R compared to A-NEC (Figure 6A-D). Although sample numbers obtained from preterm control infants were limited, expression of LYZ, TRY2, PLA2G2A, and DEFA5 tended to be higher in Preterm CO-R samples (n=5) compared to Preterm CO (n=4) (Figure 6E-H).

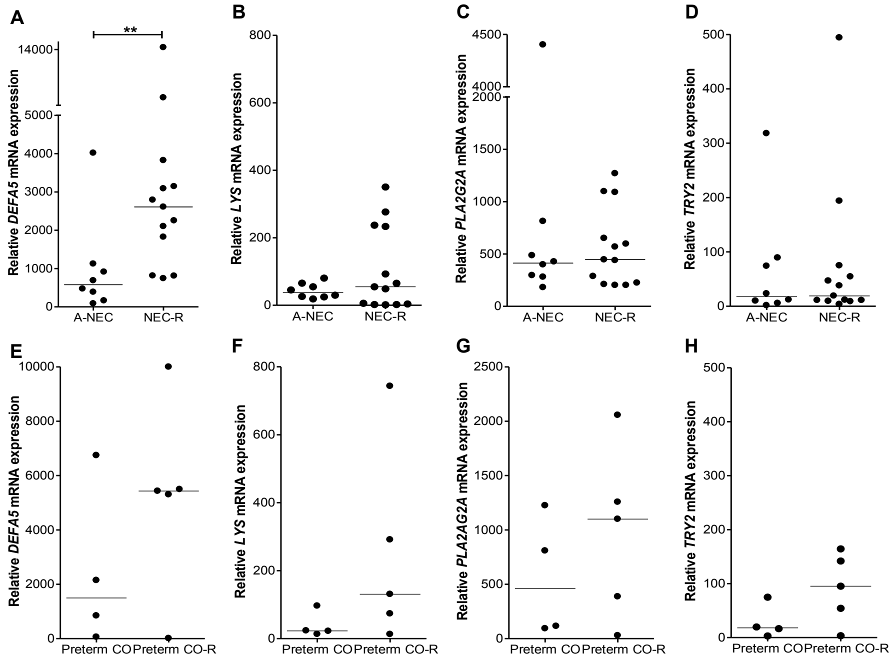


Figure 6. Expression of anti-bacterial peptide genes in A-NEC and NEC-R (A-D) and Preterm CO and Preterm CO-R (E-H): (A, E) DEFA5, (B, F) LYS, (C, G) PLA2G2A, and (D, H) TRY2. DEFA5 expression was upregulated in NEC Reanastomosis; Mann Whitney test P = .013. Lines represent medians.

Antimicrobial products in the ileostomy outflow fluid and antimicrobial activity.

Ileostomy outflow fluid from 12 patients was collected during the regenerative phase of NEC (Table 4). Isolates of the outflow fluid samples contained lysozyme, trypsin, and sPLA2. As lysozyme and trypsin are present in breast milk, we analyzed whether levels of these antimicrobial products would differ between infants receiving breast milk (n=6) or formula (n=6). There was no statistically significant difference between the groups (data not shown).

Table 4. Baseline characteristics of NEC patients sampled for ileostomy outflow fluid

No. of patients	12
Demographics	
Sex, % male	92
Gestational age (wk)	29.1 ± 3.1
Birth weight (g)	1210 ± 524
Postnatal age at 1st surgery (d)	11.5 (7.8 - 13.5)
Time of enterostomy outflow collection post-surgery (d)	22 (18.3 - 25)
Patients receiving breast milk, %	50
Patients receiving antibiotic treatment during sampling, %	33

Data provided are percentages or means ± SD. Medians (interquartile range) are provided for variables that are not normally distributed.

Bactericidal activity of the outflow fluid isolates was demonstrated by growth inhibition of *E. coli* with 52% (±18%), *L. lactis* with 81% (±20%) and *LGG* with 43% (±17%) (Figure 7A). Increasing concentrations of protein isolates (n=7) were used to determine bacterial growth inhibition capacity. Growth inhibition up to 100% was reached as presented in figure 7B. To evaluate whether antibiotic treatment influenced the observed effect, we compared bacterial growth inhibition from isolates of antibiotic-treated patients (n=4) to isolates from patients without antibiotics, but no difference was observed (Figure 7).

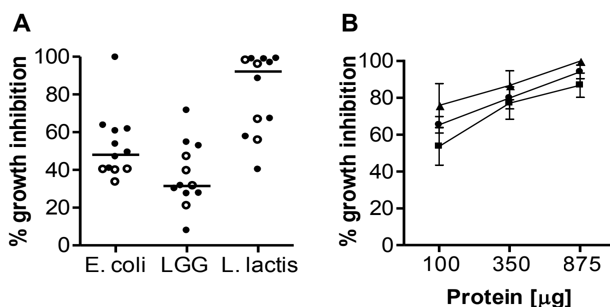


Figure 7. (A) Antimicrobial assay showing bactericidal activity of protein isolates from ileostomy outflow fluid samples (n=12) on E. coli, LGG and L. lactis. Bactericidal activity is expressed as % growth inhibition compared to non-treated controls.
Open symbols denote antibiotic-treated patients. Lines represent means. (B) Bacterial challenge assay showing up to 100 % growth inhibition of *E. coli* , *LGG* and *L. Lactis* with increasing doses of protein isolates (n=7). Bars represent means ± SEM.

Discussion

Paneth cells play a key role in the innate immune response and host defense. Although Paneth cell dysfunction has been suggested, little is known about Paneth cell presence and function in the immature gut and gastrointestinal complications of prematurity such as NEC. In the present study we analyzed Paneth cell-presence, function, and

disease-related changes in Paneth cell abundance over time in preterm infants with NEC. Our results show Paneth cell hyperplasia and metaplasia in premature infants recovering from NEC. Furthermore, Paneth cell-products from ileostomy outflow fluid of NEC patients exhibited strong bactericidal activity.

First, Paneth cell presence and protein expression in small intestinal samples of A-NEC patients vs. Preterm CO was investigated. We found that Paneth cells were present in both A-NEC and Preterm CO. Coutinho and colleagues observed an absence of lysozyme-positive Paneth cells in acute NEC, and suggested a developmental defect in Paneth cells, i.e. delayed maturation of Paneth cells, leading to lack of antimicrobial agents such as lysozyme and defensins[19]. Besides the fact that 40% of their NEC-patients were term newborns in whom the disease is thought to be different from premature infants[31-33], the absence of lysozyme-positive Paneth cells might be explained by enhanced secretion of lysozyme. Nevertheless, our study demonstrates Paneth cell-specific lysozyme and HD5 mRNA and protein in A-NEC contradicting Paneth cell deficiency in preterm infants.

Similarly to our results, Salzman et al. showed presence of Paneth cells in acute NEC patients[34]. Semi-quantitative analysis of the 6 preterm acute NEC infants investigated in that study revealed an increase in Paneth cell numbers and HD5 mRNA levels in infants with NEC compared to (near) term control infants. In our study we did not observe a difference between A-NEC and Term CO with respect to Paneth cell abundance; however we did when we compared NEC-R infants to Term CO. Another point of interest is that in the study of Salzman, intracellular peptide levels in NEC did not coincide with the observed rise in mRNA. Although we did not quantify intracellular peptide levels per Paneth cell, low HD5 mRNA levels coincided with low numbers of HD5-positive Paneth cells, whereas high HD5 mRNA levels correlated with the hyperplasia of HD5-positive Paneth cells during reanastomosis. Therefore, our study is the first to report small intestinal Paneth cell hyperplasia after recovery from NEC and strongly implies an up-regulation of Paneth cell abundance. We did not detect differences in lysozyme, sPLA2, and trypsin mRNA levels in A-NEC vs. NEC-R. However, in contrast to HD5, these products are not restricted to Paneth cells for their production and therefore don't necessarily reflect changes in Paneth cell abundance.

In concordance to the hyperplasia observed in NEC, Paneth cell numbers in Preterm CO-R were also up-regulated compared to Preterm CO. Moreover, both Preterm CO-R and NEC-R tissue showed higher numbers of Paneth cells compared to Term CO. Most likely severe or prolonged intestinal inflammation, which to a lesser extend was also seen in preterm control infants, might explain the observed hyperplasia. However, the effect of increased postnatal age in this preterm population compared to Term CO cannot be excluded.

A novel finding in our study is the occurrence of Paneth cell metaplasia in colon samples during NEC-recovery. The expression of lysozyme, trypsin, and HD5 indicated the presence of well-differentiated cells able to exert Paneth cell-defensive functions. Although Paneth cell metaplasia has not been described in NEC, this phenomenon has been reported in inflammatory bowel disease[35]. Metaplasia can occur through restitution or regeneration of tissue after intestinal mucosal surface loss, either due to resection, and/or inflammation[36]. However, as our Post-NEC stricture patients had not undergone resection during acute NEC, but did show Paneth cell metaplasia, we suggest that inflammation caused Paneth cell-metaplasia.

During recovery, lysozyme, trypsin, and sPLA2 were present in intestinal outflow fluid and outflow fluid inhibited bacterial growth in vitro. Similar outcomes were found in in vitro studies performed with human and mouse peptide isolates[37-39]. A caveat is that these antibacterial products are not Paneth cell specific. However, the presence of Paneth cells in A-NEC and the presence of these peptides in the ileal outflow fluid, imply that Paneth cells in preterm infants are present and at least partially functional by secreting antimicrobial products. Therefore, Paneth cell deficiency, i.e. a lack of Paneth cells, in preterm infants with NEC seems unlikely. However, we cannot exclude that there are abnormalities in Paneth cells that limit their functioning in infants with NEC.

A limitation of the present study is that interpretation of our findings is difficult since preterm controls also exhibit signs of inflammation, numbers of age-matched control patients are limited, and collecting material from a healthy control group is not feasible. Nevertheless, our results indicate the presence of Paneth cells in preterm infants with NEC, and imply that Paneth cell hyperplasia and metaplasia is most likely caused by inflammation. Subsequently, increased Paneth cell numbers suggests enhanced secretion of active antimicrobial products and might be indicative of an enhanced innate defense response during prolonged inflammation which might contribute to NEC-recovery. However, it still remains to be elucidated which possible cell signaling and regulatory pathways are involved in these processes to target improvement of therapy and clinical outcome.

Acknowledgements

The author’s thank J. Bouma for excellent technical assistance.

Reference

1. Paneth J. Ueber die secernirenden Zellen des Dünndarm-Epithels. Arch Mikroskop Anat 1888:113— 91.
2. Sandow MJ, Whitehead R. The Paneth cell. Gut 1979;20:420-31.
3. Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 2003;3:710-20.
4. Salzman NH, Underwood MA, Bevins CL. Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. Semin Immunol 2007;19:70-83.
5. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol 2000;1:113-8.
6. Salzman NH, Ghosh D, Huttner KM, Paterson Y, Bevins CL. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. Nature 2003;422:522-6.
7. Fernandez MI, Regnault B, Mulet C, et al. Maturation of paneth cells induces the refractory state of newborn mice to Shigella infection. J Immunol 2008;180:4924-30.
8. Wilson CL, Ouellette AJ, Satchell DP, et al. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science 1999;286:113-7.
9. Salzman NH, Hung K, Haribhai D, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol 2010;11:76-83.
10. Lin PW, Simon PO, Jr., Gewirtz AT, et al. Paneth cell cryptdins act in vitro as apical paracrine regulators of the innate inflammatory response. J Biol Chem 2004;279:19902-7.
11. Lencer WI, Cheung G, Strohmeier GR, et al. Induction of epithelial chloride secretion by channel-forming cryptdins 2 and 3. Proc Natl Acad Sci U S A 1997;94:8585-9.
12. Erlandsen SL, Parsons JA, Taylor TD. Ultrastructural immunocytochemical localization of lysozyme in the Paneth cells of man. J Histochem Cytochem 1974;22:401-13.
13. Klockars M, Reitamo S. Tissue distribution of lysozyme in man. J Histochem Cytochem 1975;23:932-40.
14. Kiyohara H, Egami H, Shibata Y, Murata K, Ohshima S, Ogawa M. Light microscopic immunohistochemical analysis of the distribution of group II phospholipase A2 in human digestive organs. J Histochem Cytochem 1992;40:1659-64.
15. Jones DE, Bevins CL. Paneth cells of the human small intestine express an antimicrobial peptide gene. J Biol Chem 1992;267:23216-25.
16. Jones DE, Bevins CL. Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel. FEBS Lett 1993;315:187-92.
17. Klockars M, Reitamo S, Adinolfi M. Ontogeny of human lysozyme. Distribution in fetal tissues. Biol Neonate 1977;32:243-9.
18. Mallow EB, Harris A, Salzman N, et al. Human enteric defensins. Gene structure and developmental expression. J Biol Chem 1996;271:4038-45.
19. Coutinho HB, da Mota HC, Coutinho VB, et al. Absence of lysozyme (muramidase) in the intestinal Paneth cells of newborn infants with necrotising enterocolitis. J Clin Pathol 1998;51:512-4.
20. Sharma R, Tepas JJ, 3rd, Hudak ML, et al. Portal venous gas and surgical outcome of neonatal necrotizing enterocolitis. J Pediatr Surg 2005;40:371-6.
21. Blakely ML, Lally KP, McDonald S, et al. Postoperative outcomes of extremely low birth-weight infants with necrotizing enterocolitis or isolated intestinal perforation: a prospective cohort study by the NICHD Neonatal Research Network. Ann Surg 2005;241:984-9; discussion 9-94.

22. Lin PW, Stoll BJ. Necrotising enterocolitis. Lancet 2006;368:1271-83.
23. Lucas A, Cole TJ. Breast milk and neonatal necrotising enterocolitis. Lancet 1990;336:1519-23.
24. Claud EC, Walker WA. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. Faseb J 2001;15:1398-403.
25. Bell MJ, Ternberg JL, Feigin RD, et al. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. Ann Surg 1978;187:1-7.
26. Renes IB, Verburg M, Bulsing NP, et al. Protection of the Peyer's patch-associated crypt and villus epithelium against methotrexate-induced damage is based on its distinct regulation of proliferation. J Pathol 2002;198:60-8.
27. Schaart MW, Yamanouchi T, van Nispen DJ, et al. Does small intestinal atresia affect epithelial protein expression in human newborns? J Pediatr Gastroenterol Nutr 2006;43:576-83.
28. Meijerink J, Mandigers C, van de Locht L, Tonnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diagn 2001;3:55-61.
29. Schaart MW, Schierbeek H, de Bruijn AC, Tibboel D, van Goudoever JB, Renes IB. A novel method to determine small intestinal barrier function in human neonates in vivo. Gut 2006;55:1366-7.
30. Tytgat KM, Klomp LW, Bovelander FJ, et al. Preparation of anti-mucin polypeptide antisera to study mucin biosynthesis. Anal Biochem 1995;226:331-41.
31. Holman RC, Stoll BJ, Clarke MJ, Glass RI. The epidemiology of necrotizing enterocolitis infant mortality in the United States. Am J Public Health 1997;87:2026-31.
32. Maayan-Metzger A, Itzchak A, Mazkereth R, Kuint J. Necrotizing enterocolitis in full-term infants: case-control study and review of the literature. J Perinatol 2004;24:494-9.
33. Sankaran K, Puckett B, Lee DS, et al. Variations in incidence of necrotizing enterocolitis in Canadian neonatal intensive care units. J Pediatr Gastroenterol Nutr 2004;39:366-72.
34. Salzman NH, Polin RA, Harris MC, et al. Enteric defensin expression in necrotizing enterocolitis. Pediatr Res 1998;44:20-6.
35. Tanaka M, Saito H, Kusumi T, et al. Spatial distribution and histogenesis of colorectal Paneth cell metaplasia in idiopathic inflammatory bowel disease. J Gastroenterol Hepatol 2001;16:1353-9.
36. Helmrath MA, Fong JJ, Dekaney CM, Henning SJ. Rapid expansion of intestinal secretory lineages following a massive small bowel resection in mice. Am J Physiol Gastrointest Liver Physiol 2007;292:G215-22.
37. Eisenhauer PB, Harwig SS, Lehrer RI. Cryptdins: antimicrobial defensins of the murine small intestine. Infect Immun 1992;60:3556-65.
38. Ouellette AJ, Hsieh MM, Nosek MT, et al. Mouse Paneth cell defensins: primary structures and antibacterial activities of numerous cryptdin isoforms. Infect Immun 1994;62:5040-7.
39. Porter EM, van Dam E, Valore EV, Ganz T. Broad-spectrum antimicrobial activity of human intestinal defensin 5. Infect Immun 1997;65:2396-401.

Chapter 9

Enteral Arginine Does Not Increase Superior Mesenteric Arterial Blood Flow But Modestly Increases Mucosal Growth in Neonatal Pigs

Patrycja J Puiman

Barbara Stoll

Johannes B. van Goudoever

Douglas G. Burrin

Accepted Journal of Nutrition

Abstract

Arginine is an essential amino acid in neonates synthesized by gut epithelial cells and a precursor for nitric oxide (NO) that regulates vasodilatation and blood flow. Arginine supplementation has been shown to improve intestinal integrity in ischemia-reperfusion models and low plasma levels are associated with necrotizing enterocolitis. We hypothesized that enteral arginine is a specific stimulus for neonatal intestinal blood flow and mucosal growth under conditions of TPN or PEN. We first tested the dose-dependence and specificity of acute (3 h) enteral arginine infusion on SMA blood flow in pigs fed TPN or PEN. We then determined whether chronic (4 d) arginine supplementation of PEN increases mucosal growth and if this was affected by treatment with the NO synthase inhibitor, L-NAME. Acute enteral arginine infusion increased plasma arginine dose-dependently in both TPN and PEN groups, but the plasma response was markedly higher (100-250%) in the PEN group than in the TPN group at the two highest arginine doses. Baseline SMA blood flow was 90% higher in the PEN (2.37 ± 0.32 $L \cdot kg^{-1} \cdot h^{-1}$) pigs than in the TPN pigs (1.23 ± 0.17 $L \cdot kg^{-1} \cdot h^{-1}$), but was not affected by acute infusion individually of arginine, citrulline, or other major gut fuels. Chronic dietary arginine supplementation in PEN induced mucosal growth in the intestine, but this effect was not prevented by treatment with L-NAME. Intestinal crypt cell proliferation, protein synthesis and phosphorylation of mTOR and p70S6K were not affected by dietary arginine. We conclude that partial enteral feeding, but not acute enteral arginine, increases SMA blood flow in the neonatal pig. Furthermore, supplementing arginine in partial enteral feeding modestly increased intestinal mucosal growth and was NO independent.

Introduction

Arginine is a nutritionally essential amino acid in neonates and required for protein synthesis and growth[1]. Although arginine production in adults occurs mainly in the kidneys, in the neonate arginine is exclusively synthesized in gut epithelial cells from amino acid precursors like citrulline, glutamine, glutamate and proline[2]. Except for serving as a building block for proteins, arginine has been shown to exert beneficial effects on intestinal integrity and function as it is the major amino acid precursor of polyamines essential for gut healing[3], an enhancer of cell migration and activator of protein synthesis[4-6], and the sole physiological precursor for nitric oxide (NO)[2].
NO is a signaling molecule that plays a central role in regulating vascular resistance and hence blood flow in the newborn intestinal circulation[7-8]. NO is a potent vasodilator that is produced by NO synthase (NOS) that catalyzes the production of NO from arginine[2]. There are three different isoforms of NOS: nNOS (type 1 NOS) which was first discovered in neuronal tissues; iNOS (type 2 NOS), which is inducible under inflammatory conditions; and eNOS (type 3 NOS), which was first identified in endothelial cells. nNOS and eNOS are expressed constitutively at low levels in a variety of cell types and tissues, whereas iNOS is normally not expressed at a significant level in cells or tissues. NO synthesis is regulated by the availability of arginine[9]. Low plasma levels of arginine have shown to lead to decreased NO synthesis and subsequently diminished blood flow in the small intestine in low grade endotoxemia in the rat[10].
Clinical studies have shown low levels of arginine in preterm infants and arginine deficiency is associated with an increased incidence of NEC[11-14]. NEC is the most common intestinal emergency in the preterm infant with reported mortality rates of up to 50%[15]. A detrimental series of pathophysiological events involving intestinal inflammation and ischemia, leads to mucosal eruption, invasion of bacteria into the intestinal wall, necrosis, and subsequently sepsis. Low circulating levels of arginine or arginine precursors result in shortage of arginine and arginine derived products like NO, which subsequently may contribute to the actual development of NEC[16].
In neonatal intensive care, most preterm infants receive full TPN or TPN with minimal or partial enteral nutrition (PEN), because of enteral feeding intolerance. Although TPN is a lifesaving therapy, arginine synthesis is greatly diminished in TPN fed pigs[17]. More importantly, we have previously shown in pigs that TPN significantly reduced iNOS activity, decreased superior mesenteric artery (SMA) blood flow and induced mucosal atrophy[17]. Lack of enteral substrate and mucosal atrophy might decrease arginine synthesis by epithelial cells leading to inadequate production of NO by the intestinal vasculature, and predispose the preterm infant to vasoconstriction and tissue injury.
Several investigators have evaluated arginine administration in animal models of ischemia-reperfusion and experimental NEC. Increased bioavailability of arginine caused a

significant increase in NO production and tissue perfusion in the rat microcirculation[18]. Arginine administration enhanced serum NO production and decreased mucosal injury in rat models of ischemia–reperfusion[19-21]. Actual blood flow measurements during intestinal ischemia-reperfusion showed an increase in blood flow upon arginine administration in both mice and pigs[22-23]. Moreover, administration of arginine has been shown to decrease the incidence of experimental NEC in an acidified casein piglet model[24] and a hypoxia–reoxygenation model in young mice[25]. Arginine administration has also been evaluated in a randomized, double-blind, placebo-controlled study where arginine supplementation to infants less than 28 weeks gestation increased plasma arginine levels and significantly decreased the incidence of NEC[26].

Several possibilities may explain how arginine supplementation reduces intestinal injury and NEC in the neonate. First, supplemental arginine may lead to increased local NO production via eNOS in the intestinal vasculature, leading to vasodilatation and preservation and/or restoration of intestinal blood flow. Second, the ability of arginine to prevent NEC and gut injury may be due to the trophic physiological effects on the intestinal epithelium. Arginine supplementation in *in vitro* and *in vivo* models stimulated intestinal protein synthesis and increased epithelial cell survival via enhanced mTOR and p70S6K signaling[4-6]. However, it is unknown whether this trophic effect of arginine on the epithelium is NO and/or blood flow dependent in the neonate. We hypothesized that enteral arginine is a specific stimulus for neonatal intestinal blood flow and subsequent mucosal growth. The objectives of this study were: 1) To establish the dose-dependency and specificity of enteral arginine infusion on SMA blood flow in neonatal pigs fed TPN or PEN; 2) To test whether enteral arginine supplementation increases intestinal mucosal growth and protein synthesis by a NO-dependent mechanism.

Materials and Methods

Animals.

The study protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and conducted in accordance with the Guide for the Care and Use of Laboratory Animals [Department of Health and Human Services publication no. (National Institutes of Health; NIH) 85–23, revised 1985, Office of Science and Health Reports, NIH, Bethesda, MD]. Three-d-old, crossbred pigs were obtained from the Texas Department of Criminal Justice (Huntsville, TX), transported to the animal facility at the Children's Nutrition Research Center (Houston, TX), and immediately placed in cages in a heated room (30°C) until surgery the following day.

Study protocol: In vivo blood flow studies.

Pigs underwent surgery under isoflurane general anesthesia. Silastic catheters were inserted into the jugular vein, carotid artery and gastric fundus as previously described[27]. An ultrasonic blood flow probe was placed around the superior mesenteric artery[17]. Pre-operatively, pigs received enrofloxacin (2.5 mg·kg^{-1}; Baytril, Bayer, Shawnee Mission, KS), and this was continued on each postoperative day. After surgery, each piglet received one dose of analgesic (0.1 mg·kg^{-1} butorphenol tartrate; Torbugesic, Fort Dodge Laboratories, Fort Dodge, IA). During the initial 24 h postoperatively, all pigs received TPN at 50% of full intake; the 100% TPN intake provided (in g·kg^{-1}·d^{-1}) 25 glucose, 13 L-amino acids, 5 lipid, and 452 kJ·kg^{-1}·d^{-1} at a volume of 120 mL·kg^{-1}·d^{-1}. The L-arginine intake during 100% TPN was 0.630 g·kg^{-1}·d^{-1}.

On d 2, pigs were assigned to receive either TPN (n=5, 240 mL·kg^{-1}·d^{-1}), or PEN (n=9, 40% enteral at 96 mL·kg^{-1}.d^{-1}) with a liquid cow's milk-replacer formula (Litter Life, Merrick, Middleton, WI) fed in three oral meals per day. Pigs were weighed daily to adjust their intake. On postoperative d 3-5 blood flow was measured in pigs of both groups (TPN and PEN). Pigs were given a continuous, intragastric infusion in a randomly assigned cross-over design with saline (0.9% NaCl; 4 mL·kg^{-1}·h^{-1}) for 1 h (baseline), followed by a primed, continuous 3-h intragastric infusion with different doses of arginine at 50, 100, 200, 400, 800 µmol·kg^{-1}·h^{-1}. The priming dose equaled the amount of amino acid infused within 2 h for the respective treatments. During enteral arginine infusions, pigs were also given TPN infused intravenously at 10 mL·kg^{-1}·h^{-1}, such that pigs received parenteral arginine at a rate of 150 µmol·kg^{-1}·h^{-1}. Pigs only received one arginine dose per day and were placed back on their respective basal diet of either TPN or PEN during the rest of the day. A third group of pigs were fed PEN (n=8, 40% enteral at 96 mL·kg^{-1}·d^{-1}) and were infused enterally with citrulline, glutamate, glutamine, or glucose as a control at 800 µmol·kg^{-1}·h^{-1}. In pigs given PEN, parenteral nutrition was infused intravenously at 6 mL·kg^{-1}·h^{-1} and enteral formula was infused intragastrically as a priming bolus (8 mL/kg) followed by a continuous infusion at 2 mL·kg^{-1}·h^{-1}. The PEN pigs received a parenteral arginine intake of 150 µmol·kg^{-1}·h^{-1}. In all three infusion groups, SMA blood flow was monitored continuously throughout the 4-h infusion protocol. Arterial blood samples (1 mL) were collected at 1 and 4 h for measurement of plasma amino acid concentrations. After completion of the protocol, pigs were killed with a venous injection of pentobarbital sodium (50 mg/kg) and sodium phenytoin (5 mg/kg, Beuthanasia-D; Schering-Plough Animal Health, Kenilworth, NJ).

Study protocol: Chronic arginine supplementation.

Pigs (n=32) underwent surgery as described above without the implantation of a gastric catheter and SMA blood flow probe, followed by 24 h of TPN as above. Beginning on postoperative d 2, pigs were weighed and assigned to one of three treatments

based on equal body weights. Pigs received PEN (20% enteral at 48 mL·kg^{-1}·d^{-1}) with a cow's milk-replacer formula (Litter Life) via an orogastric bolus 5 times per day for 4 d supplemented with 1) arginine (ARG; 800 μmol·kg^{-1}·h^{-1}; n=11), 2) arginine plus a NO synthase inhibitor N^{G}-nitro-L-arginine methyl ester (ARG+L-NAME, ARG 800 μmol·kg^{-1}·h^{-1} + L-NAME 200 μmol·kg^{-1}·h^{-1}; n=9) or 3) L-alanine (CO, 800 μmol·kg^{-1}·h^{-1}; n=11) as a control. At the end of the 4-d treatment period, 4 h prior to termination, each pig was injected with an intravenous bolus of 5-bromodeoxyuridine (BrdU; 50 mg/kg; Sigma Aldrich, St. Louis, MO) to measure the *in vivo* crypt cell proliferation index[27]. Additionally, 30 min prior to euthanasia, each animal received an intravenous flooding dose of L-phenylalanine (1.5 mmol/kg, containing 0.15 mmol/kg L-[ring-$^{13}C_6$] phenylalanine; Cambridge Isotope Laboratories, Andover, MA) to measure the rate of tissue protein synthesis[17]. Arterial blood samples were taken at 0 and 30 min of L-phenylalanine infusion. Pigs were then killed with an intravenous injection of pentobarbital sodium and sodium phenytoin. The small intestine was excised, flushed with saline, and divided into 2 segments, designated as jejunum and ileum, and weighed. Tissue sections were fixed in 10% buffered formalin for morphological and bromodeoxyuridine (BrdU) analysis. An aliquot of each segment was snap-frozen in liquid nitrogen and stored at -80°C until analysis for protein and DNA content, and isotopic tracer enrichment for protein synthesis analysis.

Plasma and tissue analyses.
Plasma samples were assayed for amino acids by reverse-phase HPLC (Pico Tag, Waters, Milford, MA), glucose by glucose oxidase (Sigma-Aldrich) and insulin by radioimmunoassay as previously described[28]. Frozen intestinal and liver tissue samples were homogenized and assayed for protein and DNA content[17].

Histology and Immunohistochemistry.
Morphometry analysis was performed on formalin-fixed, paraffin-embedded, hematoxylin and eosin-stained intestinal sections (5 μm) as described previously[28]. Villus height, crypt depth, and muscularis thickness were measured by using an Axiophot microscope (Carl Zeiss Inc, Werk Gottingen, Germany) and NIH IMAGE software, version 1.60 (National Institutes of Health, Bethesda, MD). *In vivo* crypt cell proliferation was measured by BrdU crypt-cell labeling[29].

Tissue protein synthesis.
Samples of jejunum, ileum, liver, muscle and pancreas were homogenized and deproteinized with 2 mol/L perchloric acid as described previously[17]. The perchloric acid-soluble (free amino acid pool) and acid-insoluble (protein-bound amino acid pool) fractions were subjected to mass spectrometric analysis. The acid-insoluble fraction

was hydrolyzed with 6 mol/L HCl for 24 h before gas chromatography-mass spectrometry (GC-MS) analysis. To measure the enrichment of [$^{13}C_6$] phenylalanine in the tissue protein-bound pool, hydrolyzed samples were derivatized to form *N*-pivaloyl-i-propyl esters and measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS, Thermo Finnigan Deltaplus XL GC-C-IRMS; Thermo Electron Corp.,Waltham, MA)[30].

Fractional protein synthesis rates (FSR, $\%\cdot d^{-1}$) of jejunum, ileum, liver, and muscle were calculated as follows:

$$FSR = [\,(IE_{bound} / IE_{free}) / (1440 / t)\,] \cdot 100$$

where IE_{bound} and IE_{free} are the isotopic enrichments (mol% excess) of [$^{13}C_6$]phenylalanine of the perchloric acid-insoluble and perchloric acid-soluble pool, respectively, *t* is the time of labeling (min), and 1440 is the number of minutes per day[17]. Absolute synthesis rates (ASR, $g\cdot kg^{-1}\cdot d^{-1}$) of jejunum, ileum, and liver were calculated as follows:

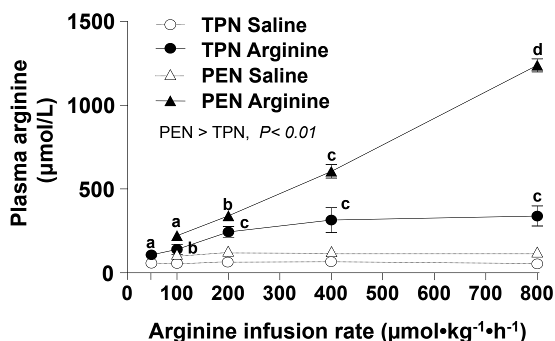
$$ASR = (FSR/100) \cdot protein$$

where protein is the protein content of the organ in gram per kg body weight.

Tissue Immunoblotting.

The tissue abundance of phosphorylated mTOR and p70s6k were measured as by immunoblotting. Frozen muscle and intestinal tissue samples (200 mg) were homogenized in buffer A containing: 50 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 5 mg/L phenylmethylsulfonyl-fluoride, 5 mg/L aprotinin, 5 mg/L chymostatin and 5 mg/L pepstatin. The homogenate was then sonicated and centrifuged at 12,000 g for 15 min at 4°C. From the resulting extracts were separated via 7-15% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS, 20 mmol/L Tris, 150 mmol/L NaOH, pH 7.4). Membranes were incubated with a primary antibody diluted in 5% non-fat milk in TBS + 0.1% Tween-20. Membranes were incubated with a secondary antibody (goat anti-rabbit IgG-HRP, or goat anti-mouse IgG-HRP, 1:5,000, Santa Cruz Biotech Inc.), and the bands were detected as described below. The membranes were probed with mTOR, phosphorylated mTOR, p70S6K or phosphorylated p70S6K antibodies (1:1000 to 3000). Bands were detected with an enhanced chemiluminescence detection kit (ECL Plus, Amersham Biosciences), and semi-quantitative data were obtained using a computer densitometer (Quantity One, Bio-Rad). Phosphorylated and total mTOR and p70S6K measurements were normalized to alpha-tubulin immunoreactivity.

1 **Statistical Analyses.**
2 Minitab statistical software (Minitab, State College, PA) was used for statistical analysis.
3 Data from acute arginine dose infusions were first analyzed by one-way ANOVA and
4 then linear regression. Data from acute substrate infusions and chronic arginine supple-
5 mentation were analyzed by one-way ANOVA, followed by a Tukey's means comparison
6 test or paired t-test when compared to saline baseline values. Differences in plasma
7 arginine concentrations between TPN and PEN groups across arginine dose levels
8 (50-800 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) were tested by two-way ANOVA, with feeding mode and arginine
9 dose as main effects, followed by a Tukey's means comparison test. Data are presented
10 as the mean ± SEM and $P < 0.05$ was considered statistically significant.
11
12
13 # Results
14
15 **In vivo blood flow studies: SMA blood flow response to acute infusion of**
16 **arginine or arginine precursors.**
17 It is important to note that during both TPN and PEN protocols, pigs were receiving
18 parenteral arginine at the rate of 150 and 90 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, respectively. Plasma arginine
19 concentrations were measured during the saline basal infusion and not significantly
20 different among the dose levels and thus have been averaged for simplicity. Plasma
21 arginine and ornithine concentrations increased upon arginine infusion in both TPN (P
22 < 0.05) and PEN ($P < 0.05$) (Fig. 1 and Table 1). In addition, the plasma arginine and
23 ornithine concentrations in PEN pigs were significantly higher compared to TPN pigs
24 during both the basal saline infusion and with arginine infusion (Fig. 1 and Table 1).
25
26
27
28
29
30
31
32
33
34
35



36 **Figure 1. Plasma arginine concentrations in TPN and PEN fed pigs infused intragastrically with arginine.**
37 Values are means ± SEM, n=4-6 pigs per dose in TPN group and n=9 pigs per dose in PEN group. Plasma arginine
38 concentrations increased ($P < 0.05$) upon arginine infusion in both TPN and PEN compared to the respective saline baseline values. Plasma arginine concentrations increased dose-dependently (linear, $P < 0.01$) with arginine infusion concentrations
39 in both TPN and PEN groups. Within a group, labeled means without a common letter differ, $P<0.05$ based on Tukey's test. Plasma arginine concentrations in PEN pigs were higher ($P < 0.05$) compared to TPN based on two-way ANOVA.

TABLE 1. Selected plasma amino acid concentrations in TPN- and PEN-fed pigs acutely infused intragastrically with saline or arginine*

			Arginine infusion rate (μmol·kg^{-1}·h^{-1})				
Amino acid	Feeding Group	Saline†	50	100	200	400	800
				μmol/L			
Arginine	TPN‡	66 ± 7^{a}	124 ± 25^{b}	186 ± 35^{b}	283 ± 37^{c}	336 ± 105^{c}	404 ± 87^{c}
	PEN‡	119 ± 6^{a}	nd	252 ± 21^{b}	385 ± 30^{c}	685 ± 43^{d}	1394 ± 38^{e}
Ornithine^	TPN‡	25 ± 3^{a}	34 ± 7^{a}	47 ± 7^{b}	71 ± 10^{bc}	82 ± 20^{bc}	113 ± 25^{c}
	PEN‡	51 ± 5^{a}	nd	75 ± 7^{b}	108 ± 7^{bc}	178 ± 42^{c}	252 ± 12^{d}
Citrulline^	TPN	113 ± 17	118 ± 17	105 ± 8	125 ± 12	113 ± 17	111 ± 14
	PEN	142 ± 4	nd	152 ± 9	134 ± 9	153 ± 8	165 ± 12
Proline^	TPN	640 ± 100	574 ± 65	556 ± 37	610 ± 52	596 ± 61	677 ± 90
	PEN	493 ± 14^{a}	nd	352 ± 9^{b}	397 ± 33^{b}	387 ± 26^{b}	389 ± 25^{b}
Glutamine^	TPN	811 ± 125	619 ± 61	665 ± 42	645 ± 81	666 ± 92	628 ± 74
	PEN	447 ± 45	nd	392 ± 49	396 ± 45	410 ± 53	341 ± 34
Glutamate^	TPN	353 ± 82	302 ± 36	256 ± 39	334 ± 45	295 ± 35	310 ± 31
	PEN	234 ± 7	nd	188 ± 12	198 ± 19	190 ± 17	194 ± 16
Threonine	TPN	1161 ± 333	1151 ± 125	1188 ± 152	1196 ± 180	1121 ± 169	936 ± 198
	PEN	1089 ± 32	nd	989 ± 67	905 ± 87	986 ± 85	937 ± 82
Lysine	TPN	295 ± 85	318 ± 46	307 ± 33	305 ± 43	254 ± 41	261 ± 48
	PEN#	365 ± 10	nd	273 ± 27	237 ± 11	251 ± 22	250 ± 10

*Means ± SEM, n=4-6 pigs per dose in TPN group and n=9 pigs per dose in PEN group. Means in a row with superscripts without a common letter differ, $P < 0.05$. nd = not determined.
†Values for saline represent overall mean from each arginine dose level (n=24 for TPN group and n=36 for PEN group)
^Significant effect of treatment (TPN vs. PEN) P < 0.05 based on two-way ANOVA.
‡Significant arginine dose effect, linear $P < 0.01$ based on one-way ANOVA.
#Significant arginine dose effect, linear $P < 0.05$ based on one-way ANOVA.

The plasma arginine and ornithine concentrations during the basal saline infusion were 80 to 100% higher in PEN vs. TPN pigs; this may have been due to either the arginine absorbed from the formula or endogenous synthesis by enterocytes. Plasma concentrations of citrulline were higher, whereas those of glutamine, glutamate, and threonine were lower in PEN pigs than in TPN pigs across all arginine doses. Plasma glutamine, glutamate, and lysine were unaffected while concentrations of proline were decreased during arginine infusion compared to the basal saline infusion, but in PEN pigs only. Plasma glucose and insulin concentrations were measured in PEN pigs, but were not affected by the enteral arginine infusion rate (Supplemental Table 1). In general, the SMA flow rates within a given arginine dose treatment were not significantly different during the 3 hr treatment period (Supplemental Table 2). SMA blood flow was continuously measured in TPN and PEN pigs during intragastric arginine infusion. Interestingly, baseline SMA blood flow during saline infusion was higher in PEN (2.48 ± 0.32 L•kg^{-1}•h^{-1}) vs. TPN (1.35 ± 0.17 L•kg^{-1}•h^{-1}) ($P < 0.05$). However, despite increased plasma arginine concentrations, arginine infusion did not change SMA blood flow in either TPN or PEN pigs (Fig. 2 AB).

TABLE 2. Selected plasma amino acid concentrations in PEN pigs acutely infused intragastrically with either saline or various arginine precursors and substrates*

Substrate†	Saline‡	Citrulline	Glutamine	Glutamate	Glucose
Arginine	115 ± 19[a]	561 ± 32[b]	206 ± 32[c]	150 ± 10[ac]	107 ± 9[a]
Citrulline	125 ± 5[a]	2481 ± 100[b]	233 ± 22[c]	134 ± 12[a]	153 ± 12[a]
Glutamine	291 ± 17[a]	249 ± 10[a]	842 ± 76[b]	400 ± 15[c]	311± 21[a]
Glutamate	172 ± 16[a]	178 ± 14[a]	285 ± 18[b]	282 ± 29[b]	189 ± 12[a]
Ornithine	48 ± 2[a]	150 ± 11[b]	68 ± 6[a]	85 ± 7[b]	46 ± 3[a]
Proline	411 ± 12[a]	432 ± 20[ab]	483 ± 18[b]	490± 27[ab]	447 ± 16[a]
Glucose, mmol/L	6.27 ± 0.14	6.64 ± 0.39	6.01 ± 0.28	6.85 ± 0.18	6.54 ± 0.26

*Means ± SEM, n=8 pigs per substrate group. Means in a row with superscripts without a common letter differ, $P < 0.05$. Intragastric infusion rate of citrulline, glutamine, glutamate, and glucose was 800 μmol•kg^{-1}•h^{-1}.
†Amino acid plasma concentrations, μmol/L except where noted otherwise.
‡Values for saline represent overall mean from each arginine dose level (n=32).

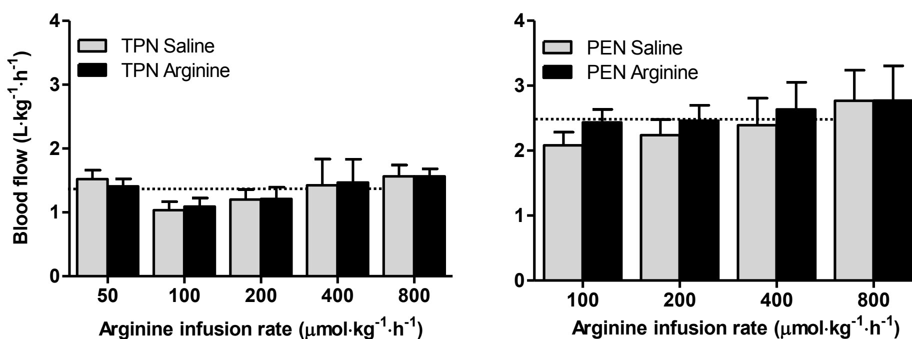


Figure 2. Mean SMA blood flow during enteral arginine infusion for 3 hours in TPN (A) and PEN (B) pigs infused intragastrically with saline or arginine.
Values are means ± SEM, n=3-5 pigs per dose in TPN group and n=5-7 pigs per PEN group. Baseline SMA blood flow in PEN-fed pigs was 90% higher compared to TPN (P <0.01) based on two-way ANOVA; the mean baseline SMA flow in TPN and PEN groups is shown as dashed line for comparison.

We also measured plasma arginine concentrations and SMA blood flow in PEN pigs during acute intragastric infusion of citrulline, glutamine, glutamate, or glucose. Plasma concentrations of glutamine, glutamate, and citrulline were increased when they were acutely infused intragastrically (Table 2). Interestingly, the arterial plasma arginine concentration increased during citrulline and glutamine, but not glutamate infusion. Equimolar infusion of citrulline and arginine resulted in plasma arginine concentrations of 561 and 1394 μmol/L (Table 1 and 2). However, none of the amino acids or glucose affected SMA blood flow (Fig. 3) despite the increased plasma arginine concentrations.

In vivo response to chronic arginine supplementation.

In the ARG+L-NAME group, one piglet died post-surgery and three pigs were euthanized before the end of the study because of clinical signs of sepsis and breathing difficulties. In the remaining pigs there were no complications although occasional diarrhea was observed

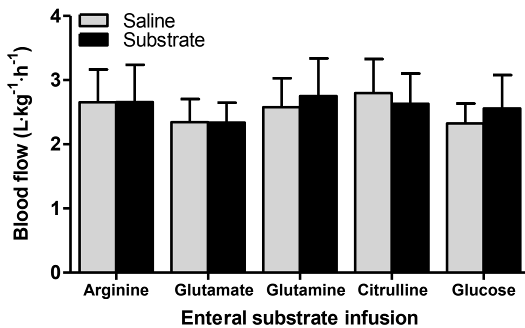


Figure 3. SMA flow in PEN pigs acutely infused intragastrically with citrulline, glutamine, glutamate, or glucose during the 3 hr infusion protocol.
Values are means ± SEM, n=6-7 pigs per group.

but not quantified. Arginine supplementation markedly increased plasma arginine and ornithine concentrations, whereas glutamate, glutamine and lysine levels were decreased (Table 3). Concentrations of other amino acids were similar in all groups (Data not shown). Weight gain during the treatment period was lower in the ARG and ARG+L-NAME groups compared to CO group (Table 4). However, ARG increased proximal intestinal weight, protein and DNA mass compared to the CO group, but not in the distal intestine. Interestingly, jejunal weight, protein mass, but not DNA, were higher in the ARG+L-NAME group than in the CO group. In contrast to ARG, ARG+L-NAME increased distal intestinal weight and protein mass compared to CO pigs. There was no treatment effect on liver growth, protein and DNA mass (Table 4). Furthermore, the weights of kidney, stomach or spleen were also not different amongst groups (Data not shown). Despite the observed changes in jejunal weight, protein and DNA mass, there were no differences in mucosal villous height, crypt depth, muscularis thickness, or crypt cell proliferation between any of the three groups (Table 4).

TABLE 3. Plasma amino acid concentrations in pigs fed PEN supplemented with CO, ARG, or ARG+L-NAME*

Amino acid	CO	ARG	ARG+L-NAME
		μmol/L	
Alanine	993 ± 84[a]	434 ± 53[b]	479 ± 64[b]
Arginine	52 ± 7[a]	647 ± 85[b]	375 ± 66[c]
Citrulline	60 ± 12	70 ± 22	75 ± 17
Glutamine	659 ± 70[a]	371 ± 60[b]	440 ± 93[b]
Glutamate	238 ± 36[a]	134 ± 9[b]	121 ± 11[b]
Ornithine	18 ± 2[a]	290 ± 58[b]	223 ± 50[b]
Proline	524 ± 27	488 ± 89	497 ± 44
Lysine	254 ± 25[a]	173 ± 15[b]	228 ± 44[ab]

*Means ± SEM, n=9-11 pigs per group. Means in a row with superscripts without a common letter differ, *P* < 0.05.

Table 4. Weight gain, organ weights and tissue analyses in pigs fed PEN supplemented with CO, ARG, or ARG+L-NAME*.

	CO	ARG	ARG+L-NAME
Body weight final, g	3057 ± 120	2846 ± 114	2671 ± 173
Weight gain, g·kg^{-1}·d^{-1}	80.6 ± 4.5^{a}	59.7 ± 4.0^{b}	49.5 ± 4.3^{b}
Proximal small intestine			
Weight, g·kg^{-1}	11.7 ± 0.5^{a}	14.7 ± 0.6^{b}	14.2 ± 0.9^{b}
Protein mass, mg·kg^{-1}	1140 ± 54^{a}	1497 ± 63^{b}	1365 ± 60^{b}
DNA mass, mg·kg^{-1}	80.5 ± 4.6^{a}	103.9 ± 5.4^{b}	89.6 ± 6.2^{a}
Villus height, µm	569 ± 46	590 ± 35	615 ± 65
Villus area, mm	42.8 ± 2.7	44.5 ± 2.1	45.7 ± 4.4
Crypt depth, µm	140.3 ± 3.9	143.5 ± 6.9	143.2 ± 9.6
Muscularis thickness, µm	149.4 ± 4.0	147.6 ± 6.7	153.6 ± 10.6
BrdU positive crypt cells, %	18.0 ± 1.5	15.3 ± 1.4	16.8 ± 1.9
Distal small intestine			
Weight, g·kg^{-1}	14.4 ± 0.9^{a}	16.3 ± 1.0^{ab}	17.0 ± 1.3^{b}
Protein mass, mg·kg^{-1}	1346 ± 106^{a}	1577 ± 115^{ab}	1609 ± 155^{b}
DNA mass, mg·kg^{-1}	94.7 ± 5.2	113.5 ± 8.2	107.5 ± 12.7
Liver			
Weight, g·kg^{-1}	35.7 ± 0.6	33.8 ± 1.1	36.5 ± 1.4
Protein mass, mg·kg^{-1}	5213 ± 160	5159 ± 197	5714 ± 203
DNA mass, mg·kg^{-1}	172.5 ± 5.1	182.2 ± 7.7	181.3 ± 10.7

*Means ± SEM, n=9-11 pigs per group. Means in a row with superscripts without a common letter differ, $P < 0.05$.

Protein FSR measured in the jejunum, ileum, liver, muscle and pancreas and the protein ASR's (data not shown) were not significantly different among the treatment groups (Table 5). We performed immunoblotting to determine whether arginine supplementation activated p70S6K and phospho-mTOR. However, levels of p70S6K and mTOR phosphorylation in gut and muscle tissue were similar among the groups (data not shown).

Table 5. Fractional protein synthesis rates in PEN pigs supplemented with CO, ARG, or ARG+L-NAME*

	CO	ARG	ARG+L-NAME
		%/d	
Jejunum	48.8 ± 3.1	44.2 ± 3.1	41.6 ± 6.1
Ileum	40.3 ± 3.0	42.9 ± 2.8	45.5 ± 7.9
Liver	69.1 ± 3.9	73.3 ± 3.6	71.2 ± 6.7
Muscle	12.5 ± 0.8	14.3 ± 1.1	13.9 ± 1.5
Pancreas	65.7 ± 3.5	80.8 ± 7.7	60.4 ± 6.1

*Means ± SEM, *n*=9-11 pigs per group.

Discussion

In neonatal intensive care, full TPN or TPN combined with partial enteral feeding is standard treatment in preterm infants because of enteral feeding intolerance. However, TPN has shown to significantly decrease SMA blood flow and induce mucosal atrophy in neonatal pigs[17]. During TPN, the absence of enteral nutrition reduces luminal arginine availability, which could limit NO synthesis and predispose the neonatal intestine to vasoconstriction and ischemia contributing to the development of mucosal atrophy and NEC. We hypothesized that provision of enteral arginine under conditions of TPN or partial enteral nutrition would increase small intestinal blood flow and mucosal growth and that these effects would be mediated by increased NO production. Our results demonstrate that over a wide range of enteral infusion rates, arginine does not acutely affect blood flow in neonatal pigs fed TPN or partial enteral nutrition. Moreover, we show that chronic dietary arginine supplementation during partial enteral nutrition only modestly increased intestinal growth and that this response was NO independent.

The main aim of our study was to address whether arginine stimulates blood flow in a clinically relevant situation of parenteral or partial enteral nutrition in neonatal pigs. We previously showed that a minimum of 40% enteral nutrition is necessary to stimulate intestinal growth[29]. Interestingly, the current study showed that providing 40% partial enteral nutrition almost doubled the SMA blood flow compared to that in TPN pigs. However, despite markedly increased circulating arginine concentrations, we found that neither enteral arginine nor the arginine precursor citrulline, or glutamine, glutamate or glucose acutely increased SMA blood flow in partially enterally fed pigs. The effect of enteral arginine on SMA blood flow has not yet been investigated in a neonatal model. Three studies performed in adult mice, rat and pigs reported increased intestinal blood flow upon arginine administration[18, 22-23]. Studies in similar models of ischemia-reperfusion injury have reported a positive effect of arginine on NO synthesis thereby suggesting increased blood flow[19, 21]. Thus it seems that in a state of injury and influx of inflammatory cells, increased amounts of plasma arginine might increase arginine availability for NO production and as a consequence increase blood flow. However, these studies were performed in adult animals where intestinal circulation, NO concentration, NO-response and eNOS expression is more developed than in neonates[7, 31].

Several factors may explain the lack of response of enteral arginine on SMA blood flow. First, blood flow stimulation may be limited by the availability of co-factors necessary to produce NO from arginine[9]. Our partially enterally fed pigs were only fed 40% of total nutrient requirements enterally, so it might be possible that some factors were limiting for NO synthesis, namely tetrahydrobiopterin (BH4). Tetrahydrobiopterin is an essential cofactor for all three NOS isoforms and BH4 bioavailability within the endothelium is a critical factor in regulating the balance between NO and superoxide production by

eNOS[32-34]. Arginine can increase BH4 synthesis and thus given the marked increase in luminal and plasma arginine with enteral supplementation, BH4 availability would likely be increased. In support of this is the fact that SMA flow was higher in partial enteral nutrition versus TPN suggesting that the mucosal mechanisms that sense luminal nutrients were functional. Second, NO synthesis may be linked to the rate of arginine transport by cationic amino acid transporter CAT-1 in endothelial cells and thus not dependent on plasma arginine concentration[35]. Third, endogenous inhibitors of NOS isoforms such as asymmetric dimethylarginine (ADMA) may play a limiting role in NO production[36]. Fourth, and most likely, endothelin (ET)-1 (vasoconstrictor) and NO (vasodilator) dysregulation might account for the lack of response. Insufficient supply of luminal nutrients undermining epithelial integrity, might induce influx of proinflammatory cytokines resulting in ET-1 mediated vasoconstriction[12, 37-38]. Interestingly, submucosal arterioles harvested from human intestine resected for NEC did not demonstrate evidence of eNOS function and showed vasoconstriction, presumably by lack of eNOS derived NO[39].

Despite the lack of effect on SMA blood flow, we observed remarkable changes in circulating amino acid concentrations in response to enteral amino acid infusions in TPN- and PEN-fed neonatal pigs. Studies in neonatal pigs have shown that the small intestine is a major determinant of arginine synthesis and catabolism and these are substantially decreased in pigs fed TPN compared to enteral nutrition[40-41]. We show here that the circulating arterial arginine concentrations were dose-dependently increased with increasing enteral arginine level. However, there were significantly greater increases in plasma arginine in pigs fed partial enteral nutrition than in TPN-fed pigs. We suspect that this occurred because of reduced arginine catabolism during first-pass through the intestinal mucosa associated with partial enteral nutrition; we would not expect intestinal absorption of free arginine to be a limiting process. It is also possible that PEN increased the mucosal epithelial mass and/or capacity of enterocytes to synthesize arginine, which led to increased intestinal arginine release. It also appeared that the major product of intestinal arginine catabolism was ornithine based on the marked increase circulating ornithine concentration, whereas citrulline and proline were unchanged, with increasing enteral arginine intake. We also found that citrulline and glutamine, but not glutamate, increased the plasma arginine concentration. Interestingly, the plasma arginine concentration was 1394 and 561 μM during equal molar infusions of arginine and citrulline, respectively, suggesting that dietary arginine was a better precursor than citrulline for maintaining blood arginine. This finding is contrary to recent reports indicating that dietary citrulline may be more effective than arginine in increasing circulating arginine levels[42-43]. Yet, these results confirm the fact that citrulline is a more important precursor for de novo arginine synthesis compared to glutamine or

glutamate and highlights the differences in first-pass splanchnic metabolism between arginine and citrulline.

In addition to acute effects of arginine, we next investigated the effects of longer term arginine administration on the intestinal growth. The ability of arginine to prevent NEC and gut injury may be due to trophic physiological effects on the intestinal epithelium. Arginine supplementation in *in vitro* and *in vivo* models stimulated intestinal protein synthesis and increased epithelial cell survival through the mTOR mediated pathway[4-6]. However, it is unknown whether this trophic effect of arginine on the epithelium is NO dependent. Our current results demonstrate that chronic arginine supplementation with partial enteral feeding only modestly stimulated gut growth.

Enteral arginine for 4 days increased the mass of tissue, protein and DNA in the proximal small intestine but not the distal small intestine. Despite the increase in intestinal mass, we found no change in intestinal protein synthesis, villus height or cell proliferation. In addition, we found no change in intestinal phosphorylation of mTOR or p70S6K. The fact that we didn't find an effect on protein synthesis or cell proliferation suggests that the observed trophic effect was due to a decrease in intestinal protein breakdown. These results are in contrast to others that have found a positive effect of arginine on protein synthesis and mTOR signaling[5]. Another important finding was that the addition of L-NAME in the formula did not reduce the trophic effect of enteral arginine on intestinal growth, suggesting that the effects were not NO-dependent.

Finally, we found that body weight gain was reduced in arginine supplemented pigs. This finding is in contrast to previously published studies in pigs where arginine resulted in increased weight gain in weanling pigs[44-46]. These previous studies provided fully enterally fed weanling pigs with arginine resulting in modest increases in plasma arginine concentrations within the physiological range. In the present study we used supraphysiological concentrations (3 times arginine requirement of 1.08 $g \cdot kg^{-1} \cdot d^{-1}$)[47] since endogenous arginine synthesis in our partially enterally fed pigs was expected to be greatly decreased[40-41]. The plasma arginine concentration in supplemented pigs was more than tenfold higher than controls. This markedly high intake of arginine may have negatively impacted protein metabolism. However, plasma concentrations of lysine were in the normal range suggesting that lysine-arginine competition for cell-entry did not occur and hence does not explain decreased weight gain. Moreover, the protein synthesis rate in other organs, including the liver, pancreas and skeletal muscle were numerically higher but not statistically different from control pigs. This would imply pharmacological effects of arginine to suppress weight gain occurred by proteolytic mechanisms. It is not clear why supplementing L-NAME with arginine resulted in lowered plasma arginine compared to arginine alone; it is possible that L-NAME blocked intestinal absorption or increased the catabolism of L-arginine.

In summary, this study aimed to further investigate the effects of enteral arginine on intestinal blood flow and mucosal growth in neonates fed TPN or partial enteral nutrition. Our results show that partial enteral feeding increased SMA blood flow compared to TPN. However, enteral arginine infusion did not affect SMA blood flow across a wide range of doses, including physiological and pharmacological. We also found that enteral infusion of arginine precursors, citrulline and glutamine did not affect SMA flow, despite the fact that they resulted in increased circulating arginine levels. Consistent with the lack of effect on SMA blood flow, we found that a pharmacological dose of enteral arginine only marginally increased intestinal mucosal growth and without significant changes in protein synthesis, cell proliferation, or activation on mTOR signaling. Our results were unexpected and contrary to considerable literature reports that increased circulating arginine via exogenous infusion increases blood flow. We postulate that the immature vascular mechanisms involved in intestinal NO synthesis and blood flow may explain our results, however, this question warrants further study in neonatal animals.

Acknowledgements

The authors wish to thank Liwei Cui and Xiaoyan Chang for their expert technical assistance.

References

1. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids 2009;37:1-17.
2. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336 (Pt 1):1-17.
3. McCormack SA, Johnson LR. Role of polyamines in gastrointestinal mucosal growth. Am J Physiol 1991;260:G795-806.
4. Ban H, Shigemitsu K, Yamatsuji T, et al. Arginine and Leucine regulate p70 S6 kinase and 4E-BP1 in intestinal epithelial cells. Int J Mol Med 2004;13:537-43.
5. Corl BA, Odle J, Niu X, et al. Arginine activates intestinal p70(S6k) and protein synthesis in piglet rotavirus enteritis. J Nutr 2008;138:24-9.
6. Rhoads JM, Niu X, Odle J, Graves LM. Role of mTOR signaling in intestinal cell migration. Am J Physiol Gastrointest Liver Physiol 2006;291:G510-7.
7. Nankervis CA, Giannone PJ, Reber KM. The neonatal intestinal vasculature: contributing factors to necrotizing enterocolitis. Semin Perinatol 2008;32:83-91.
8. Nankervis CA, Reber KM, Nowicki PT. Age-dependent changes in the postnatal intestinal microcirculation. Microcirculation 2001;8:377-87.
9. Wu G, Meininger CJ. Regulation of nitric oxide synthesis by dietary factors. Annu Rev Nutr 2002;22:61-86.
10. Prins HA, Houdijk AP, Wiezer MJ, et al. The effect of mild endotoxemia during low arginine plasma levels on organ blood flow in rats. Crit Care Med 2000;28:1991-7.

11. Becker RM, Wu G, Galanko JA, et al. Reduced serum amino acid concentrations in infants with necrotizing enterocolitis. J Pediatr 2000;137:785-93.
12. Richir MC, Siroen MP, van Elburg RM, et al. Low plasma concentrations of arginine and asymmetric dimethylarginine in premature infants with necrotizing enterocolitis. Br J Nutr 2007;97:906-11.
13. Wilmore D. Enteral and parenteral arginine supplementation to improve medical outcomes in hospitalized patients. J Nutr 2004;134:2863S-7S; discussion 95S.
14. Zamora SA, Amin HJ, McMillan DD, et al. Plasma L-arginine concentrations in premature infants with necrotizing enterocolitis. J Pediatr 1997;131:226-32.
15. Blakely ML, Lally KP, McDonald S, et al. Postoperative outcomes of extremely low birth-weight infants with necrotizing enterocolitis or isolated intestinal perforation: a prospective cohort study by the NICHD Neonatal Research Network. Ann Surg 2005;241:984-9; discussion 9-94.
16. Wu G, Flynn NE, Flynn SP, Jolly CA, Davis PK. Dietary protein or arginine deficiency impairs constitutive and inducible nitric oxide synthesis by young rats. J Nutr 1999;129:1347-54.
17. Niinikoski H, Stoll B, Guan X, et al. Onset of small intestinal atrophy is associated with reduced intestinal blood flow in TPN-fed neonatal piglets. J Nutr 2004;134:1467-74.
18. Vukosavljevic N, Jaron D, Barbee KA, Buerk DG. Quantifying the L-arginine paradox in vivo. Microvasc Res 2006;71:48-54.
19. Fu TL, Zhang WT, Zhang L, Wang F, Gao Y, Xu M. L-arginine administration ameliorates serum and pulmonary cytokine response after gut ischemia-reperfusion in immature rats. World J Gastroenterol 2005;11:1070-2.
20. Sukhotnik I, Helou H, Mogilner J, et al. Oral arginine improves intestinal recovery following ischemia-reperfusion injury in rat. Pediatr Surg Int 2005;21:191-6.
21. Ward DT, Lawson SA, Gallagher CM, Conner WC, Shea-Donohue T. Sustained nitric oxide production via l-arginine administration ameliorates effects of intestinal ischemia-reperfusion. J Surg Res 2000;89:13-9.
22. Fukatsu K, Ueno C, Maeshima Y, et al. Effects of L-arginine infusion during ischemia on gut blood perfusion, oxygen tension, and circulating myeloid cell activation in a murine gut ischemia/reperfusion model. JPEN J Parenter Enteral Nutr 2004;28:224-30; discussion 30-1.
23. Spanos CP, Papaconstantinou P, Spanos P, Karamouzis M, Lekkas G, Papaconstantinou C. The effect of L-arginine and aprotinin on intestinal ischemia-reperfusion injury. J Gastrointest Surg 2007;11:247-55.
24. Di Lorenzo M, Bass J, Krantis A. Use of L-arginine in the treatment of experimental necrotizing enterocolitis. J Pediatr Surg 1995;30:235-40; discussion 40-1.
25. Akisu M, Ozmen D, Baka M, et al. Protective effect of dietary supplementation with L-arginine and L-carnitine on hypoxia/reoxygenation-induced necrotizing enterocolitis in young mice. Biol Neonate 2002;81:260-5.
26. Amin HJ, Zamora SA, McMillan DD, et al. Arginine supplementation prevents necrotizing enterocolitis in the premature infant. J Pediatr 2002;140:425-31.
27. Stoll B, Price PT, Reeds PJ, et al. Feeding an elemental diet vs a milk-based formula does not decrease intestinal mucosal growth in infant pigs. JPEN J Parenter Enteral Nutr 2006;30:32-9.
28. Wray-Cahen D, Nguyen HV, Burrin DG, et al. Response of skeletal muscle protein synthesis to insulin in suckling pigs decreases with development. Am J Physiol 1998;275:E602-9.

29. Burrin DG, Stoll B, Jiang R, et al. Minimal enteral nutrient requirements for intestinal growth in neonatal piglets: how much is enough? Am J Clin Nutr 2000;71:1603-10.
30. Metges CC, Petzke KJ, Hennig U. Gas chromatography/combustion/isotope ratio mass spectrometric comparison of N-acetyl- and N-pivaloyl amino acid esters to measure 15N isotopic abundances in physiological samples: a pilot study on amino acid synthesis in the upper gastro-intestinal tract of minipigs. J Mass Spectrom 1996;31:367-76.
31. Reber KM, Su BY, Clark KR, Pohlman DL, Miller CE, Nowicki PT. Developmental expression of eNOS in postnatal swine mesenteric artery. Am J Physiol Gastrointest Liver Physiol 2002;283:G1328-35.
32. Alp NJ, Channon KM. Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. Arterioscler Thromb Vasc Biol 2004;24:413-20.
33. Alp NJ, Mussa S, Khoo J, et al. Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. J Clin Invest 2003;112:725-35.
34. Wu G, Meininger CJ. Nitric oxide and vascular insulin resistance. Biofactors 2009;35:21-7.
35. Mann GE, Yudilevich DL, Sobrevia L. Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. Physiol Rev 2003;83:183-252.
36. Dayoub H, Achan V, Adimoolam S, et al. Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis: genetic and physiological evidence. Circulation 2003;108:3042-7.
37. Woods M, Mitchell JA, Wood EG, et al. Endothelin-1 is induced by cytokines in human vascular smooth muscle cells: evidence for intracellular endothelin-converting enzyme. Mol Pharmacol 1999;55:902-9.
38. Yoshizumi M, Kurihara H, Morita T, et al. Interleukin 1 increases the production of endothelin-1 by cultured endothelial cells. Biochem Biophys Res Commun 1990;166:324-9.
39. Nowicki PT, Caniano DA, Hammond S, et al. Endothelial nitric oxide synthase in human intestine resected for necrotizing enterocolitis. J Pediatr 2007;150:40-5.
40. Bertolo RF, Brunton JA, Pencharz PB, Ball RO. Arginine, ornithine, and proline interconversion is dependent on small intestinal metabolism in neonatal pigs. Am J Physiol Endocrinol Metab 2003;284:E915-22.
41. Urschel KL, Rafii M, Pencharz PB, Ball RO. A multitracer stable isotope quantification of the effects of arginine intake on whole body arginine metabolism in neonatal piglets. Am J Physiol Endocrinol Metab 2007;293:E811-8.
42. Lassala A, Bazer FW, Cudd TA, et al. Intravenous administration of L-citrulline to pregnant ewes is more effective than L-arginine for increasing arginine availability in the fetus. J Nutr 2009;139:660-5.
43. Urschel KL, Shoveller AK, Uwiera RR, Pencharz PB, Ball RO. Citrulline is an effective arginine precursor in enterally fed neonatal piglets. J Nutr 2006;136:1806-13.
44. Kim SW, Wu G. Dietary arginine supplementation enhances the growth of milk-fed young pigs. J Nutr 2004;134:625-30.
45. Tan B, Li XG, Kong X, et al. Dietary L-arginine supplementation enhances the immune status in early-weaned piglets. Amino Acids 2009;37:323-31.
46. Yao K, Yin YL, Chu W, et al. Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. J Nutr 2008;138:867-72.
47. Wu G, Meininger CJ, Knabe DA, Bazer FW, Rhoads JM. Arginine nutrition in development, health and disease. Curr Opin Clin Nutr Metab Care 2000;3:59-66.

SUPPLEMENTAL

SUPPLEMENTAL TABLE 1. Plasma arterial glucose and insulin concentrations in PEN pigs infused intragastrically with saline or arginine[1].

	Arginine infusion rate							
	100		200		400		800	
	μmol•kg⁻¹•h⁻¹							
Substrate	Saline	Arginine	Saline	Arginine	Saline	Arginine	Saline	Arginine
Glucose, mmol/L	7.15 ± 0.21	7.22 ± 0.23	7.48 ± 0.36	7.61 ± 0.23	6.80 ± 0.11	7.12 ± 0.41	7.27 ± 0.15	7.02 ± 0.31
Insulin, nmol/L	32 ± 7	21 ± 5	54 ± 12	25 ± 4	24 ± 4	27 ± 5	32 ± 6	22 ± 4

[1]Means ± SEM, n= 9-11 pigs per group.

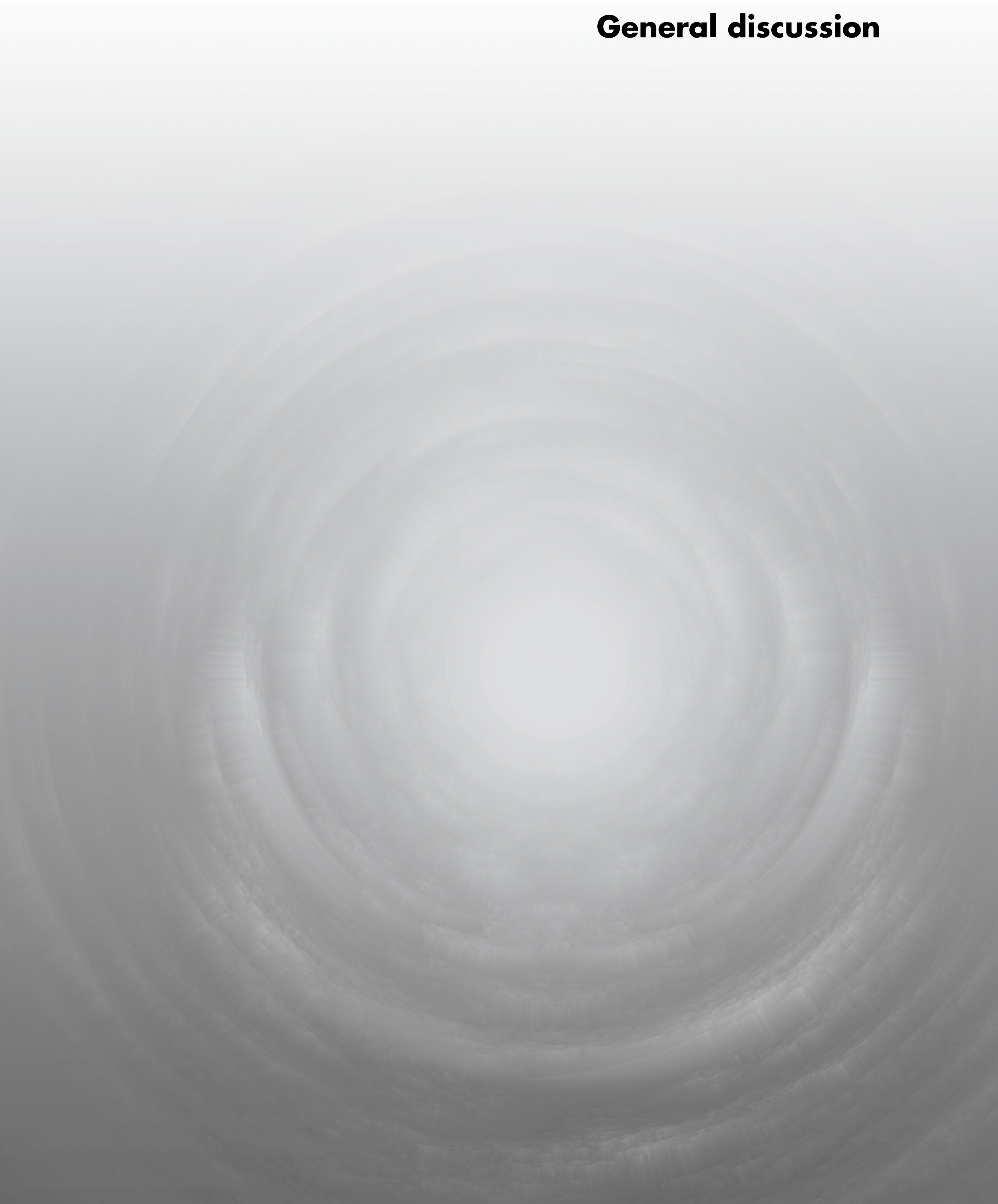
SUPPLEMENTAL TABLE 2. SMA flows during saline infusion and each time interval in response to arginine dose in pigs TPN or PEN pigs[1,2]

Treatment	Saline	1 h	2 h	3 h
	L•kg⁻¹•h⁻¹			
TPN group				
Arginine dose				
50	1.52 ± 0.34	1.48 ± 0.20	1.35 ± 0.26	1.41 ± 0.29
100	1.04 ± 0.13	1.09 ± 0.13	1.07 ± 0.13	1.11 ± 0.15
200	1.20 ± 0.18	1.30 ± 0.24	1.17 ± 0.22	1.17± 0.19
400	1.42 ± 0.48	1.48 ± 0.42	1.49 ± 0.43	1.43 ± 0.42
800	1.57 ± 0.23	1.53 ± 0.11	1.48 ± 0.18	1.68 ± 0.20
PEN group				
Arginine dose				
100	2.08 ± 0.21	2.34 ± 0.35	2.41 ± 0.21	2.55 ± 0.31
200	2.24 ± 0.35	2.49 ± 0.47	2.40 ± 0.46	2.50 ± 0.53
400	2.39 ± 0.39	2.87 ± 0.51	2.71 ± 0.63	2.32 ± 0.43
800	2.77 ± 0.56	2.77 ± 0.60	2.89 ± 0.63	2.65 ± 0.54

[1]Means ± SEM, n=3-5 pigs per TPN group and n=5-7 pigs per PEN group.

Chapter 10

General discussion



'Necrotizing enterocolitis (NEC) is the most common gastro-intestinal emergency in preterm neonates and affects 2-7% of all premature infants[1,2]. Unfortunately, treatment is still limited to immediate restriction of enteral feeds and broad-spectrum antibiotics while an estimated 20-40% of infants require additional surgery[3-5]. Mortality rates from NEC range from 15-30% but are as high as 50% in infants requiring surgery[6]. Survivors of NEC are at increased risk for complications such as short bowel syndrome and impaired neurodevelopment[7, 8]. Therefore, extensive research is necessary to gain insight in the prevention and treatment of this devastating disease.
Prematurity, enteral formula feeding and bacterial colonization are proposed risk factors for NEC. The responses of the immature gastro-intestinal tract in preterm infants with respect to motility and digestive ability, intestinal barrier function, immune defense, and circulatory regulation, in relation to enteral formula feeding and bacterial colonization are all thought to play key roles in the development of NEC[1,9,10]. However, the exact etiology of NEC remains to be elucidated. Therefore, this thesis aimed to determine the impact of nutrition and the intestinal microbiota on intestinal defense mechanisms and gut barrier function. The findings presented in this thesis provide tools for the development of strategies to prevent NEC.

Overall, four main conclusions can be drawn from the work presented in this thesis:
1. Animal models are a valuable tool to study neonatal nutrition (Chapter 2).
2. Nutrition impacts gut barrier function and intestinal circulation (Chapters 3, 4, 5 and 9).
3. The intestinal microbiota influence gut barrier function and protein metabolism (Chapters 3, 6 and 7).
4. Paneth cells enhance the innate defense response in NEC (Chapter 8).

Animal models are a valuable tool to study neonatal nutrition.

Animal models are a valuable tool to identify mechanisms that mediate the effect of nutrition on neonatal development and metabolic function. This is underlined in chapter 2 that discusses recently developed animal models that are being used to study neonatal human nutrition. Techniques for rearing newborn mice, preterm rats and preterm pigs have been developed to study the impact of neonatal nutrition on the intestine. Mice have great potential for mechanistic and genomic research in postnatal nutrition and related diseases as is presented in chapter 3. The neonatal piglet model most closely resembles the human neonate regarding intestinal growth and function, and provides us with an excellent model to investigate the nutritional impact on gut func-

tion and NEC as well as acute and chronic effects of parenteral and enteral nutrition on whole-body metabolism and specific tissues. However, the results obtained from animal models cannot be translated directly into the human situation and need to be validated. Studies using the (preterm NEC-like) piglet model are presented in chapters 4, 5, 7 and 9. Further development of neonatal animal models related to nutrition is required for the advancement of research in early postnatal nutrition as improvement of nutritional support during this critical period of development will enhance immediate clinical outcomes and possibly prevent diseases later in life.

Nutrition impacts gut barrier function and intestinal circulation.

As discussed in chapter 1, preterm infants are at high risk for NEC because of their immature gastro-intestinal functions. The studies described in this thesis show that nutrition affects certain aspects of these gut functions, in particular intestinal gut barrier function and intestinal circulation, and subsequently influences the clinical course of intestinal inflammation and NEC.

Nutrition impacts gut barrier function

An important feature of gut barrier function and the front line of innate host defense is the mucus layer coating the intestinal epithelium. Goblet cells synthesize large gel-forming mucin glycoproteins of which mucin MUC2 is the most predominant secretory mucin in the human intestinal tract[11,12]. Within the mucus gel components such as secreted defensins and immunoglobulins reside, producing a physical and chemical barrier that protects the epithelium from luminal pathogens and toxic substances[13,14]. The impact of nutritional factors on MUC2 synthesis is poorly investigated but may alter gut barrier function and play a significant role in the development of NEC.

A valuable model to study the impact of nutritional factors on the intestine at risk for inflammation is the Muc2-deficient mouse. This mouse model lacks an adequate mucus layer and spontaneously develops colitis[15]. As shown in chapter 3, a purified diet, containing a different type of protein and a low amount of insoluble fibers, markedly decreased intestinal inflammation and improved body growth in comparison to standard rodent chow in Muc2-deficient mice. In wild type mice, no difference was observed between the two feeding groups. Muc2-deficient mice, fed the purified diet, exhibited decreased signs of inflammation such as crypt lengthening, influx of Cd3 -positive T cells, expression of TNF- and interleukin 12 related cytokines, and abundance of S100a8 and S100a9-positive cells. Furthermore, abundance of Muc4-positive Goblet

cells was decreased in Muc2-deficient mice fed the standard chow compared to the purified diet, pointing towards a lower need for Muc4 to enhance epithelial protection. These findings indicate the importance of adequate nutrition in a condition of diminished barrier function by decreased mucin synthesis such as colitis or NEC. At least two plausible explanations can be provided for the observed prevention of severe colitis in Muc2-deficient mice. First, the purified diet contained casein-derived protein that might have increased the digestion and hydrolysis of proteins and nutrient uptake compared to plant-derived proteins. Protein digestion and hence absorption of dietary amino acids by the gut varies according to the type of ingested dietary protein[16]. Improved availability and utilization of amino acids affects postprandial protein metabolism and thus boosts intestinal and whole body growth. This hypothesis is supported by our observations made in chapter 4 where an increased intestinal utilization of the amino acid threonine coincided with an increase in intestinal protein and MUC2 synthesis. This matter is discussed in greater detail below.

Second, the lower amount of insoluble fibers might provide another explanation for improved gut barrier function in the Muc2-deficient mice fed the purified diet. The amount and type of fibers can modulate the gut microbiota and hence change the composition of the short chain fatty acids produced by the intestinal bacteria. As shown in chapter 6, short chain fatty acids regulate *MUC2* gene expression influencing mucin synthesis and protection of the epithelial barrier. This matter is discussed in greater detail in paragraph "The intestinal microbiota influence gut barrier function and protein metabolism".

The impact of type of nutrition on intestinal protein and MUC2 synthesis affecting gut barrier function becomes evident in chapter 4. We demonstrated that infant formula feeding in preterm piglets decreased intestinal first pass threonine utilization when compared to bovine colostrum feeding. Impaired threonine availability decreases intestinal protein and mucin synthesis in piglets[17]. Similarly, our study showed that the lower intestinal threonine utilization in formula fed piglets was concomitant with lower synthesis rates of intestinal proteins and MUC2 when compared to colostrum fed piglets. Lower MUC2 synthesis rates will diminish gut barrier function and might accounts for the higher incidence of NEC observed in the formula fed pigs. From the presented data we suggested that formula-induced reduction of MUC2 synthesis and intestinal threonine utilization will cause a breach in the intestinal barrier and might provide a possible explanation for the predisposition of the preterm neonate to develop NEC. However, the question remains which characteristic(s) of formula feeding are responsible for the hampered MUC2 synthesis and utilization of threonine. Three potential properties are discussed:

First, formula lacks various growth factors and cytokines, which are present in colostrum and breast milk[18,19]. The capacity of these specific proteins to persist and exert

their activity in the neonatal gut, affect maturation of the immune function and exert a trophic effect on the intestinal epithelium. This will increase nutrient utilization for growth, protein synthesis, and hence increased synthesis of defensive products such as defensins or MUC2. The lack of these factors in formula might therefore negatively affect gut immune and barrier function. Different studies in preterm pigs have shown that formula compared to colostrum feeding increases NEC incidence comparable to the results presented in chapter 4[20,21]. The above discussed results might therefore provide evidence that in preterm infants at risk for NEC, formula feeding reduces MUC2 synthesis because of lower enteral threonine absorption and decreased splanchnic utilization due to decreased gut maturation and growth. Alternatively, decreased enteral threonine absorption and splanchnic utilization by formula feeding might also directly lead to decreased MUC2 synthesis.

Another possible explanation for decreased MUC2 synthesis and threonine utilization in formula fed piglets might be the preferential site of threonine uptake for MUC2 synthesis. The results from chapter 5 show that threonine uptake for MUC2 synthesis in colostrum fed piglets is preferred from the luminal side, whereas threonine uptake in formula fed piglets is preferred from the basolateral side, i.e. threonine coming from the systemic pool. Other studies have reported that enteral presented nutrients are preferentially used over parenteral supply of nutrients for the synthesis of glutathione and arginine[22,23]. However, the fact that the *type* of enteral nutrition affects the preferred site of absorption is a novel finding. Additionally, in chapter 5 we have investigated the preferred site of threonine uptake for MUC2 synthesis in preterm infants with an ileostomy and recovering from gastro-intestinal surgery. Preterm infants on combined enteral and parenteral nutrition preferably used basolateral threonine (91%) over luminal threonine (9%) for synthesis of MUC2. This was in contrast to preterm piglets on full enteral nutrition, where luminal uptake of threonine for MUC2 synthesis was 40-60% depending on the type of nutrition. Under these conditions, MUC2 synthesis was lower in the preterm infants than in the preterm pigs. Although the data from our pigs and infants cannot be directly compared, these data might suggest that preterm infants on TPN with partial enteral nutrition have lower MUC2 synthesis rates due to lower luminal threonine uptake. Hence, the above discussed findings not only imply an important role for enteral nutrition over parenteral nutrition, but also for the type of enteral nutrition provided.

The third explanation confers a more indirect effect of the type of nutrition on the mucosa. Type of nutrition is a major determinant for the composition of the intestinal microbiota, which at least partially contributes to the results discussed above. In breast fed human infants the intestinal microbiota is markedly different from formula-fed infants[24,25]. Similarly, between colostrum and formula fed piglets differences in intestinal bacterial composition have been observed[21]. The composition of the intestinal microbiota affects

short chain fatty acid (SCFA) production and composition[26]. We described in chapter 6, that higher butyrate concentrations, as found in formula fed preterm infants[26], limit MUC2 expression and therefore restrict MUC2 synthesis.

Collectively, the data presented provide possible mechanisms for the suggested benefits of feeding breast milk to human preterm infants, other than the long known beneficial immunomodulatory effects. However, future studies investigating splanchnic utilization of threonine and possibly other amino acids in preterm infants fed breast milk compared to formula will have to confirm the data obtained from the preterm pig. Furthermore, illumination of the proposed mechanisms responsible for the influence of type of nutrition on MUC2 synthesis, i.e. altered threonine metabolism, preferential site of threonine uptake, and/or bacterial colonization, provide a challenge for future research. Moreover, unraveling these aspects will likely provide nutritional tools for enhancement of gut barrier function and the prevention of NEC in preterm infants.

Nutrition impacts intestinal blood flow

In neonatal intensive care, many extremely preterm infants receive full total parenteral nutrition (TPN) or parenteral nutrition combined with partial enteral nutrition because of feeding intolerance. Although TPN is considered a lifesaving therapy when enteral nutrition is not tolerated, deprivation of enteral feeding during TPN administration has shown to decrease intestinal growth, blood flow and nitric oxide (NO) synthesis in neonatal pigs[27]. In chapter 9 we reported that TPN fed piglets had markedly lower superior mesenteric arterial (SMA) blood flow compared to pigs fed partial enteral nutrition. Enteral nutrition showed to be a major determinant for blood flow as only partial (40%) enteral feeding induced a twofold increase in SMA blood flow compared to pigs only receiving TPN.

In the same study, we investigated the effect of acute enteral arginine administration in a supraphysiological dose on SMA blood flow. Arginine is an essential amino acid in neonates and the sole precursor for NO, a signaling molecule that plays a central role in regulating vascular resistance and hence blood flow in the newborn intestinal circulation[28,29]. Clinical studies have shown that preterm infants who developed NEC had significant lower plasma arginine than those who did not[30]. Low circulating levels of arginine or its precursors might result in shortage of NO and a decrease in intestinal blood flow, and subsequently may contribute to the development of NEC. The results presented in chapter 9 show that enteral arginine supplementation acutely increased arginine plasma concentrations although more dramatically in partial enterally fed pigs than in TPN fed pigs. However, enteral arginine supplementation did not increase SMA blood flow in either group. Chronic enteral arginine supplementation for 4 days in TPN

fed piglets with 20% partial enteral feeding modestly affected gut growth and interestingly, this response was NO independent.

Arginine supplementation in preterm infants less than 28 weeks gestation increased plasma arginine levels and decreased the incidence of NEC[31]. However, the authors of this study also included 'stage 1 NEC', which is hard to differentiate from feeding intolerance, which could explain the high incidence of NEC in the control group. Therefore, the data from this single study are insufficient to support a recommendation for arginine supplementation as standard practice. A multicentre randomized controlled trial of arginine supplementation in preterm neonates is needed, focusing on the incidence of stage 2 and 3 NEC. The results from chapter 9 do support a positive effect of supraphysiological arginine supplementation on plasma concentrations and a modest effect on gut growth that might enhance the epithelial barrier. However, a blood flow mediated effect of arginine supplementation for prevention of NEC cannot be supported from our study results. Finally, despite the ambiguous results of arginine supplementation, our study showed the importance of enteral nutrition as a major determinant for SMA blood flow. Hence it can be concluded that enteral feeding stimulates nutritive blood flow and is essential to support intestinal function.

The intestinal microbiota influence gut barrier function and protein metabolism

Colonization of the intestinal microbiota in the preterm infant is delayed and negatively affected because of the use of broad-spectrum antibiotics and the environmental flora on the NICU[25]. Although NEC has not been associated with a specific pathogen, interactions between the immature intestinal cells and pathogenic bacteria might lead to exaggerated immune responses and increased susceptibility to inflammation[9]. In breast fed neonates *Bifidobacterium* and *Lactobacillus* species dominate the intestinal microbiota, whereas a more adult type microbiota in which *Enterobacteriaceae*, *Clostridium*, and *Bacteroides* predominate is seen in formula-fed infants[24,32]. The potential mechanisms for the protective effects of a commensal or *Bifidobacterium* dominated microbiota are reduction of pathogenic strains, modulation of inflammatory reactions, and maturation of the gut, all leading to enhancement of the intestinal barrier and possibly reduction of NEC[9,33-35].

Impact of the intestinal microbiota on gut barrier function

Colonization by commensal bacteria is limited to the upper loose mucus layer, and interacts with the diverse oligosaccharides of mucin glycoproteins[36]. The intestinal mi-

Chapter 10

crobiota and the intestinal mucus layer have an extensive interplay. Studies in germ free animals show differences in mucus-related indexes[37]. Certain specific probiotic strains have shown to induce different mucins such as *MUC2 in vitro*, although direct regulation of *MUC2* through bacterial-host interactions *in vivo* remains uncertain[38]. Indirectly, the microbiota can impact *MUC2* regulation via production of short chain fatty acids via fermentation of undigested carbohydrates[39-41]. Our results, discussed in chapter 6, have shown that butyrate and propionate in physiological concentrations, similar to that found in stools of breastfed infants[26], induce *MUC2* expression via activation of an AP-1 (c-Fos/C-Jun)*cis*-element on the promoter. Interestingly, higher concentrations of butyrate, similar to that found in stools of formula-fed infants, were tested and found to downregulate *MUC2* expression. This might implicates that the intestinal microbiota, influenced by the type of feeding, i.e. breast milk or formula, indirectly regulate *MUC2* expression and thus MUC2 synthesis and hence impact epithelial protection of the mucosa by the mucus layer.

The beneficial impact of *Bifidobacterium* bacteria on MUC2 synthesis is supported by our findings in chapter 7. We observed an increased MUC2 protein expression and tendency to increase MUC2 synthesis rates in probiotic supplemented pigs that exhibited a bifidogenic flora. In a study performed in rats, a probiotic mix containing *Bifidobacterium* bacteria, showed increased *MUC2* gene expression and mucin secretion[42]. Hence, we suggest that regulation of MUC2 expression by butyrate, via induction of a bifidogenic flora, improves epithelial protection and defense by an enhanced mucus layer.

Other beneficial effects of butyrate shown in *in vitro* and animal models involve enhanced intestinal growth and differentiation[43,44], regulation of apoptosis[45], and inflammatory suppression[46,47]. Described in chapter 3, Muc2-deficient mice exhibit epithelial crypt lengthening as a sign of intestinal inflammation. The reduction in crypt lengthening in Muc2-deficient mice supplemented with *Bifidobacterium* bacteria might have been due to butyrate as a mediating factor.

In summary, induction of a bifidogenic microbiota in preterm infants might enhance short-chain fatty acid-mediated regulation of *MUC2*, epithelial cell proliferation and apoptosis, and immune functions, and subsequently improve gut barrier function that could provide an advantage in reducing intestinal inflammation and NEC.

Impact of the intestinal microbiota on protein metabolism

Short-chain fatty acids are not the only bacterial products that are thought to provide a health benefit to the host. Intestinal bacteria digest nitrogenous secretions and ingested carbohydrates and produce nutrients such as amino acids for their own sustenance. These bacterial amino acids are also available to the host upon bacterial degradation or secretion and have shown to contribute to human amino acid requirements[48,49]. The

potential of the intestinal microbiota to support protein metabolism in neonates is unknown. Therefore, as described in chapter 7, we investigated the impact of a modulated intestinal microbiota by antibiotics and probiotics on both intestinal and whole body protein metabolism in neonatal pigs. Although we hypothesized that antibiotics would decrease intestinal and whole body protein metabolism by diminishing the intestinal microbiota, we observed only in the proximal small intestine decreased protein synthesis rates whereas in the colon, most abundant in bacteria, no effect was observed. Surprisingly, probiotics also decreased proximal small intestinal protein synthesis and did not affect the distal small intestine or colon. Although our study was not designed to investigate the etiology responsible for this effect, two plausible hypotheses can be deducted from the literature. First, both antibiotics and probiotics may have diminished the need for defensive proteins and thus reduced protein synthesis. Second, an altered inflammatory cytokine expression, due to administration of the antibiotics or probiotics, could have moderated protein translation-initiation and hence protein synthesis[50-54]. However, future studies will have to confirm these results and further illuminate the responsible mechanism(s).
On a whole body level, antibiotic treated piglets showed an increased proteolysis rate but also an increased protein synthesis rate resulting in a similar net balance compared to control pigs. Probiotic treatment in our neonatal pigs did not affect amino acid homeostasis.

Meta-analyses have shown that administration of probiotics reduces the incidence and severity of NEC in preterm infants[55]. However, the data could not be extrapolated to extremely low birth weight infants and hence call for a confirmatory trial. Furthermore, determination of the optimal dose, duration, and the type of probiotic strains to use for supplementation in preterm infants as well as the specific mechanisms by which probiotics confer their protective effect to the intestinal epithelium have yet to be determined fully. Overall, the results from the studies presented in this thesis support the protective effects of supplementation of *Bifidobacterium* bacteria on improved gut barrier function via *MUC2* regulation. However, we did not find a direct nutritional benefit from probiotic supplementation with *Bifidobacterium* bacteria.

Paneth cells enhance the innate defense response in NEC.

Paneth cells are an important feature of the innate immune system by the secretion of endogenous antibiotics such as lysozyme, phospholipase A2, and human defensins[56] 5 and 6[57-60]. Paneth cells secrete their bactericidal products in response to microbial

stimuli and regulate the composition and distribution of bacterial populations[61,62]. Paneth cell numbers and HD5 and -6 mRNA expression are lower in premature infants at 24 weeks of gestation compared to term infants, and up to 200-fold lower than in adults[63]. Furthermore, the preterm infant is often exposed to nosocomial pathogens and has delayed colonization with beneficial commensals[25]. Therefore, decreased Paneth cell abundance could result in higher susceptibility to bacterial infection and inflammation. We performed an extensive study investigating intestinal histopathological specimens from children with acute NEC and after recovery from NEC, which is described in chapter 8. We showed that in acute NEC, Paneth cell abundance in small intestinal tissue was not significantly different from preterm controls. After recovery from NEC, Paneth cell hyperplasia was observed concomitant with elevated HD5 mRNA levels. Surprisingly, metaplastic Paneth cells were found in colonic tissue after recovery from NEC. Furthermore, ileostomy fluid contained Paneth cell proteins and inhibited bacterial growth implying secretion of functional Paneth cell products in the small intestine.
From the results described above we can conclude that NEC is unlikely to be caused by Paneth cell deficiency, but that the gut recovering from intestinal inflammation augments its innate defense system by increasing Paneth cell abundance and hence Paneth cell protein expression and secretion. Future studies concerning *in vitro* and animal models will have to provide further insight into the mechanism responsible for the occurrence of Paneth cell hyperplasia and metaplasia. Understanding these mechanisms could lead towards the development of a direct therapeutic intervention to enhance the innate defense in a state of inflammation. Another clinically relevant intervention might be to improve maturation of the immature intestine by nutrition and hence indirectly increase Paneth cell abundance and thus innate defense. However, suitable *in vitro* experiments and animal models would have to be designed to study the effect of nutrition on Paneth cell development and Paneth cell protein expression.

Recommendations & Future perspectives

Although the studies described in this thesis contributed to the understanding of nutritional and microbial factors affecting the preterm gut, there are still several issues to resolve in future research. Most importantly, the impact of type and route of nutrition and supplementation of specific nutrients on NEC development still need further research before they can be used for NEC prevention or therapy. Additionally, there are some promising topics we want to highlight in the following paragraphs.

Optimizing nutritional support as a tool for prevention of NEC

Minimal enteral or trophic feeding

In a state of parenteral feeding such as during bowel rest for active NEC, initiation of trophic feeds containing various nutrients has been advocated rather than extended bowel rest which can lead to gut atrophy. Theoretically, trophic feeds are thought to improve the activity of digestive enzymes, enhance the release of digestive hormones, and increase intestinal blood flow in premature infants[64,65]. Studies suggest that infants receiving early trophic feeds have better feeding intolerance, improved growth, reduced days of hospitalization, and decreased episodes of sepsis in comparison to infants not receiving trophic feeds[66]. However, a recent meta-analysis of the available data did not provide any strong evidence that early trophic feeding affects feeding tolerance or growth rate in very low birth weight infants[67]. Importantly, early trophic feeding does not increase susceptibility to NEC[67]. It could be worth investigating enteral threonine supplementation as a factor in trophic feeding on the novo synthesis of MUC2 in preterm neonates during TPN administration. Supplementation of other MUC2 precursors to trophic feeds such as proline, serine and cysteine might also enhance MUC2 synthesis[68]. In addition, it could be interesting to investigate the effect of arginine or arginine precursors as a factor in trophic feeding to stimulate endogenous arginine synthesis. Although arginine supplementation in supraphysiological levels only modestly affected gut growth in our neonatal pigs, studies using lower amounts of arginine supplementation have shown beneficial effect on intestinal protein synthesis and body growth and has been suggested to decrease the incidence of NEC[31,69].

Enteral feeding

Mother's milk is considered to be the best type of nutrition for preterm infants. However, provision of sufficient mother's milk to meet their infants' requirement is often not feasible for mothers of extremely premature infants[70]. In addition, the nutrient composition often does not meet the quantitative requirements of rapid growing preterm infants and is the basis for our practice to fortify breast milk. Donor milk could be an attractive substitution for mother's milk. Donor breast milk is associated with a lower risk of NEC, but the quality of the evidence is limited[71]. Recently, a study performed in extremely preterm infants showed that an exclusive human milk-based diet with donor human milk-based breast milk fortifier was associated with a significant lower rate of NEC and surgical NEC when compared to mother's milk-based diet that also included bovine milk-based breast milk fortifier[72]. Although a promising future perspective, the results of this study will need a large confirmatory randomized control trial and should compare: a) infants fed either formula or human donor milk supplemented mother's milk; and b)

human donor milk or mother's milk fortified with human vs. bovine milk-based breast milk fortifier, starting immediately from birth onwards.
To optimize infant formula we need to determine the breast milk-composition and investigate the effect of breast milk factors on the intestine and more specifically on intestinal threonine metabolism and gut barrier function. The differences between formula or colostrum feeding in preterm pigs with respect to NEC incidence and intestinal threonine utilization for protein and MUC2 synthesis we observed in our studies raise further research questions. First, the exact role of formula and colostrum feeding in threonine metabolism and MUC2 synthesis in early stages of NEC development need to be further elucidated. Second, possible changes in intestinal threonine utilization in preterm infants fed either formula or mother's milk will need to be confirmed in order to translate our findings into clinical recommendations or therapeutic interventions.

Dietary supplementation: Probiotics, Prebiotics, and Postbiotics
Probiotics are emerging as a new preventive strategy against NEC development. Clinical trials have shown reduction of incidence and severity of NEC with probiotic supplementation[55,73]. However, the data could not be extrapolated to the extremely low birth weight infant and there is continuing debate about the administration of the optimal probiotic strain, dosage, timing and safety. Although probiotics convey a promising future dietary supplement to promote intestinal health also in extremely low birth weight neonates, the mechanisms of bacterial-host interactions need to be further illuminated. Often *Bifidobacterium*, *Lactobacillus* and *Streptococcus* species are used as a probiotic supplement. Consumption of *Bifidobacterium* bacteria can contribute to barrier function by enhancing the physical impediment of the mucus layer as shown in this thesis. Additionally, various effects of probiotics on the epithelial barrier in *in vitr*o and animal models have been shown in terms of increased antimicrobial peptides and sIgA production, competitive adherence for pathogens and increased tight junction integrity of epithelial cells[38]. However, conclusive evidence is impeded owing to the wide range of candidate strains and doses. Understanding the specific effects that probiotic strains have on the host should ultimately lead to a scientifically-based choice for the best strain(s) to use and facilitate the use of probiotics in extremely low birth weight infants.

The predominance of beneficial bacteria in the gut microbiota of breast-fed infants is thought to be supported by the fermentation of oligosaccharides, i.e. non-digestible dietary carbohydrates that selectively promote proliferation of beneficial enteric bacteria, present in human breast milk. Therefore, optimizing infant formula with prebiotics might be another potential strategy to prevent NEC in preterm infants. Oligosaccharides are not absorbed by the small intestine and reach the colon where they are fermented by bifidobacteria to short-chain fatty acids and lactic acid and create a pH of approximately

5.7 in the colonic lumen. In contrast, the microbiota in formula-fed infants produces a different profile of short-chain fatty acids concomitantly with a pH of 7.0 in the luminal environment. Besides indirect effects of prebiotics on the intestinal microbiota and short-chain fatty acid production[32], prebiotics also exert direct effects on the gastro-intestinal tract such as enhancement of gastric emptying in preterm infants, possible improvement of intestinal tolerance to enteral feeding, increased intestinal transport, and reduction of pathogens by prevention of pathogen binding to the epithelium[74-76]. Recently, an infant formula with prebiotic supplementation has been developed. However, investigating the function of the complex oligosaccharides present in human breast milk will improve our understanding of the prebiotic effects of human breast milk on the intestine and subsequently improve infant formulae.

Bacterial metabolites or postbiotics, such as butyrate supplementation, have been suggested as a possible therapy for intestinal inflammation because of the beneficial effects of butyrate shown in various *in vitro* and animal studies concerning intestinal growth and differentiation[43,44], inflammatory suppression[46,47], and apoptosis[45]. However, our own data suggest that butyrate supplementation leading to high intraluminal concentrations of butyrate could lead to decreased MUC2 synthesis and hence diminish the protective mucus layer. Furthermore, butyrate supplementation passing the small intestine is unlikely to exert a similar effect to butyrate production by the intestinal microbiota. Therefore, it might be better to use short-chain fatty acid profiling, i.e. the determination of short-chain fatty acid composition, as a tool for monitoring and adjustment of nutritional therapy to establish a beneficial intestinal microbiota similar to that observed in breast-fed infants.

Concluding remarks

The results presented in this thesis illuminated the impact of nutritional and bacterial factors, and disease on innate defensive mechanisms of the neonatal gut. Until the exact cause for NEC has been pinpointed, we need to gain further understanding of the dietary influence on the immature gut and the interaction of the immature cells of the host with intestinal bacteria colonizing the gut. The use of animal models will continue to increase our physiological knowledge and provide an opportunity for development of new strategies optimizing nutritional support as a tool for prevention of NEC. In this thesis the importance of optimal enteral feeding for the improvement of nutrient absorption, utilization and favorable bacterial colonization is stressed. However, we are in great need of further research to continue in our determination to optimize prevention and therapy of NEC in preterm infants on the neonatal intensive care unit.

References

1. Lin PW, Stoll BJ. Necrotising enterocolitis. Lancet 2006;368:1271-83.
2. Holman RC, Stoll BJ, Curns AT, Yorita KL, Steiner CA, Schonberger LB. Necrotising enterocolitis hospitalisations among neonates in the United States. Paediatr Perinat Epidemiol 2006;20:498-506.
3. Guthrie SO, Gordon PV, Thomas V, Thorp JA, Peabody J, Clark RH. Necrotizing enterocolitis among neonates in the United States. J Perinatol 2003;23:278-85.
4. Luig M, Lui K, Nsw, Group AN. Epidemiology of necrotizing enterocolitis--Part II: Risks and susceptibility of premature infants during the surfactant era: a regional study. J Paediatr Child Health 2005;41:174-9.
5. Sankaran K, Puckett B, Lee DS, et al. Variations in incidence of necrotizing enterocolitis in Canadian neonatal intensive care units. J Pediatr Gastroenterol Nutr 2004;39:366-72.
6. Blakely ML, Lally KP, McDonald S, et al. Postoperative outcomes of extremely low birth-weight infants with necrotizing enterocolitis or isolated intestinal perforation: a prospective cohort study by the NICHD Neonatal Research Network. Ann Surg 2005;241:984-9; discussion 9-94.
7. Stoll BJ, Hansen NI, Adams-Chapman I, et al. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. Jama 2004;292:2357-65.
8. Petty JK, Ziegler MM. Operative strategies for necrotizing enterocolitis: The prevention and treatment of short-bowel syndrome. Semin Pediatr Surg 2005;14:191-8.
9. Claud EC, Walker WA. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. Faseb J 2001;15:1398-403.
10. Anand RJ, Leaphart CL, Mollen KP, Hackam DJ. The role of the intestinal barrier in the pathogenesis of necrotizing enterocolitis. Shock 2007;27:124-33.
11. Van Klinken BJ, Tytgat KM, Buller HA, Einerhand AW, Dekker J. Biosynthesis of intestinal mucins: MUC1, MUC2, MUC3 and more. Biochem Soc Trans 1995;23:814-8.
12. Tytgat KM, Buller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J. Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology 1994;107:1352-63.
13. Specian RD, Oliver MG. Functional biology of intestinal goblet cells. Am J Physiol 1991;260:C183-93.
14. Hecht G. Innate mechanisms of epithelial host defense: spotlight on intestine. Am J Physiol 1999;277:C351-8.
15. Van der Sluis M, De Koning BA, De Bruijn AC, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006;131:117-29.
16. Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL, Beaufrere B. Slow and fast dietary proteins differently modulate postprandial protein accretion. Proc Natl Acad Sci U S A 1997;94:14930-5.
17. Nichols NL, Bertolo RF. Luminal threonine concentration acutely affects intestinal mucosal protein and mucin synthesis in piglets. J Nutr 2008;138:1298-303.
18. Field CJ. The immunological components of human milk and their effect on immune development in infants. J Nutr 2005;135:1-4.
19. Kelly GS. Bovine colostrums: a review of clinical uses. Altern Med Rev 2003;8:378-94.
20. Bjornvad CR, Thymann T, Deutz NE, et al. Enteral feeding induces diet-dependent mucosal dysfunction, bacterial prolif-

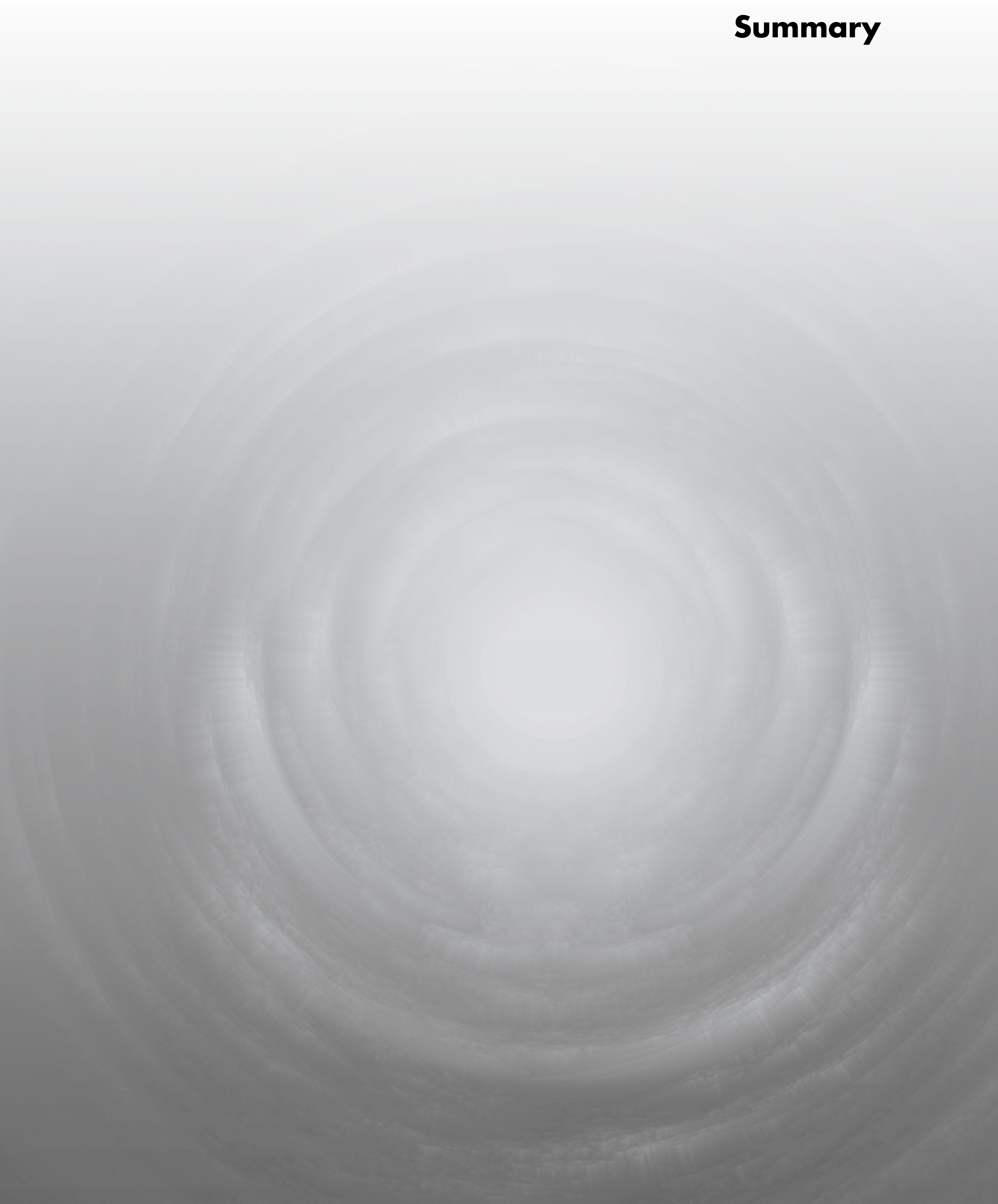
eration, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. Am J Physiol Gastrointest Liver Physiol 2008;295:G1092-103.
21. Sangild PT, Siggers RH, Schmidt M, et al. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. Gastroenterology 2006;130:1776-92.
22. Reeds PJ, Burrin DG, Stoll B, et al. Enteral glutamate is the preferential source for mucosal glutathione synthesis in fed piglets. Am J Physiol 1997;273:E408-15.
23. Ligthart-Melis GC, van de Poll MC, Vermeulen MA, et al. Enteral administration of alanyl-[2-(15)N]glutamine contributes more to the de novo synthesis of arginine than does intravenous infusion of the dipeptide in humans. Am J Clin Nutr 2009;90:95-105.
24. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr 2000;30:61-7.
25. Westerbeek EA, van den Berg A, Lafeber HN, Knol J, Fetter WP, van Elburg RM. The intestinal bacterial colonisation in preterm infants: a review of the literature. Clin Nutr 2006;25:361-8.
26. Knol J, Scholtens P, Kafka C, et al. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. J Pediatr Gastroenterol Nutr 2005;40:36-42.
27. Niinikoski H, Stoll B, Guan X, et al. Onset of small intestinal atrophy is associated with reduced intestinal blood flow in TPN-fed neonatal piglets. J Nutr 2004;134:1467-74.
28. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336 (Pt 1):1-17.
29. Nankervis CA, Giannone PJ, Reber KM. The neonatal intestinal vasculature: contributing factors to necrotizing enterocolitis. Semin Perinatol 2008;32:83-91.
30. Becker RM, Wu G, Galanko JA, et al. Reduced serum amino acid concentrations in infants with necrotizing enterocolitis. J Pediatr 2000;137:785-93.
31. Amin HJ, Zamora SA, McMillan DD, et al. Arginine supplementation prevents necrotizing enterocolitis in the premature infant. J Pediatr 2002;140:425-31.
32. Knol J, Boehm G, Lidestri M, et al. Increase of faecal bifidobacteria due to dietary oligosaccharides induces a reduction of clinically relevant pathogen germs in the faeces of formula-fed preterm infants. Acta Paediatr Suppl 2005;94:31-3.
33. Penna FJ, Peret LA, Vieira LQ, Nicoli JR. Probiotics and mucosal barrier in children. Curr Opin Clin Nutr Metab Care 2008;11:640-4.
34. Hooper LV. Bacterial contributions to mammalian gut development. Trends Microbiol 2004;12:129-34.
35. Neu J. Gastrointestinal maturation and implications for infant feeding. Early Hum Dev 2007;83:767-75.
36. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A 2008;105:15064-9.
37. Deplancke B, Gaskins HR. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. Am J Clin Nutr 2001;73:1131S-41S.
38. Ohland CL, Macnaughton WK. Probiotic bacteria and intestinal epithelial barrier function. Am J Physiol Gastrointest Liver Physiol 2010;298:G807-19.
39. Barcelo A, Claustre J, Moro F, Chayvialle JA, Cuber JC, Plaisancie P. Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. Gut 2000;46:218-24.
40. Hatayama H, Iwashita J, Kuwajima A, Abe T. The short chain fatty acid, butyrate, stimulates MUC2 mucin production

in the human colon cancer cell line, LS174T. Biochem Biophys Res Commun 2007;356:599-603.
41. Shimotoyodome A, Meguro S, Hase T, Tokimitsu I, Sakata T. Short chain fatty acids but not lactate or succinate stimulate mucus release in the rat colon. Comp Biochem Physiol A Mol Integr Physiol 2000;125:525-31.
42. Caballero-Franco C, Keller K, De Simone C, Chadee K. The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 2007;292:G315-22.
43. Tsukahara T, Iwasaki Y, Nakayama K, Ushida K. Stimulation of butyrate production in the large intestine of weaning piglets by dietary fructooligosaccharides and its influence on the histological variables of the large intestinal mucosa. J Nutr Sci Vitaminol (Tokyo) 2003;49:414-21.
44. Bartholome AL, Albin DM, Baker DH, Holst JJ, Tappenden KA. Supplementation of total parenteral nutrition with butyrate acutely increases structural aspects of intestinal adaptation after an 80% jejunoileal resection in neonatal piglets. JPEN J Parenter Enteral Nutr 2004;28:210-22; discussion 22-3.
45. Avivi-Green C, Polak-Charcon S, Madar Z, Schwartz B. Apoptosis cascade proteins are regulated in vivo by high intracolonic butyrate concentration: correlation with colon cancer inhibition. Oncol Res 2000;12:83-95.
46. Yin L, Laevsky G, Giardina C. Butyrate suppression of colonocyte NF-kappa B activation and cellular proteasome activity. J Biol Chem 2001;276:44641-6.
47. Venkatraman A, Ramakrishna BS, Shaji RV, Kumar NS, Pulimood A, Patra S. Amelioration of dextran sulfate colitis by butyrate: role of heat shock protein 70 and NF-kappaB. Am J Physiol Gastrointest Liver Physiol 2003;285:G177-84.
48. Metges CC, El-Khoury AE, Henneman L, et al. Availability of intestinal microbial lysine for whole body lysine homeostasis in human subjects. Am J Physiol 1999;277:E597-607.
49. Jackson AA, Gibson NR, Bundy R, Hounslow A, Millward DJ, Wootton SA. Transfer of (15)N from oral lactose-ureide to lysine in normal adults. Int J Food Sci Nutr 2004;55:455-62.
50. Cooney RN, Kimball SR, Vary TC. Regulation of skeletal muscle protein turnover during sepsis: mechanisms and mediators. Shock 1997;7:1-16.
51. Lang CH, Frost RA, Vary TC. Regulation of muscle protein synthesis during sepsis and inflammation. Am J Physiol Endocrinol Metab 2007;293:E453-9.
52. Madsen K. Probiotics and the immune response. J Clin Gastroenterol 2006;40:232-4.
53. Madsen K, Cornish A, Soper P, et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 2001;121:580-91.
54. Vary TC, Kimball SR. Regulation of hepatic protein synthesis in chronic inflammation and sepsis. Am J Physiol 1992;262:C445-52.
55. Alfaleh K, Anabrees J, Bassler D. Probiotics reduce the risk of necrotizing enterocolitis in preterm infants: a meta-analysis. Neonatology 2010;97:93-9.
56. Hintz SR, Kendrick DE, Stoll BJ, et al. Neurodevelopmental and growth outcomes of extremely low birth weight infants after necrotizing enterocolitis. Pediatrics 2005;115:696-703.
57. Erlandsen SL, Parsons JA, Taylor TD. Ultrastructural immunocytochemical localization of lysozyme in the Paneth cells of man. J Histochem Cytochem 1974;22:401-13.
58. Jones DE, Bevins CL. Paneth cells of the human small intestine express an antimicrobial peptide gene. J Biol Chem 1992;267:23216-25.

59. Jones DE, Bevins CL. Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel. FEBS Lett 1993;315:187-92.
60. Kiyohara H, Egami H, Shibata Y, Murata K, Ohshima S, Ogawa M. Light microscopic immunohistochemical analysis of the distribution of group II phospholipase A2 in human digestive organs. J Histochem Cytochem 1992;40:1659-64.
61. Salzman NH, Hung K, Haribhai D, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol 2010;11:76-83.
62. Salzman NH, Underwood MA, Bevins CL. Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa. Semin Immunol 2007;19:70-83.
63. Salzman NH, Polin RA, Harris MC, et al. Enteric defensin expression in necrotizing enterocolitis. Pediatr Res 1998;44:20-6.
64. McClure RJ, Newell SJ. Randomised controlled trial of trophic feeding and gut motility. Arch Dis Child Fetal Neonatal Ed 1999;80:F54-8.
65. McClure RJ, Newell SJ. Randomized controlled study of digestive enzyme activity following trophic feeding. Acta Paediatr 2002;91:292-6.
66. McClure RJ, Newell SJ. Randomised controlled study of clinical outcome following trophic feeding. Arch Dis Child Fetal Neonatal Ed 2000;82:F29-33.
67. Bombell S, McGuire W. Early trophic feeding for very low birth weight infants. Cochrane Database Syst Rev 2009:CD000504.
68. Faure M, Mettraux C, Moennoz D, et al. Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. J Nutr 2006;136:1558-64.
69. He Q, Kong X, Wu G, et al. Metabolomic analysis of the response of growing pigs to dietary L-arginine supplementation. Amino Acids 2009;37:199-208.
70. Schanler RJ, Lau C, Hurst NM, Smith EO. Randomized trial of donor human milk versus preterm formula as substitutes for mothers' own milk in the feeding of extremely premature infants. Pediatrics 2005;116:400-6.
71. Boyd CA, Quigley MA, Brocklehurst P. Donor breast milk versus infant formula for preterm infants: systematic review and meta-analysis. Arch Dis Child Fetal Neonatal Ed 2007;92:F169-75.
72. Sullivan S, Schanler RJ, Kim JH, et al. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. J Pediatr 2010;156:562-7 e1.
73. Deshpande G, Rao S, Patole S. Probiotics for prevention of necrotising enterocolitis in preterm neonates with very low birthweight: a systematic review of randomised controlled trials. Lancet 2007;369:1614-20.
74. Indrio F, Riezzo G, Raimondi F, et al. Prebiotics improve gastric motility and gastric electrical activity in preterm newborns. J Pediatr Gastroenterol Nutr 2009;49:258-61.
75. Mihatsch WA, Hoegel J, Pohlandt F. Prebiotic oligosaccharides reduce stool viscosity and accelerate gastrointestinal transport in preterm infants. Acta Paediatr 2006;95:843-8.
76. Newburg DS. Oligosaccharides in human milk and bacterial colonization. J Pediatr Gastroenterol Nutr 2000;30 Suppl 2:S8-17.

Chapter 11

Summary



In **chapter 1** a general overview is provided of the incidence of necrotizing enterocolitis (NEC), the risk factors associated with the occurrence of NEC, and the pathophysiological mechanisms in the premature intestine that are thought to play a role in the development of NEC. The increase in preterm births and improved survival rates of (extremely) low birth weight preterm infants, challenges us with the continuing battle against NEC. Although prematurity, enteral formula feeding, and bacterial colonization are identified as risk factors for NEC, the exact etiology of NEC remains to be fully elucidated. Treatment for NEC is limited and the morbidity as well as the mortality rate remains high. Therefore, extensive research is warranted to gain insight in this devastating disease. The overall goal of this thesis was to achieve a better understanding of factors regulating intestinal defense. This thesis aimed to determine to which extent various nutritional factors and the intestinal microbiota impact intestinal defense mechanisms and gut barrier function. The studies described in this thesis comprised of in vitro studies, studies performed in various well established animal models, and studies undertaken in preterm infants recovering from NEC.

Chapter 2 of this thesis describes and emphasizes the use of animal models as an invaluable tool to study human neonatal nutrition and related diseases such as NEC. Mice have great potential for mechanistic and genomic research in postnatal nutrition and related diseases as described in chapter 3. The neonatal piglet model most closely resembles the human neonate regarding intestinal development and function, and provides us with an excellent model to investigate the nutritional impact on gut function and NEC. The (preterm) piglet model was used in various studies in this thesis as described in chapters 4, 5, 7 and 9.

Chapter 3 describes the dietary impact on intestinal inflammation in Muc2-deficient mice that exhibit a compromised barrier function by a diminished mucus layer. The aim of this study was to determine the effect of standard rodent chow versus a purified diet, and dietary supplementation of probiotics, on growth and intestinal inflammation in wild type and Muc2-deficient mice. The purified diet markedly decreased intestinal inflammation and improved body growth in comparison to standard rodent chow in Muc2-deficient mice. This underlines the importance of optimal nutrition for maintenance and improvement of gut barrier function and overall health, especially under conditions that increase the risk of intestinal inflammation. Furthermore, supplementation of probiotics to standard chow in the Muc2-deficient mice improved intestinal epithelial morphometry and decreased inflammation. These beneficial effects of probiotics provide evidence for enhanced epithelial protection and increased gut barrier function in the compromised intestine.

Chapter 4 describes the dietary impact of infant formula vs. colostrum on the premature gut. The aim of this study was to determine intestinal threonine utilization necessary for intestinal protein and mucin MUC2 synthesis in preterm pigs fed either formula or colostrum. The results of the study depict the negative effect of formula feeding on growth, NEC incidence, intestinal protein synthesis, and intestinal MUC2 synthesis when compared to colostrum feeding. These findings provide a possible explanation for the predisposition of the formula-fed preterm neonate to develop NEC.

Chapter 5 shows that both luminal and systemic threonine are used for mucin MUC2 synthesis secreted by goblet cells in preterm pigs and preterm infants with an ileostomy. In this study we determined the preferential site of threonine uptake for MUC2 synthesis. Both luminal threonine uptake and MUC2 synthesis seemed to be influenced by the amount and type of enteral nutrition. In preterm infants on partial enteral nutrition, 91% of threonine absorption for MUC2 synthesis was extracted basolaterally. In the preterm pigs, threonine uptake switched from predominantly basolateral in formula fed piglets to luminal in colostrum fed piglets. Concomitantly, the colostrum fed piglets with higher luminal threonine absorption showed increased MUC2 synthesis rates compared to formula fed piglets. Based on these results, we stressed the importance of optimal enteral feeding for the enhancement of mucin synthesis and hence improvement of gut barrier function in preterm neonates.

Chapter 6 illuminates the regulation of intestinal mucin *MUC2* expression by short-chain fatty acids. We hypothesized that short-chain fatty acids, fermentation products of bacteria, affect *MUC2* expression and hence alter epithelial protection. The aim of the study was to investigate the mechanisms that regulate short-chain fatty acid-mediated effects on MUC2 synthesis. The results clearly show that butyrate and propionate in physiological concentrations, similar to that found in stools of breastfed infants, induce *MUC2* expression via activation of an AP-1 (c-Fos/C-Jun) *cis*-element within the promoter. Higher concentrations of butyrate, similar to that found in stools of formula-fed infants, downregulate *MUC2* expression. This implicates that the intestinal microbiota, influenced by the type of feeding, i.e. breast milk or formula, indirectly regulate *MUC2* expression leading to MUC2 synthesis and hence impact epithelial protection of the mucosa by the mucus layer.

Chapter 7 describes the impact of an altered intestinal microbiota on amino acid and protein metabolism in piglets. We hypothesized that modulation of the intestinal microbiota by antibiotics or probiotics affect both intestinal and whole body protein metabolism. In this study we showed that antibiotics reduced the amount of intestinal bacterial species, decreased urea synthesis, and elevated threonine plasma concentrations and

turnover, as well as whole body protein synthesis and proteolysis. Furthermore, antibiotics decreased protein synthesis rate in the proximal small intestine and liver. Probiotic supplementation of *Bifidobacterium* bacteria induced a bifidogenic microbiota, decreased plasma urea concentrations but did not affect whole body threonine or protein metabolism. Probiotics decreased protein synthesis in the proximal small intestine, increased MUC2 synthesis rate, and enhanced MUC2 protein expression in the colon. We concluded that alteration of the microbiota by antibiotics or probiotics differentially impacted urea and threonine kinetics as well as intestinal protein synthesis and MUC2 protein expression. Therefore, alteration of the microbiota might have implications for protein homeostasis in human neonates treated with antibiotics or probiotics.

Chapter 8 provides insight into Paneth cell presence and function in NEC. Paneth cells are a major component of intestinal innate defense and enhance the gut barrier by secretion of endogenous antibiotic peptides. We found that in infants with acute NEC, Paneth cell numbers tended to be lower in small intestinal tissue compared to preterm controls. After recovery from NEC, Paneth cell hyperplasia was observed in the small intestine concomitant with elevated human alpha-defensin 5 (HD5) mRNA levels but Paneth cell numbers were not different from control preterm infants. A novel finding was the presence of metaplastic Paneth cells in the colon after recovery from NEC. Furthermore, analysis of ileostomy fluid containing Paneth cell proteins inhibited bacterial growth suggesting secretion by and functionality of Paneth cells. Collectively, our results indicate the presence of Paneth cells in preterm infants with NEC, and imply that Paneth cell hyperplasia and metaplasia is most likely caused by inflammation. The increase in Paneth cell numbers suggests enhanced secretion of active antimicrobial products and might be indicative of an enhanced innate defense response during prolonged inflammation which might contribute to the recovery from NEC. However, it still remains to be elucidated which possible cell signaling and regulatory pathways are involved in these processes to target improvement of therapy and clinical outcome.

Chapter 9 describes the effects of enteral arginine on intestinal blood flow and mucosal growth in neonatal pigs fed total parenteral nutrition (TPN) or partial enteral nutrition. Our results show that partial enteral feeding increased superior mesenteric arterial (SMA) blood flow compared to TPN. However, enteral arginine infusion did not affect SMA blood flow across a wide range of doses, including physiological and pharmacological. We also found that enteral infusion of arginine precursors, citrulline and glutamine did not affect SMA flow, despite the fact that they resulted in increased circulating arginine levels. Despite the lack of effect of arginine or precursors on SMA blood flow, we did find that a pharmacological dose of enteral arginine, although only marginally, increased

intestinal mucosal growth. However, this was observed without significant changes in protein synthesis, cell proliferation, or activation of mTOR signaling.

The main results of the studies described in this thesis are highlighted and discussed in **chapter 10**. With the gained knowledge from these studies we contributed to the understanding of the factors that affect the preterm gut and render the preterm infant at increased risk for NEC. The thesis is finalized by the provided recommendations for future research projects to further investigate the mechanisms of intestinal defense and gut barrier function in the preterm lumen. Ultimately, this might illuminate the predisposition of the preterm neonate to NEC and lead to improvement of nutritional care.

Chapter 12

Nederlandse Samenvatting

Necrotizerende enterocolitis (NEC) is de meest voorkomende darm-aandoening bij premature pasgeborenen die zich kenmerkt door een zich snel ontwikkelende ernstige ontsteking van de dunne en/of dikke darm. Van alle prematuur geboren baby's krijgt 2-7% NEC. Helaas zijn er slechts weinig behandelingsopties en is men beperkt tot darmrust door te stoppen met voeden via de darm en het geven van antibiotica om de ontsteking te beperken. Bij ongeveer 20-40% van alle kinderen met NEC is de ontsteking zodanig ernstig dat chirurgische verwijdering van het aangedane deel van de darm noodzakelijk is. Sterfte ten gevolge van NEC komt voor bij 15-30% van de kinderen maar loopt op tot 50% bij kinderen met een ernstige vorm van NEC waarvoor een operatie nodig is. Kinderen die NEC overleven hebben veelal complicaties zoals voedings- en groeiproblemen door een verkorte darm na operatieve verwijdering waardoor voedingsstoffen niet meer opgenomen kunnen worden. Ook is er een verhoogd risico op een vertraagde ontwikkeling van het kind. Gezien de ernst van NEC, de complicaties, en de hoge sterfte is uitgebreid onderzoek noodzakelijk om meer inzicht te verschaffen in het voorkómen en behandelen van deze ziekte.
Inmiddels is bekend dat vroeggeboorte, kunstvoeding, en de bacteriële kolonisatie van de darm risicofactoren zijn voor het ontstaan van NEC. De onrijpheid van het darmstelsel in te vroeg geboren kinderen zorgt voor verminderde darmpassage en vertering van voeding, verlaagde barrière van het darmweefsel voor schadelijke bacteriën en stoffen, slechte functie van het afweersysteem, en een nog niet goed werkzame regulatie van de bloedsomloop in de darm. Deze factoren, in combinatie met kunstvoeding en darmbacteriën, worden gedacht een belangrijke rol te spelen in het ontstaan van NEC. Echter, wat nu precies de oorzaak is van NEC is onbekend en dient verder te worden onderzocht. Daarom is met dit proefschrift getracht om de invloed van voeding en darmbacteriën op de beschermingsmechanismen van de darm toe te lichten. De resultaten die gepresenteerd worden in dit proefschrift leveren handvaten voor de verdere ontwikkeling van strategieën om NEC te voorkomen.

Over het algemeen kunnen 4 conclusies worden getrokken uit dit proefschrift:

1. Diermodellen zijn onmisbaar voor het bestuderen van voeding voor pasgeborenen (Hoofdstuk 2).
2. Voeding heeft invloed op de barrière functie en de bloedsomloop van de darm (Hoofdstukken 3, 4, 5 en 9).
3. Darmbacteriën hebben een effect op de barrière functie en stofwisseling van dedarm (Hoofdstukken 3, 6 en 7).
4. Paneth cellen in de darm vergroten de aangeboren afweer als reactie op het ontstaan van NEC (Hoofdstuk 8).

In **hoofdstuk 1** wordt een algemeen overzicht gegeven van het vóórkomen van NEC, van de risicofactoren geassocieerd met het ontstaan van NEC en van de mechanismen in de premature darm die verondersteld worden een rol te spelen in de ontwikkeling van NEC. Door de toename van het aantal extreem te vroeg geboren kinderen en de verbeterde overlevingskansen worden we voortdurend uitgedaagd NEC te voorkómen en bestrijden. Ondanks het feit dat prematuriteit, kunstvoeding en bacteriële kolonisatie van de onrijpe darm geïdentificeerd zijn als risicofactoren, is de precieze oorzaak van NEC nog niet volledig opgehelderd. De behandeling van NEC is beperkt en de kans op complicaties en sterfte door NEC blijven hoog. Daarom is uitgebreid onderzoek gerechtvaardigd om beter inzicht in deze verwoestende ziekte te verkrijgen. Het algemene doel van dit proefschrift was om meer kennis te vergaren van de factoren die invloed hebben op de bescherming van de darm. Met dit proefschrift werd gepoogd om te bepalen in welke mate diverse voedingsfactoren en de darmflora de intestinale afweermechanismen en barrière functie van de darm beïnvloeden.

Hoofdstuk 2 van dit proefschrift beschrijft en benadrukt het belang van het gebruik van proefdieren als hulpmiddel voor het bestuderen van voeding bij pasgeborenen en darmziekten zoals NEC. Studies verricht met muizen bieden veel mogelijkheden voor onderzoek naar mechanistische en genetische factoren gepaard gaande met postnatale voeding en geassocieerde ziekten zoals beschreven in hoofdstuk 3. De big lijkt het meest op de humane pasgeborene als het gaat om de ontwikkeling en functie van de darm, en voorziet ons daarom van een uitstekend model om effecten van voeding op darmfunctie en NEC te onderzoeken. De big als model voor de humane pasgeborene werd gebruikt in diverse studies in dit proefschrift zoals beschreven in hoofdstukken 4, 5, 7 en 9.

Hoofdstuk 3 beschrijft het effect van voeding op ontsteking van de darm in muizen die een gecompromitteerde barrièrefunctie van de darm hebben door een verminderde aanmaak van de slijmlaag die de darm normaliter beschermt. Het doel van deze studie was om het effect van standaard knaagdiervoer tegenover een synthetisch dieet te bepalen op de groei en darmontsteking in normale muizen en muizen met een verminderde slijmlaag, alsmede het effect van toevoeging van probiotica aan het standaard dieet. In muizen met een verminderde slijmlaag ontbreekt het gen om het slijmeiwit Muc2 aan te maken waardoor ze gevoelig zijn voor het ontwikkelen van darmontsteking. Het synthetische dieet verminderde duidelijk de darmontsteking en verbeterde de lichaamsgroei in de Muc2-deficiente dieren in vergelijking met dieren die het standaard voer kregen. Dit onderstreept het belang van optimale voeding voor zowel het onderhouden als het verbeteren van de barrière functie van de darm en de algemene gezondheid, vooral in aanwezigheid van factoren die het risico op darmontsteking verhogen. De toevoe-

ging van probiotica, dat wil zeggen bacteriën waaraan een gezondheidsbevorderende werking wordt toegeschreven, op het standaardvoer in de Muc2-deficiënte muizen verbeterde de structuur van het darmweefsel en verminderde de darmontsteking. Deze gunstige effecten leveren bewijs voor een verbeterde bescherming van de gevoelige darm door probiotica.

Hoofdstuk 4 beschrijft het effect van kunstvoeding voor pasgeborenen op de onrijpe darm in vergelijking met colostrum, oftewel biest. Het doel van deze studie was het verbruik van het aminozuur threonine in de darm te bepalen in premature biggen gevoed met kunstvoeding of met colostrum. Threonine is een belangrijke bouwsteen van de eiwitten en de slijmlaag in de darm en daarom noodzakelijk voor een goede aanmaak van deze producten en bescherming van de darm. De resultaten van deze studie laten een negatief effect zien van kunstvoeding op de groei, het ontstaan van NEC, de eiwitaanmaak in de darm, en de aanmaak van het slijmeiwit MUC2 in de darm in vergelijking met colostrum. Deze bevindingen geven een mogelijke verklaring voor de aanleg van premature en kunstgevoede pasgeborenen om NEC te ontwikkelen.

Hoofdstuk 5 toont aan dat zowel threonine aanwezig in de darmholte, oftewel het darm lumen, als threonine in het circulerende bloed voor de aanmaak van het slijmeiwit MUC2 gebruikt worden. Dit vindt plaats in de goblet cellen gelokaliseerd in het darmweefsel. Met deze studie hebben we onderzocht wat de voorkeur van de darm voor de opname van threonine is; opname vanuit het lumen of opname vanuit het bloed. Dit hebben wij gedaan in prematuur geboren biggen als ook in prematuur geboren kinderen met een stoma na een darmoperatie. De resultaten laten zien dat threonine van beide kanten wordt opgenomen voor aanmaak van MUC2, maar dat dit beïnvloed wordt door de hoeveelheid voeding als ook het type voeding. In de premature kinderen die na de operatie gedeeltelijke enterale voeding kregen aangevuld met voeding via het infuus, werd 91% van de threonine uit het bloed opgenomen. In de premature biggen die alleen enteraal gevoed werden, was de threonine opname voornamelijk uit het darm lumen wanneer ze gevoed werden met colostrum en voornamelijk uit het bloed wanneer ze gevoed werden met kunstvoeding. Tevens was er bij de biggen gevoed met colostrum sprake van een verhoogde aanmaak van het slijmeiwit MUC2 in vergelijking met kunstvoeding gevoede biggen. Gebaseerd op deze resultaten, benadrukken wij het belang van optimale enterale (via de darm) voeding voor de verhoging van slijmaanmaak en daarmee verbetering van de barrière functie van de darm in premature pasgeborenen.

Hoofdstuk 6 geeft inzicht in de regulatie van het slijmeiwit MUC2 in de darm door korte keten vetzuren. Onze hypothese was dat korte keten vetzuren, dit zijn producten onder andere afkomstig van bacteriën, effect hebben op de regulatie van het slijmeiwit

MUC2 in de darm en zo de bescherming van het darmweefsel kunnen beïnvloeden. Het doel van de studie was de mechanismen te onderzoeken die zorg dragen voor het effect van de korte keten vetzuren op MUC2 aanmaak. De resultaten tonen duidelijk aan dat de vetzuren butyraat en propionaat in concentraties zoals die gevonden zijn in de ontlasting van borstgevoede zuigelingen MUC2 stimuleren via activatie van het ap-1 (c-Fos/C-Jun) *cis*-element op het gen van MUC2. Hogere concentraties van butyraat, gelijkend op concentraties in de ontlasting van zuigelingen gevoed met kunstvoeding, onderdrukken MUC2 regulatie. Dit suggereert dat darmbacteriën, die qua samenstelling beïnvloed worden door het type voeding, d.w.z. moedermelk of kunstvoeding, indirect de aanmaak van MUC2 reguleren en zodoende de bescherming van het darmweefsel door de slijmlaag beïnvloeden.

Hoofdstuk 7 beschrijft het effect van de darmbacteriën op de aminozuur- en eiwitstofwisseling in pasgeboren biggen. Wij veronderstelden dat een verandering in de samenstelling van darmbacteriën door het geven van antibiotica of probiotica zowel de eiwitstofwisseling van de darm als die van het gehele lichaam zou beïnvloeden. In deze studie toonden wij aan dat het toedienen van antibiotica de hoeveelheid soorten darmbacteriën vermindert en de aanmaak van ureum vermindert. Tevens werd de concentratie en het verbruik van threonine in het bloed verhoogd, evenals de aanmaak maar ook afbraak van eiwitten in het lichaam. Echter, in de dunne darm en lever verminderde het geven van antibiotica de aanmaak van eiwitten. Het geven van probiotica stimuleerde kolonisatie van de darm met Bifidobacterien die een gunstig effect op de darm hebben. Tevens was er sprake van een verminderde ureum concentratie in het bloed maar zagen wij geen effect op de stofwisseling van threonine of eiwitten in het lichaam. Wel verminderde het geven van probiotica de aanmaak van eiwitten in de dunne darm, maar verhoogde het de aanmaak en de hoeveelheid van het slijmeiwit MUC2 in de dikke darm. Wij concludeerden dat het veranderen van de darmflora door het geven van antibiotica of probiotica van invloed is op ureum en threonine in het bloed en op aanmaak van eiwitten en MUC2 in de darm. Kortom, wanneer pasgeboren kinderen met antibiotica of probiotica worden behandeld leidt dit tot een verandering van de darmflora wat gevolgen zou kunnen hebben voor de eiwitstofwisseling in het lichaam.

Hoofdstuk 8 geeft inzicht in de hoeveelheid en functionaliteit van Paneth cellen in prematuur geboren kinderen met NEC. Paneth cellen zijn een belangrijk onderdeel van de afweer van de darm en beschermen het darmweefsel door uitscheiding van eiwitten met een antibacteriële werking. Echter, de hoeveelheid Paneth cellen en de werking van Paneth cellen in de onrijpe darm van prematuur geboren kinderen is niet bekend. Wij toonden aan dat in pasgeborenen die NEC ontwikkelden en geopereerd werden, het aantal Paneth cellen in de dunne darm minder leek te zijn in vergelijking met kinderen

zonder NEC. In de herstelfase van NEC, observeerden wij een sterke toename van Paneth cellen gepaard gaande met een verhoogde hoeveelheid antibacteriële eiwitten. Een nieuwe bevinding was de aanwezigheid van Paneth cellen in de dikke darm herstellende van NEC die normaliter deze cellen niet bevat. Analyse van stomavloeistof van de kinderen liet zien dat Paneth cellen in deze prematuren functioneel zijn door eiwitten uit te scheiden en de bacteriële groei te remmen. Deze resultaten bewijzen dat Paneth cellen aanwezig zijn in de darm van te vroeg geboren zuigelingen met NEC, en suggereren dat de toename van Paneth cellen in de dunne en dikke darm door de ernstige ontsteking wordt veroorzaakt. De toename van Paneth cellen leidt naar alle waarschijnlijkheid tot een verbeterde uitscheiding van actieve antibacteriële eiwitten. Dit is indicatief voor een verbeterde bescherming tijdens langdurige ontsteking en draagt bij aan het herstel van NEC. Desalniettemin dient nog verder te worden onderzocht hoe deze toename van Paneth cellen tot stand komt zodat mogelijkheden kunnen worden geboden voor verbetering van de behandeling van NEC.

Hoofdstuk 9 beschrijft het effect van het aminozuur arginine op de bloedsomloop en het darmweefsel in pasgeboren biggen. Arginine wordt in pasgeborenen gemaakt in cellen van het darmweefsel en is belangrijk voor de aanmaak van stikstofmonoxide welke de bloedsomloop reguleert. In premature pasgeborenen zijn lage arginine concentraties in het bloed gevonden en dit was geassocieerd met het ontstaan van NEC. Het extra toedienen van arginine zou de bloedtoevoer naar de darm kunnen verbeteren en zo NEC mogelijk kunnen helpen voorkomen. Onze resultaten in pasgeboren biggen tonen aan dat het toedienen van voeding in het algemeen, en niet zozeer het geven van arginine in het bijzonder, de bloedstroom naar de darm doet toenemen in vergelijking met biggen die voeding via het infuus ontvingen. Wij vonden ook geen effect van toediening van arginine voorloperproducten citrulline en glutamine op de bloedtoevoer naar de darm, ondanks het feit dat het toedienen van deze producten wel een verhoogde concentratie van arginine bewerkstelligde. Wel werd er een effect van hoge dosis arginine gevonden op groei van het darmweefsel, hoewel marginaal, maar dit ging niet gepaard met veranderingen in de aanmaak van eiwitten of vermeerdering van cellen.

Ten slotte worden de belangrijkste resultaten van de studies beschreven in dit proefschrift besproken in **hoofdstuk 10**. Door het uitvoeren van deze studies hebben we onze kennis vergroot van factoren die de darm van de pasgeborene beïnvloeden en een risico vormen op het ontstaan van NEC. Dit proefschrift wordt afgerond met aanbevelingen voor toekomstig wetenschappelijk onderzoek om de mechanismen die een rol spelen bij de afweer en de barrièrefunctie van de premature darm verder te onderzoeken. Uiteindelijk zou dit moeten leiden tot het vinden van een verklaring voor het ontstaan van NEC en bijdragen aan het ontwikkelen van optimale voeding voor te vroeg geboren kinderen.

Dankwoord – Words of thanks

De afgelopen 5 jaar waren een groot wetenschappelijk avontuur waarin ik door velen ben vergezeld, opgeleid, gestimuleerd en gesteund. Als ik terugdenk aan dit avontuur komen vele mooie herinneringen boven, vergeet ik de frustraties, en voel ik mij een gelukkig mens.

First I would like to thank my promotors Prof. van Goudoever, Prof. Boehm and my co-promotor Dr. Renes for the opportunity to perform this research.
Prof. van Goudoever, beste Hans, dank voor je positivisme en besmettelijke wetenschappelijke enthousiasme. Ook wil ik je danken voor mijn uitzending naar Houston en Kopenhagen. De onderzoeks- en levenservaringen die ik daar heb opgedaan zijn mij erg dierbaar en waren een 'once in a lifetime opportunity'. Je bent niet alleen wetenschappelijk gezien een goede promotor maar zorgt ook goed voor je promovendi-kroost. Ik zeg Aloha en iedereen weet wat ik bedoel! Dat siert je en maakte dit proefschrift niet alleen maar een mooi onderzoeksproject maar ook een heel mooi avontuur.
Prof. Boehm, or to me just 'professor', thank you for your guidance and experience derived research-lessons. I very much appreciated your long car trips to Rotterdam, your fast email replies and your feedback, especially these last few months when everything came together in this opus.
Dr. Renes, Ingrid, Bossy, er is een wereld voor me opengegaan op het lab. Ik heb me regelmatig afgevraagd waar ik in hemelsnaam aan begonnen was maar jij wist dan weer rust in de tent te brengen en het vertrouwen te geven dat het echt wel ging lukken. Dankjewel voor je geduld en begeleiding, en voor de DE momenten.

Graag wil ik de kleine commissie, Prof. van der Heijden, Prof. Lafeber en Prof. Sauer bedanken voor hun deelname in de promotiecommissie en voor hun snelle beoordeling van dit proefschrift. Ook Prof. Kuipers wil ik danken voor het plaatsnemen in de promotie-commissie. I would like to express my gratitude to Prof. Burrin, Prof. Sangild and Prof. Castillo for coming over and taking part in my defense committee.

Dan 'de 15e', lab kindergeneeskunde, waar ik de kop en staart van mijn promotie heb doorgebracht. Allereerst wil ik natuurlijk mijn collega's van het neo-lab bedanken: Janneke, ik zal onze miljarden cellen nooit vergeten! Je hebt me ontgroend op het lab en veel dingen geleerd om nooit meer te vergeten zoals vortexen, precipiteren en transfecteren. En ook Ad, jij bedankt voor je kennis en ervaring, inzet, nauwkeurigheid en vooral fijne samenwerking. We waren een goed team! Maria, je stond altijd klaar om te helpen en mee te denken; Anita, bedankt voor de prachtige glaasjes; Peng and Laura, thanks for your international input. En natuurlijk Nanda, mijn promotie-maatje en lotgenoot als experimenten weer eens niet lukten of onze muizen 'het' niet deden. We did it!

Van ‘de 15e’ naar beneden, zoals elke dinsdag, naar het massaspec-lab: Henk, Gardi en Kristien. Dank jullie voor jullie hulp, met name gedurende de laatste loodjes en de mooie secure data. Met name de doppen zullen me nog lang bijblijven.

SK 2210 is voor mij ingedeeld in 2 tijdperken. De eerste is vóór Houston met Chris, Frans en Maaike: We wisten de Sophia-‘de 15e’ afstand te overbruggen. Van lunchen, congressen en borrelen geen genoeg en door de jaren heen naast collega’s vrienden geworden. Thanks! Na Houston en Kopenhagen waren daar Denise, Hester en Willemijn. Jullie bedankt voor de gezelligheid en veel succes met jullie mooie projecten. En natuurlijk Margriet, succes met ook een beetje mijn dikke vriendjes.
Ook Ineke, ons onderzoeksorakel als het gaat om de wirwar van MECT / CCMO reglementen en administratie, dankjewel voor je alwetendheid. En lieve Daniella, Daantje, als rechterhand van Hans was je absoluut onmisbaar maar je interesse, je steun en je inzet ook voor onze belangen maken je uniek .

Then Part II, Houston, Texas, USA. It was a great adventure that I cherish in my heart. Dearest B, you thought me how to take care of your ‘babies’ and I feel privileged you trusted me and took me under your wing. I would state that you were my ‘la condition essentielle du succèss d’une expérience’, with P-boxes in the fridge to keep me going. Even when we saw each other 3 weeks for almost 24/7, I would not think of missing out on our Adobe Gay-night with Mr. Bob. And I will not forget our sleep-deprived Texas Martini’s after finishing our experiments. The Houston-, Lake Bob-, and especially the running-experience would not have been the same without you two. I love you for who and what you are: so intense, sweet and a little nuts! Doug, you have been a great example of how to be a good researcher, teacher, and coach. Thank you for having me in your lab. I felt it was in Houston that I became a true researcher, and I would not have accomplished all of this without your invaluable expert scientific guidance.
I would also like to thank Xiaoyan and Liwei for helping me out in the lab, and my office-mates Nancy, Caroline and Steven for the US-European cultural experience, and I am sorry if I was being too ‘Dutch’ every now and then.

The CNRC was not only a place to work but also a place where I have found some special friends. Marta, guapa, thanks for being there at the 11th floor, for your enthusiasm and your indestroyable joy in life. You are one strong woman, mother, and talented researcher. Gerrit, wat een geluk dat jij er ook was! We hebben met, en vaak ook dankzij jou zoveel super herinneringen: Chipotle, Gingerman, Breckenridge, Huntsville, Crawfish, the Gay Parade, en Noddy met Afie in de living…. Monique and Robert, thanks for showing us that ‘other’ side of Houston, our great conversations, the unforgettable ‘Manneke Pis’-till-we-drop-G’man afternoons, the most inspiring Sinterklaas ever, and

the great and delicious home-made dinners. Of course I should not forget Murali, Jean, Mahmoud, Trinna, Luisa and Steven for opening up to the Dutch Sinterklaas and Friday afternoon experience on Sinterklaas and the Friday afternoons.

Part III was in Copenhagen, I came with one mission and left after completing two. Thank you Per for helping me out, giving me the opportunity to work in your group and your confidence in our research. It was inspiring and turned out into a great success. Mikkel, it was great to have you in Rotterdam joining me to work against the clock. It payed off! Mette and Lars, thank you for having me in your lab and helping me with your expertise on the TRFLP. Also many thanks to the rest of the lab, you made me feel closer to home with your morning 'party' bread and cake-days, and a special thanks to Sophia for helping me out. And dearest Malene, both in Houston and in Copenhagen I was fortunate you were around. We had lots of fun, you showed me your Copenhagen life and you were there for me when I needed a friend. I hope I can return the favor soon!

Lieve vrienden, collega's, familie en buurtjes, dank jullie voor jullie begrip, interesse, steun en peptalks. De wetenschap dat jullie er ook na deze periode nog zouden zijn maakt dat er nog veel gezelligheid in te halen is om naar uit te kijken. Lieve Nens, Fies en Pien, ik heb jullie of liever gezegd 'het klavertje vier' gemist. Jullie zijn meer dan mijn vriendinnen en dat zal ook altijd zo blijven.

Dan mijn paranimfen, Nancy en Maaike, met jullie aan mijn zijde moet het lukken! Maaike, jouw medeleven en vriendschap zijn me ontzettend dierbaar en een voorbeeld dat doet volgen. Nancy, je bent mijn geneeskunde maatje vanaf het eerste uur, en zelfs daar voor. Promoveren zonder jou naast me is gewoonweg ondenkbaar.

Henk en Lian, ik bof met jullie als schoonouders. Zonder jullie hulp met het huis, Isa en Jet was dit ons nooit gelukt.

Papa en mama, door jullie genen, opvoeding en stimulans ben ik geworden wie ik ben, en heb ik tot nu toe datgene bereikt wat ik wilde bereiken. Jullie liefde, vertrouwen, steun en trots hebben me altijd gesterkt. Ik hou van jullie.

Lieve Isa, jij bent mijn kleine grote wonder en het is onvoorstelbaar maar je wordt steeds mooier.
Sas, lieve lieveling, jij de een! Mijn leven met jou is een mooie en avontuurlijke reis: Rotterdam, Afrika, trouwen, Houston, Kopenhagen, Jetje, Isa, opleiding en nu samen promoveren. Je bent mijn grote liefde en mijn grootste drijfveer. Er is niemand zoals jij bent voor mij.

Affiliation of co-authors

Author	Affiliation
Boehm, Günther	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands Danone Research, Centre for Specialised Nutrition, Friedrichsdorf, Germany
Bouma, Janneke	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Burger-van Paassen, Nanda	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Burrin, Douglas	Children Nutrition Research Center, Baylor College of Medicine, Houston, TX, USA
Bruijn, Adrianus de	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Dorst, Kristien	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Korteland-van Male, Anita	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Krijger, Ronald de	Department of Pathology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, the Netherlands
Lars Mølbak	National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark
Le Polles, Nicolas	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Lu, Peng	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Lykke, Mikkel	Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, Denmark

Renes, Ingrid	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Sangild, Per	Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, Denmark
Schierbeek, Henk	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Schaart, Maaike	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Schmidt, Mette	National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark
Sluis, Maria van der	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
Stoll, Barbara	Children Nutrition Research Center, Baylor College of Medicine, Houston, TX, USA
Tibboel, Dick	Department of Pediatric Surgery, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands
van Goudoever, Hans	Department of Pediatrics, Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands Department of Pediatrics, Academisch Medisch Centrum – Emma Children's Hospital, Amsterdam, The Netherlands Department of Pediatrics, VU Medisch Centrum, Amsterdam, The Netherlands
van Seuningen, Isabelle	Inserm, U837, Centre de Recherche Jean-Pierre Aubert, Lille, France
Vincent, Audrey	Inserm, U837, Centre de Recherche Jean-Pierre Aubert, Lille, France

List of publications

Dietary influence on colitis-development in Muc2-deficient mice: diet matters!
N Burger-van Paassen, PJ Puiman, P Lu, N Le Polles, J Bouma, AM Korteland-van Male, G Boehm, JB van Goudoever, IB Renes
Manuscript in preparation

Intestinal threonine utilization for protein and mucin synthesis is decreased in preterm pigs fed formula.
PJ Puiman, M Lykke, B Stoll, IB. Renes, ACJM de Bruijn, K Dorst, H Schierbeek, M Schmidt, G Boehm, DG Burrin, PT Sangild, and JB van Goudoever
Submitted

Intestinal threonine uptake routes for mucin synthesis in preterm pigs and infants
PJ Puiman, N Burger-van Paassen, B Stoll, ACJM de Bruijn, K Dorst, H Schierbeek, PT Sangild, G Boehm, IB Renes, JB van Goudoever
Submitted

Modulation of the gut microbiota with antibiotic or probiotic treatment suppresses body urea production and stimulates mucosal mucin production in neonatal pigs.
PJ Puiman, B Stoll, Lars Mølbak, ACJM de Bruijn, H Schierbeek, M Boye, G Boehm, I Renes, JB van Goudoever, DG Burrin.
Submitted

Enteral arginine does not increase superior mesenteric arterial blood flow, but modestly increases mucosal growth in neonatal pigs.
PJ Puiman, B Stoll, JB van Goudoever, DG Burrin
Accepted J Nutr

Paneth cell hyperplasia and metaplasia in necrotizing enterocolitis.
PJ Puiman, N Burger-van Paassen, MW Schaart, ACJM de Bruijn, RR de Krijger, D Tibboel, JB van Goudoever, IB Renes.
Accepted Ped Res

The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection.
N. Burger-van Paassen, A. Vincent, P.J. Puiman, M. van der Sluis, J. Bouma, G. Boehm, J.B. van Goudoever, I. van Seuningen, I.B. Renes.
Biochem J. 2009;420(2):211-9.

Animal models to study neonatal nutrition in humans.
P.J. Puiman, B. Stoll
Curr Opin Clin Nutr Metab Care. 2008;11(5):601-6.

A cognitive behavioral therapy program for overweight children.
Puiman PJ, van den Akker EL, Groen M, Timman R, Jongejan MT, Trijsburg W.
Journal of Pediatrics 2007;151(3):280-3.

Een zeldzame vorm van hartfalen: Peripartum Cardiomyopathie.
P.J. Puiman, S. Strikwerda, R. Heydanus, G.P. Verburg.
Hart Bulletin 2005; 3; 64-68.

Een zeldzame vorm van hartfalen: Postpartum Cardiomyopathie.
P.J. Puiman, S. Strikwerda, R. Heydanus, G.P. Verburg.
Nederlands Tijdschrift voor Obstetrie en Gynaecologie 2004; 17; 196-199.

Total body water in children with cystic fibrosis using bioelectrical impedance.
P.J. Puiman, P. Francis, H. Buntain, C. Wainwright, B. Masters, P.S.W. Davies.
Journal of Cystic Fibrosis 2004;3:243-7

Curriculum Vitae

Patrycja Puiman was born on the 6th of December 1977 in Velp, the Netherlands. After finishing high school (Stedelijk Gymnasium Arnhem), she started her medical training at the University of Maastricht in 1997. During her study she was active at the students association S.V. KoKo where she became a board member in the year 1999-2000. She obtained her medical degree in September 2004, after which she started working as a pediatric resident (ANIOS) at the Sint Franciscus Gasthuis Hospital in Rotterdam. In 2006 she started her residency at the neonatology department at the Erasmus MC-Sophia Children's Hospital in Rotterdam, which quickly resulted in the start of her PhD project on 'Intestinal defense mechanisms in the neonate' at the laboratory of pediatrics at the Erasmus MC in 2006 under supervision of Prof. van Goudoever, Prof. Boehm and Dr. Renes. In June 2006 she married her husband Sascha Verbruggen and in the summer of 2007 they moved to Houston, Texas, where she started working at the Children's Nutrition Research Center at Baylor College of Medicine for 15 months with Prof. Burrin and Dr. Stoll. Thereafter, she continued her studies for 3 months in Copenhagen, Denmark, at the Life Sciences University with Prof. Sangild and at the Veterinary Institute at the Technical University of Copenhagen with Prof. Boye. In December 2008 she returned to Rotterdam to finalize her dissertation. In June 2009 she gave birth to a beautiful daughter named Isa. The following year in July 2010 she started her clinical training as a pediatric resident (AIOS) at the Erasmus MC – Sophia Children's Hospital in Rotterdam under guidance of Dr. de Hoog.

PhD Portfolio
Summary of PhD training and teaching

Name PhD student: Patrycja J. Puiman	PhD period:	2006 - 2010
Erasmus MC Department: Pediatrics	Promotors:	Prof. J.B. van Goudoever, MD. PhD.
Research School: Erasmus MC		Prof. H.J.G. Boehm, MD. PhD.
	Supervisor:	Dr. I.B. Renes, PhD.

1. PhD training

	Year	Workload ECTS
General courses		
Cursus Proefdiergeneeskunde, Erasmus MC	2006	1.0
Animal Science Course, Baylor College of Medicine, Houston, USA	2007	0.8
BROK 'Basiscursus Regelgeving Klinisch Onderzoek', Erasmus MC	2009	1.0
Specific courses		
Isotope Tracers in Metabolic Research; University of Arkansas for Medical Sciences, Little Rock, USA	2007	2.0
Seminars and workshops		
Research Seminars, Children's Nutrition Research Center, Baylor College of Medicine, Houston, USA	2007- 2008	1.0
Research Fellow Symposium, Baylor College of Medicine, Houston, USA	2007- 2008	0.8
Research bespreking kindergeneeskunde, Erasmus MC	2008 - 2010	0.6
Research bespreking 'Moeder en Kind Centrum', Erasmus MC	2008 - 2010	0.6
(Inter)national conferences		
European Society of Pediatric Research, Barcelona, Spain	2006	1.0
Federation of American Societies for Experimental Biology, Washington, USA	2007	1.0
Society of Pediatric Research, Research meeting, Woodlands, USA	2007	1.2
Society of Pediatric Research, Honolulu, USA	2008	1.0
European Society of Pediatric Research, Nice, France	2009	1.0
Federation of American Societies for Experimental Biology, New Orleans, USA	2009	1.0
Benelux Association of Stable Isotope Scientist, Brugge, Belgium	2009	0.4
European Society of Pediatric Research, Hamburg, Germany	2009	1.0
Nederlands Vereniging van Gastro-Enterologie, Veldhoven, The Netherlands	2010	0.4
Society of Pediatric Research, Vancouver, Canada	2010	1.0

Poster Presentations		
Paneth cells in preterm infants with necrotizing enterocolitis, Society of Pediatric Research, Research Conference, Woodlands, USA	2007	1.0
The effect of short chain fatty acids on mucin MUC2 synthesis: implications for epithelial protection?, European Society of Pediatric Research, Prague, Czech Republic	2007	1.0
Paneth cells in preterm infants with necrotizing enterocolitis, Federation of American Societies for Experimental Biology, Washington, USA	2007	1.0
Intestinal trophic effect of enteral arginine is independent of blood flow in neonatal piglets, Society of Pediatric Research, Honolulu, USA	2008	1.0
Modulating the gut flora by pro- and antibiotics alters threonine metabolism in neonatal pigs, European Society of Pediatric Research, Nice, France	2008	1.0
Modulating the gut flora alters amino acid metabolism in neonatal pigs, Federation of American Societies for Experimental Biology, New Orleans, USA	2009	1.0
Intestinal trophic effect of enteral arginine is independent of blood flow in neonatal piglets, European Society of Pediatric Research, Hamburg, Germany	2009	1.0

Oral Presentation		
Paneth cells in preterm infants with necrotizing enterocolitis, European Society of Pediatric Research, Prague, Czech Republic	2007	1.4
Intestinal Metabolism is influenced by Antibiotics and Probiotics in Neonatal Pigs, Benelux Association of Stable Isotope Scientist, Brugge, Belgium	2009	1.4
Improved gut barrier function via increased threonine utilization may explain enhanced resistance to necrotizing enterocolitis in preterm pigs fed colostrum, European Society of Pediatric Research, Hamburg, Germany	2009	1.4
Probiotics and antibiotics change microbial diversity and decrease gut growth in neonatal pigs, European Society of Pediatric Research, Hamburg, Germany	2009	1.4
Increasing intestinal threonine metabolism improves gut barrier function and resistance to necrotizing enterocolitis in preterm pigs fed colostrum, Nederlands Vereniging van Gastro-Enterologie, Veldhoven, The Netherlands	2010	1.4
Increasing Intestinal Threonine Metabolism Improves Gut Barrier Function and NEC Resistance in Preterm Pigs Fed Colostrum, Society of Pediatric Research, Vancouver, Canada	2010	1.4

2. Teaching

	Year	Workload ECTS
Supervising Master's theses		
Mikkel Lykke, Faculty of Life Sciences, University of Copenhagen	2009	3.0

Portfolio