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PhD Thesis

DIARRHEA IN EARLY LIFE: PROGRESS IN DIAGNOSIS AND CONTROL OF DISEASE

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INTRODUCTION

Diarrheal diseases occurring in early life (< 3 years) are an heterogeneous group of abnormalities including gastrointestinal infections, food hypersensitivity and allergy, immune dysregulation, and primary abnormalities of the enterocyte.

Diarrhea is a leading cause of illness and death in children younger than 3 years worldwide (Figure 1), causing 1·3 million deaths every year (1). Despite defining etiology of diarrhea is critical to decide therapy and prevention strategy, the overall prevalence of early onset diarrhea was not clearly defined because of the large spectrum of etiologies and difficulties in patient selection.

From a clinical point of view, diarrhea needs to be classified taking into account certain characteristics such as trends over time (acute or chronic, using a limit of 4 week to separate the two conditions) and the characteristics of the feces (2,3). Acute diarrheal diseases occurring in early life, usually due to infectious agent or food allergy, were burdened by an increased mobility and mortality, whereas chronic evolution of early onset diarrhea frequently suggests a congenital disorder determined by genetic defects. Using this classification a pediatrician can decide upon diagnosis and therapy more rationally. However, acute diarrhea may be also a symptom of the onset of chronic organic or functional disease.

Acute diarrhea in early life

In younger children acute diarrhea will lead to severe dehydration. Fluid loss and dehydration are the cause of death in nearly all children with acute diarrhea. Over 3 decades ago, the discovery of mechanisms of intestinal electrolytes transport, which was the basis

for the development of oral treatment of dehydration, was hailed as the most important medical advance of the 20th century (4). Complications can be prevented by the early and adequate oral administration of a rehydration solution, by normal food for the child's age, and through induction of benefical intestinal microbiota composition (4). The evidence-based guidelines of the ESPGHAN and Cochrane analyses, based also on studies reported in this manuscript, indicate zinc and probiotics as useful therapeutic aids for children younger than 3 years with acute diarrhea (5-9).

Chronic diarrhea in early life

Chronic diarrhea in early life includes a group of rare chronic enteropathies characterized by a heterogeneous etiology, which in most cases is related to an identified or to an as yet unidentified genetic defect, generally inherited as an autosomal recessive trait. These congenital diarrheal disorders (CDD, OMIM 251850) represent one of the most challenging clinical conditions for pediatric gastroenterologists because of the severity of the clinical picture and the broad range of conditions in its differential diagnosis (10). Early in life, patients affected by CDD usually present with severe diarrhea that within a few hours leads to a life-threatening condition secondary to massive dehydration and metabolic acidosis (10). Consequently, children affected by CDD require a prompt diagnosis and assistance. Clinical manifestations are variable from severe conditions leading to intestinal failure, to milder forms with subtle clinical signs that may remain undiagnosed until adulthood, when patients have just developed irreversible complications. Intestinal failure may lead to the necessity of total parenteral nutrition with further complications for the health of the subject affected by these disorders. The number of conditions included within the CDD group has gradually increased over the years (10). Now it is clear that CDD depend on defects in the structure and function of absorptive, enteroendocrine or inflammatory cells of the gut, determined by mutations in genes expressed throughout the gastrointestinal tract involving different segments and different cells. Therefore, as shown in Figure 2, we have proposed that CDD could be classified into 4 groups: (i) Absorption and transport of nutrients and

electrolytes; (ii) Enterocyte differentiation and polarization; (iii) Enteroendocrine cell differentiation; and (iv) Modulation of the intestinal immune response. In recent years, many new genes have been identified. Such molecular techniques as positional candidate genes and genome-wide linkage analysis have clarified the role of these genes in the physiology of the gastrointestinal tract. Understanding the function of this genes may open new diagnostic and therapeutic perspectives for chronic and acute forms of early onset diarrhea.

Progress for diagnosis and control of early onset diarrheal diseases

The early postnatal period represents a critical window during which the evolving intestine is programmed by intrinsic (genetic programming) and extrinsic factors (microbe, nutrition, drugs). Intestinal content may modify pretranslational and post-translational gene expression. These ‘‘epigenetic’’ mechanisms are involved in the development of gut enzymes, hormones, transporters, and immunity. Occurrence of diarrheal diseases and related treatments, during this temporarily window, may influence the entire body health status in the short and long-term period (11,12) (Figure 3). In this scenario, accurate identification and adequate control of diarrheal disorders occurring in early life are crucial for reducing morbidity and mortality.

Finally, advances made in the pathophysiology of chronic early onset diarrheal disorders could contributed to a better understanding the mechanisms also of the more common acute diarrheal diseases and to identify novel strategy for disease control.

Starting from these considerations, we elaborate selected items focusing on the new diagnostic, therapeutic and preventive strategies for diarrheal diseases occurring in early life.

INVESTIGATIONAL PLAN

The PhD project was focused on 4 closely integrated research area:

Research Area 1. Epidemiology and clinical features of diarrhea in early life

A key to understanding the treatment and control of early onset diarrhea is establishment of epidemiology and etiology. Early diagnosis and timely treatment are crucial because diarrhea in the first phase of the life may rapidly lead to life-threatening dehydration and malnutrition (15). Clinical and epidemiologic studies defining severity and etiology are needed in order to improve diagnostic and therapeutic approaches for diarrhea in early life.
Frequently diarrhea is associated with intestinal failure during neonatal age, imposing clinician to recur to artificial nutrition. Few studies have addressed the incidence, prognostic factors, diagnosis, and nutritional aspects of intestinal failure occurring early in life (14). Identification of the risk factors for dependence on parenteral nutrition is of critical importance for rapid and appropriate preventive strategies application (15).

Planned studies. Two nationwide multicenter studies investigating (i) epidemiology, etiology, clinical features, and management of diarrhea observed in hospitalized newborns, and (ii) epidemiology and natural course of neonatal onset intestinal failure, were performed.

Research Area 2. New approaches for control of acute diarrhea in early life

The most common causes of acute diarrhea involve gastrointestinal infection and food allergy. Although diarrhea acts as a defense mechanism in the body, quickly eliminating infective organisms and antigens, the most serious sequelae is dehydration. Oral rehydration solution (ORS) is the first-line therapy for the management of children with acute gastroenteritis worldwide (4). However, ORS neither reduces the severity nor the duration of symptoms. Several substrates and substances acting on transepithelial fluid transport have been proposed in order to enhance clinical efficacy of ORS (5-9). Such evidences indicated that zinc-fortified ORS and probiotics could be effective in reducing diarrhea duration and severity in children with acute gastroenteritis (4).

Planned studies: (i) Prospective study evaluating efficacy of new oral rehydration solution in children with acute gastroenteritis; and (ii) multicenter study comparing the therapeutic effects of different probiotics in children with infectious diarrhea, were performed.

Research Area 3. Diagnosis and treatment of chronic diarrhea in early life

Early diagnosis of this condition is necessary in infancy because hyponatremic episodes may result in mental and psychomotor impairment and the chronic contraction of the intravascular space leading to renal dysfunction and gout.

Recently, the role of amylase-resistant starch has been increasingly recognized for the management of diarrheal diseases. On reaching the colon, amylase-resistant starch are fermented by resident bacteria into the short-chain fatty acids (SCFA), including acetate, propionate, and butyrate. As already shown, SCFA have a great capacity for stimulating ion and water absorption; they provide energy and induce a trophic effect on both colonic and

small bowel mucosa (10). The important regulatory role of SCFA on fluid and electrolyte absorption has led to the hypothesis that butyrate treatment could reduce diarrhea in patients with such forms of chronic diarrhea occuring in early life.

Planned studies: Prospective studies (i) defining the genotype of children with early onset chronic diarrhea, and (ii) evaluating the clinical efficacy of butyrate in children affected by congenital diarrhea, were performed.

Research Area 4: Prevention of diarrhea in early life

In the previous century, more than 100 countries had established national programs for control of diarrheal disease. In developed countries, these programs should be taken into account that the first two causes of diarrhea in early life are infections and food allergy. Gastric acidity inhibitors the mainstay of therapy of gastrointestinal acid-related disorders, has been associated with increased risk of acute gastroenteritis (16). Reduction of the use of GAI may reduce the risk of gastrointestinal infections occurring in early life. The prevalence of food allergy has increased worldwide, especially in children (17). Data suggest that the onset of food allergy may be prevented by nutritional intervention early in life (18).

Planned studies: (i) prospective studies evaluating the risk of intestinal and extraintestinal infections associated with the gastric acidity inhibitors use, and (ii) multicenter audit study measuring the implementation of prevention program for control of food allergy, were performed .

OBJECTIVES OF THE PhD

The objectives of PhD project were divided per research area:

1. Epidemiology and clinical features of diarrhea in early life

1. To study epidemiology and etiology of diarrhea occurring in early life
2. To define natural course of diarrhea associated with intestinal failure occurring in early life and to verify the efficacy of strategies for control of complications related to early onset intestinal failure

2. New approaches for control of acute diarrhea in early life

1. To evaluate efficacy of new oral rehydration solution in children with acute gastroenteritis
2. To compare the therapeutic effects of different probiotic formulations in children with infectious diarrhea

3. Diagnosis and treatment of chronic diarrhea in early life

1. To define the genotype of children with early onset chronic diarrhea
2. To evaluate the clinical efficacy of new therapeutic strategy by *in vivo/in vitro* approach

4. Prevention of diarrhea in early life

1. To prevent the risk of acute diarrhea reducing the use of gastric acidity inhibitors
2. To evaluate the efficacy of preventive strategy for control of early onset diarrhea due to food allergy

ETHICS

Ethical Conduct of the Study

The study was conducted in accordance with the Declaration of Helsinki (6th revision 2008) and its amendments. All researchers involved into the study disclosed any financial and personal relationships with organizations that could inappropriately influence their work. Examples of financial conflicts included employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications, and travel grants, all within at least 3 years of the beginning the study.

Patient Informed Consent

The parents of all eligible subjects invited to participate into the study received an information sheet and consent form. Consent form was illustrated by researchers involved into the care of the patient.

PROJECTS

In this section were reported design and main results of the studies performed during the PhD divided per research project section and aims. Results were discussed separately in each section.

Research Area 1:
Epidemiology and clinical features of diarrhea in early life

Epidemiology

Results of this research were published on World Journal of Gastroenterology 2010; 16:2664-8 Web link

Diarrhea and neonatal age represent two major conditions responsible for pediatric mortality worldwide (19,20). First weeks of the life are characterized by an increased susceptibility to the complications related to diarrhea because of immaturity of the systems that regulate fluid homeostasis and immunologic response (21). Early diagnosis and timely treatment are crucial because diarrhea early in life may rapidly lead to life-threatening dehydration and malnutrition (22,23). Clinical and epidemiologic studies defining severity and etiology are needed in order to improve diagnostic and therapeutic approaches for early onset diarrhea. Starting from these considerations, in collaboration with the Working Group on Intestinal Infections of the Italian Society of Pediatric Gastroenterology Hepatology and Nutrition (SIGENP), on behalf of the Italian Society of Neonatology (SIN), a nationwide study aiming to investigate frequency, etiology, clinical features, nutritional management, therapeutic approach, and outcomes of diarrhea observed in hospitalized newborns was designed.

STUDY DESIGN

A multicenter, retrospective study was planned. The study design and aims were presented and discussed during two meetings of the SIGENP and of the SIN. We invited to participate the chiefs of Neonatal Intensive Care Units (NICUs) of urban children's hospitals, university medical center or large community hospitals, observing at least 100 newborns per year and having the following diagnostic facilities: determination of fecal electrolytes concentration, full microbiological examination, food allergy tests, gastrointestinal endoscopy and histology, metabolic tests, genetic counseling. The neonatologists operating in participating centers were invited to review data of 3 consecutive years (i.e., 2000-2002). Inclusion criteria were: a) age at hospitalization ≤ 28 days; b) gestational age at birth ≥ 24 weeks; c) clinical chart and hospital records available for review; d) presence of diarrhea, defined on the basis of increased frequency and watery consistency of stool along with dehydration. This definition that was adopted in previous study in neonatal age (22) is in accordance with the traditional definition employed in pediatric gastroenterology (24) and to the more recent guidelines for the management of acute gastroenteritis of the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN)/European Society for Pediatric Infectious Diseases (ESPID) (4). Data were recorded in a specific reporting form articulated in 5 sections: *i.* demographic characteristics: sex, gestational age, birth weight, age at hospitalization and at diarrhea onset; *ii*. anamnesis: intrauterine growth restriction, polyhydramnios, risk for allergy according to American Academy of Pediatrics (AAP) guidelines (18), familiarity for chronic diseases including diarrhea, surgery, diet before diarrhea onset; *iii.* characteristics of diarrhea: number of bowel movements, stool consistency, presence of blood and mucus in the stools, severity of dehydration, complications, development of chronic diseases, diarrhea duration and presence of other intestinal or extra-intestinal symptoms; *iv.* etiology of diarrhea; *v.* nutritional and therapeutic management, clinical outcomes.

To better define study population and to establish diarrhea occurrence rate, the number of hospital admissions and the main demographic characteristic of all subjects observed during

the study period, in each participating NICU, were also evaluated. Diarrhea was classified as acute or chronic if it was lasting <14 or > 14 days, respectively according to the definition of World Health Organization (25,26). The study protocol was approved by Ethics Committee of our institution. No competing interest was declared by the investigators involved in the study.

Statistical analysis

Patients were classified according to the duration of diarrhea in acute and chronic group. For continuous variables the t-test for equality of means was used. For categorical variables the χ^2 test and Fisher's exact test were used. Possible correlations between the duration of diarrhea with gestational age, birth weight, sex, number of bowel movements, antibiotic use, age at diagnosis, severity of dehydration, were investigated by linear regression analysis. The level of significance for all statistical tests was 2-sided $p < 0.05$. Statistical analysis was performed by software SPSS (Version 14.0 for Windows, SPSS Inc, Chicago, IL).

RESULTS

Sixteen NICUs were invited and 7 accepted to participate into the study. The clinical charts of 5801 were reviewed, and 39 cases of diarrhea were reported. Occurrence rate of diarrhea was estimated in 6.72 per 1.000 hospitalized newborns. The number of cases observed during the study period was: 12 in the first, 14 in the second and 13 in the third year of the study. Diarrhea was the main cause of hospital admission in 14 out of 39 subjects (35.9%). Diarrhea was classified as acute in 36 patients (92.3%) and chronic in 3 neonates (7.7%). The etiology of diarrhea was identified in 29 out of 39 patients (74.3%) (Table 1).

A diagnosis of cow's milk allergy (CMA) was reported in 8 cases. Five of them were to be considered at risk for allergic diseases according to the AAP definitions (18). In patients with CMA, diarrhea was associated with eczema or vomiting in 6 and in 5 subjects, respectively. Specific IgE titers against milk proteins resulted positive in 6 out of 8 patients

(>5.0 KU/L) (27). In each case of CMA, the diagnosis was confirmed by observation that antigen elimination diet with extensively hydrolyzed casein formula (Nutramigen®, Mead-Johnson Nutritionals, Italy) resulted in symptomatic improvement, and re-introduction of cow's milk after 4 weeks caused symptoms reappearing. An open food challenge was performed in 5 out of 8 subjects during hospitalization in consultation with a pediatric allergy specialist, and in 3 out of 8 patients after the discharge, in a tertiary Center of Pediatric Gastroenterology and Food Allergy.

Seven patients were classified as affected by intestinal infection according to the results of microbiological analysis. The microorganisms responsible for diarrhea are reported in the Table 1. One patient initially classified as affected by gastrointestinal infection had familiar history of immunodeficiency and a clinical course characterized by growth delay, recurrent opportunistic infections, lymphopenia, associated with defective cellular and humoral immune responses. In this case, the molecular analysis confirmed the clinical diagnosis of adenosine deaminase deficiency (OMIM 608958) (28).

Five babies presented antibiotic-associated diarrhea. The microbiological analysis, including the search for *Clostridium difficile*, resulted negative in all these subjects.

According to clinical findings and the results of molecular analysis two patients received a diagnosis of congenital diarrheal disorders (OMIM 251850): one with glucose-galactose malabsorption (OMIM 182380); and one with congenital chloride diarrhea, (OMIM 214700) (3).

Neonatal withdrawal syndrome-induced diarrhea was reported in 2 subject birth from mothers with a history of drug abuse (heroine and methadone) during pregnancy (29). One case of Hirschsprung's disease was diagnosed according to the clinical history (not passage of meconium in the first 72 hours of life, bloating of the abdomen) and the result of diagnostic tests, including rectal manometry, barium enema, and rectal biopsy (30). This patient presented diarrhea as consequence of severe enterocolitis requiring broad-spectrum of antibiotic therapy. Parenteral diarrhea induced by extraintestinal infection determined by *Klebsiella pneumonia*-induced urinary infection was reported in one full term baby during

the first week of life. In this patient diarrhea started before antibiotic treatment, all microbiological evaluation on stools resulted negative, and diarrhea improved rapidly when urinary infection disappeared. For one subject presenting familiar history of cystic fibrosis (CF), intrauterine growth retardation, and chronic diarrhea, a final diagnosis of CF was achieved through sweat test and confirmed by identification of CFTR ΔF508 mutation, at the age of 4 months (31). Urea defect cycle was diagnosed in one patient by the presence of diarrhea together with metabolic acidosis, hyperammoniemia and protein load intolerance (32).

Main anamnestic and demographic characteristics of neonates with diarrhea are provided in Table 2. Symptoms associated with diarrhea are reported in Table 3. The newborns affected by diarrhea with unknown origin showed a birth weight significantly lower (1920 g, IQR 1418 g) compared to subjects with an established etiologic diagnosis (2870 g, IQR 705 g) ($p<0.040$). The mean number of bowel movements per day was 7.4 (95% CI 7.0-7.8) without differences between acute and chronic diarrhea. The mean number of daily bowel movements wasn't influenced by the etiology. The mean duration of diarrhea was similar for full-term (9.08 days, 95% CI 4.7-13.4) and preterm newborns (4.7 days, 95% CI 3.9-5.6) ($p = 0.953$). Linear regression analysis using a stepwise method demonstrated that diarrhea duration have a negative relationship to the age of symptoms onset (B -0.80; Beta -0.48; $p = 0.005$). Inversely, birth weight, sex, gestational age, antibiotic use, severity of dehydration, rehydration or re-feeding strategies, and assumption of breast milk not correlated with duration of diarrhea.

Data on dehydration and rehydration strategies are reported in Table 4. As re-feeding strategy in patients with acute diarrhea, extensive casein hydrolyzed formula (Nutramigen®, Mead-Johnson Nutritionals, Italy) was administered in all subjects with suspected CMA diagnosis, and in only 6 out of 31 of remaining subjects (19.3%). Nine out of 39 subjects presenting diarrhea were on breast milk, 3 of them received exclusive breast milk at the diarrhea onset. After the diarrhea onset breast milk was continued in 3 subjects, temporally withdrawal (24 hours) in 2, and definitively suspended in 4 out of 9 newborns.

The clinical outcomes of diarrhea are reported in the Table 4. Three deaths were reported among the 39 subjects (7.7%). Patients affected by adenosine deaminase deficiency, CF, and Hirschsprung's disease died for complications related to fatal systemic infections at 1, 12 and 7 months of life, respectively.

CONCLUSIVE REMARKS

This is the first systematic study describing diarrhea in patients hospitalized in NICU in an industrialized country and outside outbreak conditions. The results of our investigation showed that, in this particular setting, diarrhea is a relatively uncommon but insidious condition underlying a broad spectrum of illnesses. The list of diseases and mechanisms responsible for diarrhea in neonatal age is large and the number of possible etiologies is higher if compared to the other pediatric ages (3, 32-34). In the recent years, new diseases have been described (i.e. enteric anendocrinosis), and more accurate knowledge about neonatal enteropathies has been obtained (23,35). The pathophysiology of these conditions is extremely variable and requires a complex diagnostic work up in order to rapidly adopt adequate therapy. We believe that all these steps should be performed providing a tight collaboration between neonatologist, pediatric gastroenterologist, immunologist, geneticist, and nutritionist. The most common etiology identified in our patients was food allergy (FA). Recently, the International Study of Asthma and Allergies in Childhood reported an increase of allergic disease prevalence occurring in the younger pediatric age group(17). The 20.5% of neonates in our population presented FA-related diarrhea, and none of these were adherent to the recommendations of the American Academy of Pediatrics for primary prevention of FA (18). These data suggest the importance of a high index of suspicion for FA in neonates presenting diarrhea, and of the application of preventive strategies (18, 36,37). The most frequent infective etiology was *Rotavirus that* represents the leading cause of infectious diarrhea in childhood (38). All patients acquired *Rotavirus* infection during hospitalization. Considering that *Rotavirus* is implicated in up to 50% of nosocomial

pediatric diarrheal episodes, our data further support the importance of an accurate surveillance against infection spreading in NICU (38).

In this study we reported a lack of a univocal rehydration and re-feeding strategy for hospitalized newborn with diarrhea indicating the necessity of further prospective studies to optimize the therapeutic approaches. The vast majority of newborn with diarrhea was rehydrated by enteral route and received enteral feeding within 4-6 hour, without complications. Despite it is difficult to assess the efficacy of rehydration and re-feeding strategies in patients with such different disorders, our results suggest that the therapeutic strategies for diarrhea that are commonly adopted in older subjects, could be successfully used also in newborn hospitalized in NICU. This is of particular importance because the available guidelines for diarrhea management are mainly focused on subjects of subsequent pediatric ages (4,39,40).

To conclude, despite the limit deriving from the retrospective design, our study showed that neonatal diarrheal diseases are challenging clinical conditions because of the heterogeneous etiologies and possible severe outcomes. We believe that this study will help neonatologists to prevent diarrhea from becoming a severe clinical condition, and to recognize and correctly take in charge the rare cases that are chronic and need the assistance of specialized team dedicated to their long-term treatment. Specific guidelines for the management of diarrheal disorders in the neonatal age are advocate.

SUMMARY. To investigate frequency, etiology, and current management strategies for diarrhea early in the life. *Methods*. Retrospective, nationwide study involving 5801 subjects observed in neonatal intensive care units during 3 years. *Results.* 39 cases of diarrhea (36 acute, 3 chronic) were identified. Occurrence rate of diarrhea was 6.72 per 1000 hospitalized newborn. Etiology was defined in 29 out of 39 newborns (74.3%): food allergy (20.5%), gastrointestinal infections (17.9%), antibiotic-associated diarrhea (12.8%), and congenital defects of ion transport (5.1%), withdrawal syndrome (5.1%), Hirschsprung's disease (2.5%), parenteral diarrhea (2.5%), cystic fibrosis (2.5%), and metabolic disorders (2.5%). Three patients died for complications related to diarrhea (7.7%). In 19 out of 39

patients (48.7%), the rehydration was performed exclusively by enteral route. *Conclusions.* Diarrhea in the neonatal age is a challenging clinical condition because the possible heterogeneous etiologies and severe outcomes. Specific guidelines are advocated in order to optimize management of diarrhea in this particular setting.

Future implications. Our study showed that neonatal diarrheal diseases are challenging clinical conditions due to their heterogeneous etiology and possible severe outcomes. The list of diseases and mechanisms responsible for diarrhea in neonates is larger, and the number of possible etiologies is higher compared with older pediatric patients. Specific guidelines for the management of diarrheal disorders in neonates are advocated. The research open the way to new investigation in the area of diarrheal diseases with onset in the neonatal age. We believe that these studies will help neonatologists to prevent diarrhea from becoming a severe clinical condition, and to recognize and correctly take in charge these patients. Assistance by specialized team dedicated to their long-term treatment is advocated.

Natural course of diarrhea associated with intestinal failure

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The majority of cases of chronic diarrhea occurring in early life are associated with intestinal failure. Intestinal failure in children can be defined as the critical reduction of functional intestinal mass necessary to ensure adequate digestion and absorption for body nutrient, fluid requirements, and growth (41). A diagnosis of intestinal failure is based on the presence of a primary intestinal disease that induces the need of prolonged or persistent parenteral nutrition (PN) (42). The intestinal failure can be transitory (short-term or protracted) or permanent (irreversible), depending on the underlying cause (43). The cause of intestinal failure includes 3 broad categories: (1) intestinal epithelial defects (ie, tufting enteropathies, immune-mediated enteropathies); (2) motility disorders; and (3) short bowel syndrome (SBS), of iatrogenic nature in many cases (44).The onset of intestinal failure is most often during the neonatal period. Prompt diagnosis, adequate medical and surgical treatment, and appropriate nutritional support are crucial to avoid complications and to improve the prognosis (45). Unfortunately, few studies have addressed neonatal SBS, as assessed the incidence, (46) prognostic factors,(47-49) surgical aspects, (50-51) prenatal diagnosis, (52) or nutritional aspects of intestinal failure (53). Information regarding the epidemiology, diagnostic tools available for prenatal and postnatal diagnosis, therapy, and natural history of neonatal onset intestinal failure are lacking. Current information is derived from single-institution case series and a small cohort of patients. Early identification of the risk factors for long-lasting dependence on PN is of critical importance for rapid and appropriate referral of children with intestinal failure to a coordinated interdisciplinary team for management (54). We aimed to investigate the epidemiology, diagnostic, and therapeutic approach and natural course of intestinal failure with neonatal onset through a large nationwide multicenter study.

STUDY DESIGN

A multicenter, nationwide, retrospective study was planned in collaboration with the Italian Society of Pediatric Gastroenterology Hepatology and Nutrition and with the Italian Society of Neonatology. Clinical charts of all newborns observed in Italian tertiary center neonatal intensive care units (NICU) during the years 2003 and 2004 were reviewed. Participating centers were selected on the basis of reported characteristics: ability to take care of high-risk pregnancy and of critically ill newborns (inborn and outborn), with direct contact with a neonatal surgery service and tertiary care centers for the management of intestinal failure. The diagnosis of intestinal failure was confirmed in all cases as a primary intestinal disease that induced the need of total PN for more than 4 weeks or the need of partial PN for more than 3 months (42). SBS was defined as residual small bowel length less than 25% of that predicted for gestational age after intestinal resection or the need for PN for more than 42 days after intestinal surgery (47,48). Newborns were considered eligible for the study when they fulfilled the following criteria: (1) gestational age >24 weeks; (2) postnatal age <28 days; (3) diagnosis of intestinal failure; and (4) parental consent. Data were recorded by neonatologists operating in participating centers in a specific reporting form and included in a national database expressed in the following sections: (1) demographic and clinical characteristics; (2) intestinal failure diagnostic, nutritional and therapeutic management; and (3) complications and clinical outcome. To establish intestinal failure occurrence rate, the number of live births and hospitalizations during the study period was also provided by each center. Newborns admitted to the NICU were considered as “high-risk infants.” Data sources included the NICU patient chart and hospital records. The primary and tertiary care physician and family of each subject with intestinal failure were contacted by phone to obtain information about the long-term outcome. Written informed consent was obtained by parents of each enrolled newborn. Ethical approval was conferred by the Institution Review Board of the coordinator center after submission of the study protocol.

Statistical Analysis

For continuous variables the Mann-Whitney test was used. For categorical variables the$\times^2$ test and Fisher's exact test were used. The level of significance for all statistical tests was 2-sided *P* 0.05. Linear regression analysis with stepwise method was used to study the possible influence of different variable on the cholestasis development. The Spearman rank test was used to define coefficient of correlation *(r).* Statistical analysis was performed by software SPSS (Version 14.0 for Windows, SPSS Inc, Chicago, Illinois).

RESULTS

The total number of live births in the 7 enrolled institutions was 30 353 newborns, and 5088 were admitted to the NICU. Twenty-six patients met the criteria for intestinal failure and were included in the study (gestational age: 32.0 weeks, IQR 27.7-35.2; birth weight: 1865.0 g, IQR: 867.5-2262.5). The occurrence rate of intestinal failure was 0.1% (1:1077) and 0.5% (1:196) considering all live-birth newborns and high-risk infants, respectively. Main demographic and clinical characteristics are listed in Table 5. The causes of intestinal failure are shown in the Figure 4. The length of follow-up was 34.5 months (IQR: 28.0-41.0). Diagnosis of the disease leading to intestinal failure was suspected prenatally in 70% of infants affected by congenital intestinal defects, on the basis of ultrasound pattern suggestive of intestinal obstruction. The most common neonatal diseases that led to intestinal failure were congenital intestinal defects, necrotizing enterocolitis, and intestinal motility disorders. Twenty-two patients underwent intestinal surgery; SBS developed in 16.All 3 patients who underwent resection of the ileocecal valve were weaned off PN. After a follow-up of 36 months, 84.6% of patients achieved intestinal competence, 1 patient (3.8%) was still receiving home PN while awaiting intestinal transplantation, 1 patient (3.8%) underwent transplantation, and 2 patients (7.7%) died (Table 6). The cause of death was respiratory failure in a patient with congenital neuromuscular disease (died at 11.8 months of age) and liver failure in a patient (43 months old), who underwent intestinal resection at birth for duodenal atresia. One patient with megacystis-microcolonhypoperistalsis syndrome died at 31.6 months of age from complication of intestinal transplantation. Among the infants weaned from PN, the median length of

intravenous nutrition was 55.5 days (IQR 34-85). Cholestatic liver disease was observed in 14 of 26 children. The linear regression analysis showed that development of cholestasis was not influenced by birth weight, sex, or gestational age, but only by the diagnosis of SBS (Beta 0.698, $P < .005$). Significant correlations between the development of cholestatic liver disease and duration of total PN (r =0.449, $P = .021$) was observed. However, the difference observed in the median duration of total PN between patients with (72.0 d; IQR 46.75) and without cholestatic liver disease (40.0 d; IQR 37) as complication of intestinal failure was not a statistically significant ($P = .63$). Twelve of 14 patients with cholestatic liver disease were treated with ursodeoxycholic acid. Cholestasis improved in all the patients but one.

CONCLUSIVE REMARKS

The average incidence of intestinal failure is a 1 in 200 NICU admission. Although improved technologies and expertise have led to better outcomes, complications of long-term PN such as progressive liver disease, sepsis, and loss of venous access still represent a major source of morbidity and death for PN-dependent infants. In this Italian cohort, the major underlying disorders leading to intestinal failure by congenital intestinal defects that require bowel surgery. Intestinal atresia and abdominal wall defects are more frequent in intestinal failure neonatal patients than NEC; this contrasts with older case series available in literature (49). The low incidence of NEC is due to advances in perinatal care (exogenous surfactant availability, extensive use of antenatal steroids, centralization of high-risk delivery at tertiary perinatal centers), despite a more prolonged survival of extremely premature infants in recent years. An important finding of this study is that in certain cases intestinal failure may be a transient condition. The longest duration of PN dependence was observed in the group with severe motility disorders and those with congenital disease of enterocyte development that commonly cause irreversible intestinal failure. An understanding of the factors linked to poor outcomes allows an estimate of the need for prolonged PN support and a multidisciplinary approach to the newborn with intestinal failure (50). Intestinal failure-associated liver disease represents the major complication of prolonged PN (51). Prolonged PN-associated cholestasis is the primary indication for

combined liver and intestinal transplantation in children and is responsible for significant mortality rates (52-54). Our data confirm the high prevalence of cholestasis among PN-treated infants. Treatment with ursodeoxycholic acid in our patients appeared to be of value; however, the retrospective design of the study and the relatively small number of treated subjects precludes definitive conclusion (55). A limitation is the retrospective design that reduced our ability to assess actual incidence of intestinal failure. The multicenter approach allowed the sharing of cases between national reference centers, thereby overcoming the limitation of small case series that affects rare diseases.

Summary. To describe the natural course of intestinal failure with onset in the neonatal period to provide data regarding the occurrence and to provide a population-based survey regarding the spectrum of underlying diseases. *Methods*. We performed a retrospective chart review including infants admitted to the neonatal intensive care unit of 7 Italian tertiary care centers. Intestinal failure was defined as a primary intestinal disease that induces the need of total parenteral nutrition (PN) for more than 4 weeks or the need of partial PN for more than 3 months. *Results.* The total number of live births during the study time within the enrolled institutions was 30 353, and the number of newborns admitted to the neonatal intensive care unit was 5088. Twenty-six patients satisfied the definition of intestinal failure; thus the occurrence rate of intestinal failure was 0.1% among live-birth newborns and 0.5% among infants at high risk. The main underlying diseases leading to intestinal failure in neonatal age were congenital intestinal defects (42.3%), necrotizing enterocolitis (30.8%), severe intestinal motility disorder (11.5%), intestinal obstruction (7.7%), structural enterocyte defects (3.8%), and meconium peritonitis (3.8%). After a follow-up of 36 months, 84.6% of patients achieved intestinal competence, 1 patient was still receiving home PN, 1 patient underwent transplantation, and 2 patients died. Cholestatic liver disease was diagnosed in 54% of observed children. *Conclusion.* An understanding of the incidence, causes, and natural history of intestinal failure would be helpful to appropriately allocate resources and to plan clinical trials

Future implications. For conditions such as intestinal failure, the development of nationwide databases would allow to advanced multidisciplinary support for the infants affected by intestinal failure. Interpreting in advance the natural course of intestinal failure occurring early in life may contribute to optimize treatment of the disease and prevention of complications.

Strategy for control of complications related to intestinal failure

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Providing a safe feeding approach and appropriate nutritional in the first phases of the life remain a challenging aim in neonatal care (57). A major limit of enteral nutrition in these subjects is represented by the concern for precipitating necrotizing enterocolitis (NEC) (58,59). Parenteral nutrition (PN) is essential to meet many of the nutritional needs of children with intestinal failure occuring early in the life. In particular, it is common in the clinical practice to consider total PN as primary mean of nourishing premature infants when they show the first signs of feeding intolerance in order to reduce the risk of developing NEC (58-63). However, the basis for this practice is largely undefined. Clinical manifestations of feeding intolerance may represent a physiological conditions related to a late maturity of gut motility typical of many preterm newborns (60-62). In addition, receiving nothing by enteral route (NBE) during feeding intolerance periods predisposes neonate to the consequences of starvation and a prolonged duration of PN increases the risk of infections (56-64). On the other hand, it has been demonstrated that also a small volume of enteral feeding has several advantages when compared with total PN including maintenance of intestinal barriers and decreased risk of infections in pediatric patients (65-71). Thus we hypothesize that minimal enteral feeding (MEF) instead of NBE may alleviate the side effect of parenteral nutrition in very low birth weight infants presenting feeding intolerance without increasing risk for NEC. The aim of this study was to investigate MEF efficacy and safety in VLBW infants presenting the first signs of feed intolerance.

STUDY DESIGN

A retrospective design using data reported in the clinical charts was adopted. Eligible patients were: 1) consecutively observed in Neonatal Intensive Care Units (NICU) from September 2001 to September 2003, 2) born with weigh <1500 g, 3) presenting at least one

episode of feed intolerance, defined by the presence of a gastric residual ≥ 3 ml/Kg associated with abdominal distension (increase of abdominal circumference ≥ 2 cm) for at least 2 consecutive feeds. All infants with the following conditions were excluded: 1) Apgar score <3 at 5 min; 2) congenital heart diseases or malformations; 3) critical clinical conditions, as indicated by a blood pH < 6.8, or by the presence of hypoxia with persistent bradicardia; 4) immunodeficiency; 5) incomplete clinical data report.

Feeding protocol during study period

Enteral feeding was started on the first day of life at 10 ml/Kg/day divided in 12 feeds, using preterm formula in all stable infants. Maternal unfortified-milk was administered whenever available starting from the 24th hour of life. Aspirate residual from orogastric tube and abdominal circumference, were measured before every feed. Total amount of gastric residual was calculated daily. The nutritional strategy changed during the 2 years of study period for the patients presenting feed intolerance: in the first year of the study period when subjects presented feeding intolerance they received only total PN and NBE for 24 hours, whenever in the next study year these patients received PN plus MEF (10 ml/Kg/day) for 24 h. This change in feeding protocol derived from the increased acceptance of MEF in neonatology clinical practice. All subjects were evaluated daily. The total amount of enteral nutrition was increased of 20 ml/Kg/day in the absence of feed intolerance in the previous 24 hours. In the presence of erythematic abdominal wall, absence of bowel sounds, or blood in the stools or in aspirates associated with radiological marker of NEC-Bell stage > I (58,59) enteral nutrition was discontinued during both years of study period. Parenteral nutrition was administered through a central vascular access in all subjects to maintain adequate fluid, electrolytes and nutrients intake, until full enteral feeding (120 Kcal/Kg/day) was reached. Fluids were started at 70-100 ml/kg/day and advanced by increments of 10-20 ml/Kg/day until 150-180 ml/kg/day.

Data collection and outcomes

Main demographic and clinical characteristics of the study population, together with the critical respiratory index for babies (CRIB), were recorded in a specific reporting form.

Enrolled patients were grouped on the basis of two different nutritional strategies: 1) total PN and NBE, 2) PN plus MEF, for at least 24 h. The efficacy outcome of the two feeding strategies was determined primarily by the time to reach full enteral feeding (at least 120 Kcal/Kg/day by oral route) and by the incidence of late-onset culture proven sepsis (positive blood culture obtained after 72 hours of life) (72), secondarily by the time to regain birth weight and the length of hospital stay according to standardized criteria (73). The safety of the two different feeding approaches was assessed determining the rate of subjects presenting NEC Bell stage > II (58) and the rate of infants death. The risk factors associated with NEC occurrence including time to start enteral feeding, assumption of breast milk, rate of infants with umbilical catheter, patent ductus arteriosus (PDA), intraventricular hemorrhages (IVH) and feeding intolerance characteristics (total gastric residual as a percentage of total daily feed, maximum gastric residual volume, number of episodes of feeding intolerance), were also collected. Clinical outcomes were systematically reviewed by two independent investigators who were blinded to study aims and patient's identity. Any disagreement in opinion between investigators was subjected to a further review, including a third investigator, and final decision was based on a consensus of opinion. The study protocol followed the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983 and it was approved by the University "Federico II" Ethic Committee.

Statistical analysis

Statistical analysis was performed by a statistician blind to individual feeding strategy adopted in the two groups of preterm infants. The chi-square test was applied for categorical variables. Because of the non-Gaussian distribution of continuous variables data were expressed as median and interquartile range (IQR) and analyzed with the Mann-Whitney U test. Binary logistic regression analysis was used to predict the presence or absence of NEC in each group based on values predictor variables: patient's gestational age, birth weight, sex, time to start enteral feeding, rate of infant with umbilical catheter, occurrence of PDA, IVH, intake of breast milk at 14^{th} day of life. Kaplan-Meyer method was used to estimate the probability of hospital discharge at 30 and 60 days in each study group, and the resulting

functions were compared with the log-rank test. Statistical analysis was performed with SPSS Version 13.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

Two hundred and forty-two clinical charts were reviewed. One hundred and twelve presented at least one episode of feed intolerance and were considered eligible for the study: 15 patients were excluded (8 cardiac or intestinal malformations, 7 incomplete clinical data) and 97 were analyzed. Forty-nine subjects out of 97 were classified in the Group 1 (Total PN and NBE), and 48 in the Group 2 (PN plus MEF). The study groups were comparable for birth weight, gestational age, sex and CRIB score (Table 7); and for variables that may influence NEC development (Table 8).

The amount of gastric residual was comparable in the 2 groups: the median total gastric residual, as a percentage of total daily feed volume, was 32% in the Group 1 and 34% in Group 2; the maximum median residual was 5.0 ml/Kg (IQR 4.0 ml/Kg) and 4.5 ml/Kg (IQR 3.0 ml/Kg) in Group 1 and 2, respectively. Rate of patients that presents at least two episodes of feeding intolerance was similar between the 2 groups (Table 9).

The neonates in Group 2 showed a shorter duration of central venous access and reached full enteral intake earlier (Table 9). A significant difference between the 2 groups was observed about the incidence of culture-proven late-onset sepsis (Table 9). Pathogens identified were: *Staphylococcus aureus* (20%), *Candida albicans* (29%), *Klebsiella pneumoniae* (38%), *Serratia marcescens* (12%), and *Proteus mirabilis* (10%). One patient in Group 1 (septic shock) and 2 in Group 2 (disseminated intravasal coagulation) died because of sepsis complications. A significant difference was observed between the 2 groups about the time to regained birth weight (Table 9). Finally, Kaplan-Meyer functions showed a significant difference in the time to reach hospital discharge at day 40, 50 and 60 of life (Figure 5).

The NEC (Bell stage > II) incidence observed was similar in the 2 groups (Table 9). One patient in Group 1 died because of severe NEC (stage IV) developed after 13 days of life. In the Group 2, one newborn experienced severe NEC (stage III) at day 18, but a prompt

surgical therapy (resection of terminal ileum and of ileo-cecal valve) resulted in symptoms resolution. Number of exitus was not significantly different between the 2 groups (Table 9).

CONCLUSIVE REMARKS

This is the first study investigating MEF utility in feed intolerant VLBW subjects. Our findings suggest that MEF could be an efficacious and safe strategy also for VLBW infants presenting feed intolerance. This nutritional approach could be able to reduce the incidence of sepsis without increasing risk of NEC and death. We report also evidences that MEF reduce the time of parenteral nutrition, promote regain of birth weight, and minimize duration of hospital stay when adopted in preterm presenting feeding intolerance. In the last years minimal enteral feeding (MEF) has gained a greater consensus in neonatology (70,71). It has been demonstrated that an early introduction of MEF is helpful in VLBW infants because their ability in promoting maturation of several intestinal and immune function (64,70,71). Additionally to previous evidences we demonstrate a new role of MEF as a safe nutritional strategy for feed intolerant VLBW infants.

Bloodstream infections are the most common severe complication of parenteral nutrition (74). Numerous strategies have been attempted to prevent the risk of PN-related sepsis with varying success (74,75). Prolonged duration of intravascular access for PN increases the risk of sepsis (76,77). In addition, it has been demonstrated that total PN directly impairs immune response to bacterial infections and that small volume of enteral feeding may reverse this effect (78,79). Thus we speculate that the MEF effects observed in our study could be related to the ability of small volume of enteral nutrition to reduce risks related to parenteral nutrition.

The NEC is one of the most common gastrointestinal emergencies in neonatology (80). Pathogenesis is still unproven, treatment is difficult, and no effective prevention strategy has been agreed (58,59). The disease is especially poignant because it mainly affects VLBW infants who have survived the early neonatal period and subsequently face a disease with high morbidity and mortality (59,80,81). A recent reports (82-89) and a Cochrane meta-analysis (90), including 9 randomized clinical trials on minimal enteral nutrition in

parenterally fed VLBW neonates, showed no convincing evidence for beneficial effects of MEF. However, this meta-analysis was not designed to verify the MEF effect on NEC occurrence (90). We showed a similar incidence of NEC in feed intolerant newborn receiving total PN or MEF. Considering that impairment of intestinal microflora composition has recently outlined in the development of NEC, we speculate that introduction of MEF in feed intolerant infants may contribute to the growth of a balanced intestinal microflora, which in turn could be protective for NEC (59,91). Additionally, we speculate that the exposure of the neonatal gut to intraluminal nutrients may promote maturation of intestinal motility, barrier function and immunity, thereby decreasing the incidence of PN-induced mucosal atrophy and of bacterial overgrowth and translocation (71,92-94). Further investigations are needed to address these interestingly hypothesis.

Because the mechanisms facilitating the development of NEC are not fully understood, the identification of preventive strategy has been impaired. Clinical evidences derived by our results suggest that suspension of enteral feeding on the basis of detection of first sign of feeding intolerance would represent a cumulative risk for sepsis and not a protective strategy versus NEC. Therefore, when making decision about suspending enteral nutrition in feed intolerant preterm babies it should be remembered that diet has an important role in intestinal development and systemic defense.

Finally, different gastric residual volume and abdominal circumference increase are usually adopted by neonatologists in order to predict NEC development (58,61,62). In accordance to previous report, our data suggest that a gastric residual volume up to 40% of total daily feed could be considered safe and did not represent an increasing risk for NEC development (63).

The MEF administration in feed intolerant VLBW infants results also in cost saving through the reduction in duration of hospital care. In our country the cost of hospitalization is estimated in about 750 Euro/day for VLBW infants. The difference in median duration of hospitalization between the two groups was 10 days, resulting in a saving of about 7500 euro per patient when MEF strategy was adopted in infant presenting signs of feed intolerance.

This study is limited because of its retrospective design. However, our data supporting the feasibility and efficacy of MEF administration in feed intolerant VLBW patients could open the way for future prospective trials.

Summary. To evaluate the efficacy and safety of minimal enteral feeding (MEF) nutritional practice in parenteral nourished infants. *Methods.* Retrospective design using data reported in the clinical charts including VLBW newborns consecutively observed in Neonatal Intensive Care Units (NICU) that presents feed intolerance. During study period two feeding strategies were adopted: total parenteral nutrition (PN) (Group 1) or PN plus MEF (Group 2), for at least 24 hours. Outcomes were the time to reach full enteral feeding, the occurrence of sepsis, the time to regain birth weight, the length of hospitalization, the occurrence of NEC Bell stage > II and death. *Results.* 97 newborns were enrolled: 49 in the Group 1, and 48 in the Group 2. Neonates in the Group 2 achieved full enteral nutrition earlier (8 days, IQR 5) compared with subjects receiving total PN (11 days, IQR 5, p< .0001). A reduction of sepsis episodes was observed in Group 2 (16.6%) compared to Group 1 (34.7%, p< 0.047). Additionally, subjects in the Group 2 regained their birth weight and were discharged earlier. The occurrence of NEC and death were similar in the two groups. *Conclusions.* MEF could be an efficacious and safe strategy for VLBW infants presenting feed intolerance, reducing sepsis without increasing risk of NEC and death.

Future implications. Mechanisms trough minimal enteral feeding produce benefical effects remain largely uninvestigated. Identification of nutrient that may contribute to growth and maturation of normal intestinal functions represent the main research frontier in this field.

Research Area 2.
New approaches for control of acute diarrhea in early life

Efficacy of new oral rehydration solution in acute gastroenteritis

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Acute diarrhea, a major cause of childhood morbidity, is also a source of anxiety to families of affected children, representing a heavy economic burden for families and for society as a whole. Oral rehydration solution (ORS) is the first-line therapy for the treatment of children with acute diarrhea worldwide (4,34,95,96). Currently available ORSs efficiently cure and prevent dehydration, but are unable to reduce the duration and the severity of diarrhea. Several substrates and substances that affect transepithelial fluid transport have been added to ORS to limit diarrhea duration and severity, and the costs deriving from this condition, but conclusive clinical data about their effect are scanty (4,97,98). Studies and meta-analyses indicate that zinc fortified ORS reduces diarrhea duration and severity in children with acute diarrhea (5, 99-104). Despite the evidence of benefit, there has been little progress on widespread introduction of low osmolarity ORS and zinc for treatment of acute diarrhea. In addition, most data came from studies of malnourished children living in developing countries (99-104). Thus, at present there is not sufficient evidence to recommend either in favor or against the addition of zinc to ORS in children living in developed countries. Despite this, there is a large use of several formulations of ORS containing such substances as zinc, prebiotics, probiotics, and glutamine on the market without clear evidence of their efficacy in children living in developed countries (104,105).The aim of this study was to investigate the efficacy of a new hypotonic ORS containing zinc plus fructooligosaccharides (FOS) and xilooligosaccharides in the treatment of children observed in the pediatric office for acute diarrhea.

STUDY DESIGN

We performed a prospective, randomized, single-blind controlled trial in collaboration with family pediatricians, who care for children up to 14 years of age in the Italian Public Health System. The study protocol was illustrated and discussed during 3 meetings. The study protocol was reviewed and approved by the ethics committee of the University Federico II of Naples. From November 2007 to March 2008, all children aged 3 to 36 months consecutively observed in pediatrician offices with diarrhea lasting <24 hours with mild-moderate dehydration were considered eligible for the study. Diarrhea was defined as >3 outputs of loose or liquid stools per day (7). At the enrollment, dehydration was assessed in each patient by using standardized criteria, as previously described. Exclusion criteria were: diarrhea lasting >24 hours; malnutrition as judged by a body weight/height ratio <5th percentile; clinical signs of severe dehydration; clinical signs of a coexisting severe acute systemic illness (meningitis, sepsis, pneumonia); immunodeficiency; underlying severe chronic disease; malnutrition; cystic fibrosis; food allergy or other chronic gastrointestinal diseases; endocrinopathy; use of prebiotics/probiotics in the previous 3 weeks; and use of antibiotics or any antidiarrheal medication in the previous 3 weeks. Informed consent was obtained from the parents of all enrolled children. Microbiologic and other laboratory investigations were performed only when required for specific clinical reasons. Enrolled patients were randomly allocated to standard hypotonic ORS (group 1) or super-hypotonic ORS containing zinc and prebiotics (group 2). We used two commercial ORS preparations available on the market as sachets, with similar cost and packaging. The composition of the two ORSs is reported in Table 10. The parents were instructed to rehydrate their children orally with ORS in 3 to 4 hours and then to administer ORS for dehydration prevention until cessation of symptoms, and re-feed their child with a normal appropriate-for-age diet including full strength lactose containing formula or cow's milk. To circumvent the problems in performing a blind study on commercially available products in a large population, we used the third-part blind observer method to assess the efficacy of the ORS preparations. Patients were allocated to each group according to a computer-generated randomization list. The researchers responsible for enrolling patients allocated the next

available number on entry in the trial. To maintain the concealed randomization procedure, each number of the randomization list corresponded to the number of a closed envelope containing a written prescription of the name of the ORS product and instructions about how it should be administered. The parents of enrolled children were instructed to record daily on a specific form: (1) time and the number of fecal outputs; (2) amount of daily ORS consumed by the child; (3) occurrence of adverse events; and (4) missed days work, hospital admission, and use of other medications. To ensure unbiased efficacy assessment, the investigators collecting the reporting forms completed by the parents were blind to the patients' treatment assignments, whereas the family pediatricians in charge of treatment allocation were excluded from efficacy assessment. We previously used this procedure in a study in children affected by acute diarrhea (7). The principal outcome measure of the study was the rate of resolution of diarrhea 72 hours after starting oral rehydration therapy. We selected this time point according to an earlier study that demonstrated an increased risk of dehydration during this period and an effective use of zinc in reducing diarrhea after the first 72 hours of treatment (106). The latter finding was recently confirmed in a Cochrane meta-analysis. Diarrhea was considered to have stopped after a patient had passed the last abnormal (loose or liquid) stool preceding a normal stool output, as applied in an earlier study (7). To obtain a power of the study of 80% (type 1 error = 0.05; 2-tailed test), considering a difference of 25% (75% versus 50%) in the rate of resolution of diarrhea at 72 hours between the study groups, 57 patients in each group were required. This estimation was based on our preliminary data and on earlier results obtained in children with acute diarrhea treated with zinc (106). We decided to enroll 65 patients per group, considering a possible dropout rate as high as 15%.

Statistical Analysis

A statistician blind to individual ORS preparations received performed statistical analysis by children in the two groups. Continuous variables were expressed as means plus or minus standard deviation. For categorical variables, the Pearson c2 test or Fisher exact test were performed as appropriated. The two groups were compared for continuous variables with the t test for equality of means. The Kaplan-Meier method was used to estimate the

probability of diarrhea at 72 hours in each study group, and the resulting functions were compared with the log-rank test. Analyses were conducted on an intention-to-treat and per-protocol basis. All tests of significance were two-sided. A P value <.05 was considered to be significant. The statistical analysis was performed by using the SPSS software package for Windows (release 16.0.0; SPSS Inc., Chicago, Illinois) and Stats Direct (release 2.6.6, Altrincham, United Kingdom).

RESULTS

Figure 6 shows the flow of children through the study; 65 children in each group were allocated to intervention. The baseline, demographic, and clinical characteristics were similar in the 2 groups (Table 11). Resolution of diarrhea at 72 hours was observed in 30 of 60 children in group 1 (50.0%) and in 43 of 59 children in group 2 (72.9%, P = .010; Figure 7). The number of daily outputs was significantly reduced in group 2 compared with group 1 at 24 hours (4.5; 95% confidence interval [CI], 3.89-5.11 versus 5.9; 95% CI, 5.28-6.63; P = .002), 48 hours (4.06; 95% CI, 3.46-4.66 versus 5.11; 95% CI, 4.29-5.94; P = .037), and 72 hours (2.88; 95% CI, 2.44-3.32 versus 3.89; 95% CI, 3.13-4.65; P = .020). The total ORS intake in the first 24 hours of rehydration therapy was significantly lower in group 1 (22 mL/kg; 95% CI, 17-29) than in group 2 (50 mL/Kg; 95% CI, 41-59; P < .001). The number of missed working days was significantly higher for parents of children enrolled in group 1 (1.45; 95% CI, 1.02-1.88 versus 0.39; 95% CI, 0.08-0.70; P < .001). The rate of parents who missed at least one working day was significantly higher in group 1 (51.7% versus 15.3%, P < .001). The rate of patients requiring hospitalization because of worsening of symptoms was similar in the 2 groups (5.0% versus 1.7%). Adjunctive medications within the first 72 hours were not used by any patients in the two groups, whereas after the first 72 hours additional treatments were used by 19 of 60 patients of group 1 and by 6 of 59 patients of group 2 (P = .004). In particular, the medications used were probiotics (n = 12), diosmectite (n = 4), racecadotril (n = 2), in group 1, and probiotics (n = 4), and domperidone ((n = 2) in group 2.). No adverse events related to the use of the ORS were observed in the study groups.

CONCLUSIVE REMARKS

In this trial, we investigated the therapeutic efficacy of a new commercially available hypotonic ORS containing zinc and prebiotics in the treatment of acute diarrhea in children. The positive clinical effect exerted by this new ORS on diarrhea could be related to a synergistic effect between prebiotics and zinc. Prebiotics have been proposed for the prevention and treatment of acute diarrhea, but efficacy data of FOS and xilooligosaccharides in the treatment of acute diarrhea are still scant and conflicting (4,107-114). Many of the effects attributed to prebiotics are related to the consequences of their use on gut microbiota composition. The ability to target specific groups of organisms (ie, bifidobacteria) in the large intestine by prebiotics is increasingly seen as being of significant health value. Many studies have established that prebiotics increase bifidobacteria numbers in infant stool to levels comparable with breast-fed infants (106-114). Several investigations have demonstrated an increased sIgA response resulting from the use of prebiotics (110,111). However, trials on diarrhea have been essentially limited to FOS, and all except one have been carried out in animals. The exception is a promising study involving 244 people at increased risk of acquiring traveler's diarrhea. This investigation showed that travelers who received FOS had a reduced incidence of diarrheal events compared with the placebo group, although the reduction was not significant (113). A large body of evidence supports the use of zinc in the treatment of acute diarrhea, and the mechanisms of action of zinc are becoming clearer (5,104,116-118). Zinc is now included in the World Health Organization essential medicine list for diarrhea treatment, and in the 2008 Copenhagen Consensus, a group of leading global economists ranked zinc supplementation as the most effective intervention for advancing human development (98,119). Clinical trials, reviews, and metaanalyses have demonstrated that zinc reduces diarrhea duration, stool output, and stool frequency. In particular, a Cochrane meta-analysis demonstrated that zinc is effective in reducing the duration of diarrhea at 72 hours (5). This coincides with our finding that significantly fewer children who were treated with zinc-containing ORS had diarrhea 72 hours after symptom onset versus the group treated with standard hypotonic ORS. Although most studies reported positive effects elicited by zinc in the treatment of childhood acute diarrhea, some negative results have recently been

published (120). This discrepancy could be caused by such factors as nutritional status, zinc status, or both, age, race, sex, (121,122) and the causative pathogen (123,124). Notwithstanding the discrepancy, zinc is widely used in the treatment of acute diarrhea in developing countries, where it is responsible for saving >400 000 lives a year (121). Moreover, a universal zinc containing super-ORS has been proposed by various authors (125). These results will hopefully stimulate further investigation. Zinc supplementation induces a therapeutic effect by stimulating water and electrolyte absorption across the intestinal mucosa, thereby preventing villous atrophy and improving overall immunity (126-128). We previously demonstrated that zinc induces a pro-absorptive effect on ion transport in basal condition and inhibits the main intracellular pathways of intestinal ion secretion that are involved in acute diarrhea by directly interacting with enterocytes. We have demonstrated that zinc affects ion transport when used at concentrations (10-22 mmol/L) that are within normal plasmatic ranges and very similar to the plasma concentrations reported in clinical studies in patients with diarrhea treated with zinc. The ‘‘super-ORS’’ used in this study contains a zinc concentration of 3.75 mg/100 mL. This concentration compares well with the United Nations Children Fund and World Health Organization recommendations for the use of zinc as a universal treatment of children with acute diarrhea, namely 10 to 20 mg zinc daily. The mean intake of 49.7 mL/kg corresponds to an average daily intake between 10 and 20 mg. The positive therapeutic effects of this new ‘‘super-ORS’’containing zinc and prebiotics are probably responsible for the reduction of drug use and parental work days missed. The Italian Society of Pediatric Gastroenterology Hepatology and Nutrition estimated an average cost of approximately 137.000 per episode of acute diarrhea in ambulatory children aged <3 years, mostly related to drugs and to loss of work days of parents (33). In this light, the use of this new ‘‘super-ORS’’ could be responsible for a substantial reduction of the cost related to acute diarrhea. The results of our trial suggest that a new hypotonic ORS containing zinc and prebiotics is useful in the treatment of ambulatory children with acute diarrhea living in a developed country.

Summary. To evaluate the efficacy of a hypotonic oral rehydration solution (ORS) containing zinc and prebiotics for treatment of acute diarrhea in early in the life. *Methods*.

We conducted a single-blind, prospective, controlled trial including children (age range, 3-36 months) with acute diarrhea randomly assigned to standard hypotonic ORS (group 1) or to new hypotonic ORS containing zinc and prebiotics (group 2). The main outcome was the rate of resolution of diarrhea at 72 hours.

Results. A total of 60 children in group 1 (34male; mean age, 18.58months; 95%confidence interval [CI], 15.5-21.6) and 59 in group 2 (36 male; mean age, 19.26 months; 95% CI, 15.9-22.6) completed the study protocol. The rate of diarrhea resolution at 72 hours was higher in group 2 (50% versus 72.9%, P = .010). Total ORS intake in the first 24 hours was higher in group 2 (50 mL/kg; 95% CI, 41-59 versus 22 mL/kg; 95% CI, 17-29; P < .001). The mean number of missed working days by the parents of children in group 2 was lower (0.39; 95% CI, 0.08-0.70 versus 1.45; 95% CI 1.02-1.88; P < .001). Fewer patients in group 2 needed adjunctive drugs for the treatment of diarrhea 6/59 versus 19/60, P = .004. No adverse events were observed in either of the two groups.

Conclusion. The addition of zinc and prebiotics to ORS limits diarrhea duration in children.

Future implications. It is well known that ORS is largely underused because they are not perceived as being effective against diarrhea. The addition of zinc would not only enhance ORS efficacy, but also increase its use by introducing an active component capable of reducing water loss, rather than relying on components that merely replace fluid loss, as with standard ORS. This aspect of adding zinc to ORS could be even more important than its ion proabsorbative/antisecretory effects. We believe that the use of zinc as adjunctive therapy has the potential to improve the management of diarrhea and increase survival in children. It has been estimated that the successful implementation of the UNICEF/WHO recommendations on zinc use in the treatment of diarrhea could save nearly 400,000 lives annually. Zinc may also improve child health in the public health setting (ie, growth and development, respiratory infections, and malaria). There are several reasons to establish a unique universal ORS, and the composition of ORS has undergone many changes in the last 30 years. Perhaps the time has come to consider adding zinc to a new universal ORS.

Efficacy of different probiotic formulations

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The search for agents that reduce diarrhea severity and duration started over 20 years ago (129). Probiotics, defined as micro-organisms that exert beneficial effects on human health when they colonize the bowel, have been proposed as adjunctive therapy in the treatment of acute gastroenteritis (130). A number of micro-organisms have proven effective in reducing the severity and duration of acute gastroenteritis in children: Lactobacillus rhamnosus (formerly "Lactobacillus casei strain GG" or "Lactobacillus GG"), Lactobacillus plantarum, several Bifidobacteria strains, Enterococcus faecium SF 68, the yeast Saccharomyces boulardii and preparations containing a mix of strains (130-135). Several trials with probiotic preparations have been conducted in different settings and with different endpoints. Meta-analyses of probiotic efficacy, including a Cochrane review, are also available (136-138). However, very few of these studies meet the criteria of properly controlled trials (36). Probiotics were the most frequently prescribed medication in a recent study of Italian children with diarrhea (33). With the increasing availability and widespread use of probiotics, it is important to identify the most effective preparations. In the current study we evaluated the efficacy of 5 probiotic preparations in the treatment of acute diarrhea in children.

STUDY DESIGN

The study was performed in collaboration with family pediatricians, who in the Italian Public Health System care for children up to 12 years of age. The study design was discussed in 3 meetings with 6 family pediatricians. Diarrhea was defined as 3 or more outputs of loose or liquid stools/day. Children 3–36 months of age seen in pediatricians' offices from October 1999 to September 2000 because of diarrhea were eligible for the study. Patients presenting diarrhea lasting less than 48 hours with informed consent gives

from parents were included in the study. Exclusion criteria were: (a) malnutrition as judged by body weight/height ratio, (b) clinical signs of severe dehydration, (c) clinical signs of a coexisting acute systemic illness (meningitis, sepsis, pneumonia), (d) immunodeficiency, (e) underlying severe chronic diseases, (f) cystic fibrosis, (g) food allergy or other chronic gastrointestinal diseases, (h) use of probiotics in the previous 3 weeks, (i) use of antibiotics or any antidiarrheal medication in the previous 3 weeks and during study medication, (e) poor compliance (defined by administration of at least four doses of study medication).

All children were rehydrated orally with 60 mMol Na+ ORS for 3–6 hours and then reefed with full strength, lactose-containing formula or cow's milk, depending on age (for guidelines, see references 96,139-140). Microbiologic investigation was performed only if required for specific clinical reasons. In addition to the above supportive treatment, children were randomized to the following groups: 1. ORS alone; 2. Lactobacillus GG; 3. Saccharomyces boulardii; 4. Bacillus clausii; 5. mix of Lactobacillus Delbrueckii var. bulgaricus + Streptococcus thermophilus + Lactobacillus acidophilus + Bifidobacterium bifidum; 6. Enterococcus faecium strain SF68. Probiotic preparations were prescribed for 5 days and administered by the oral route suspended in 20 ml of water according to the manufacturers' indications. The composition and administration schedules of probiotic preparations included in the trial are listed in Table 12. A group of children receiving only ORS served as controls.

Patients were allocated to each group according to a computer-generated randomization list. Random allocation was made in blocks of six to obtain groups of similar size. The sequence was concealed until treatments were assigned. The researchers responsible for enrolling the patients allocated the next available number on entry into the trial, and the parent of each child received a written prescription to use a specific product.

The primary outcome measures were the duration of diarrhea and its severity. Secondary measures were the duration of vomiting and of fever, and hospital admission. Safety and tolerability were also investigated.

The study was performed according to a multicenter, single-blind, and controlled design. Due to the problems of performing a double-blind study of commercially available products in a large population, we used the third-part blind observer method to assess the efficacy of

the probiotic preparations. To ensure unbiased efficacy assessment, the investigators collecting the reporting forms were blinded to the patient's treatment assignment, whereas the researchers in charge of treatment allocation were excluded from efficacy assessments. The reporting forms were delivered to the coordinating Center at the Department of Pediatrics for analysis.

A clinical history and physical examination was completed upon enrolment to identify the patient, to determine the duration and severity of diarrhea, to assess associated clinical features (fever, vomiting, dehydration) and to establish nutritional status and previous therapy. The parents of enrolled children were instructed to record daily on a specific form, the number of faucal outputs and their consistency, and the daily doses of the probiotic preparation assumed by the child. This procedure was applied in previous studies of the efficacy of anti-diarrheal treatments (141,142). Informed consent was obtained from the parents of all enrolled children. The study protocol and consent form were approved by the Ethics Committee of our Institution.

Outcome measures were divided into primary and secondary. The former were total duration of diarrhea, number of stools per day and their consistency. Diarrhea duration was defined as the time in hours from the first to the last abnormal (loose or liquid) stools preceding a normal stool output. Stool consistency was evaluated through a score system, as previously described (141), and faces were graded as 1 (normal), 2 (loose), 3 (semi liquid) and 4 (liquid). Secondary outcome measures were the incidence and median duration of vomiting, fever, defined as body temperature above 37.5°C, and the number of hospital admissions in each group. Safety and tolerability were also investigated. Any pharmaceutical company did not fund the research.

Estimate of sample size

Forty-five patients in each group were required to obtain a power of the study = 95%, type 1 error = 0.05, 2-tailed test. This estimate assumes that the mean difference in duration of diarrhea is 24 h between treated and control children (corresponding to means of 120 h versus 96 h) with a within-group standard deviation of 30 h. This computation was based on the results of a preliminary open trial (141). To investigate the secondary outcome

parameters, we doubled the number of patients. Sample size estimation included a drop-out as high as 10%.

Statistical analysis

Statistical analysis was performed by a statistician blind to individual probiotic preparations received by the groups of children, as well as to the control group. The chi-square test was applied for categorical variables. For continuous variables, differences between the 6 groups were analyzed by the Kruskal-Wallis H test. All analyses were conducted on an intention-to-treat (ITT) basis. Statistical analysis was performed with SPSS version 15.0.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

Figure 8 shows the flow of children through the study. A total of 600 children with acute gastroenteritis were considered eligible for the study: 29 were excluded, 571 were randomized to receive intervention. Data from 571 subjects were considered for the intention-to-treat analysis.

The baseline features of the patients enrolled in each group were similar (Table 13). The total duration of diarrhea was significantly lower in children receiving Lactobacillus GG (group 2) and in those receiving the bacterial strain mix (group 5) than in patients receiving ORS alone (group 1) (Table 14). The other three probiotic preparations had no effect on diarrhea, and the duration of diarrhea in groups 3, 4 and 6 was similar to that in the group receiving only ORS (Table 14). Daily stool outputs were significantly lower ($p < 0.001$) in groups 2 and 5 (Table 15), and a reduction occurred the day after the first probiotic administration. Median stool outputs/day did not differ between groups 2 and 5 (Table 4). Stool consistency, as judged by the scoring system, differed significantly ($p<0.001$) between preparations 2 and 5 versus the other groups (Table 16). The median daily scores did not differ between groups 2 and 5 (Table 16). Microbiological investigations were requested in only few instances and the results did not provide useful information.

None of the secondary outcome measures evaluated in this study was significantly modified in children receiving probiotic preparations or in the control group (Table 17). The probiotic

preparations included in the study were well received by the vast majority of the children, and no adverse event was observed.

CONCLUSIVE REMARKS

Acute infectious gastroenteritis is still a major cause of childhood morbidity. Moreover, it is a source of anxiety to families of affected children, and represents a heavy economic burden for families and for society as a whole (143,144). Drugs that affect intestinal motility, ion transport and adsorptive moieties, and living bacteria have been used in the attempt to reduce diarrhea duration (144,145). Probiotics have progressively gained in credibility for the treatment of diarrheal diseases (146,147). However, in most countries, micro-organisms claiming probiotic properties are considered food additives, rather than drugs. Consequently, only safety features, and not proof of efficacy, are required for marketing (148). In addition, the term "probiotic" is often improperly used and information about specific probiotic properties of the strains contained in the products is not exhaustive (149,150). We did not conduct a qualitative and quantitative study of the microbial content of the probiotic preparations in this trial because our aim was to carry out a field trial of the clinical effectiveness of commercially available probiotic products.

In the only comparative trial reported previously, 3 preparations were tested in 46 children (151). We evaluated the efficacy of 5 widely used preparations in 554 children, and found substantial differences in their efficacy. Two preparations reduced diarrhea duration and its severity, evaluated based on the number and consistency of stool outputs, whereas the other 3 had no significant effect. Recent Cochrane meta-analysis, including 23 randomized controlled trials, found mild therapeutic benefit from probiotics that was generally reproducible regardless of organism (138). On the contrary, our trial studied in depth this issue demonstrating a clear therapeutic benefit only for selected probiotic preparations in the treatment of acute gastroenteritis.

Lactobacillus GG was associated with a shorter diarrhea duration, which was not unexpected because proof of efficacy of this strain has been obtained in various settings, including hospitalized and outpatient children in both industrialized and developing

countries (130,141,152,153). The results that we obtained with Lactobacillus GG closely resembled those obtained in a similar setting with the same strain (141). The other effective preparation was a mix of 4 strains. Ours is the first trial with this preparation. However, a formula with Streptococcus thermophilus and Bifidobacterium bifidum, 2 of the 4 bacterial species in the effective preparation, protected against diarrhea in a ward with chronically sick children below 24 months of age (133).

The other 3 preparations evaluated in our study had no or little clinical effect. This was unexpected in the case of Saccharomyces boulardii, because in a controlled, open trial, it was beneficial in children hospitalized for diarrhea (155), i.e., with a more severe condition than the mild to moderate diarrhea in the children in our trial, which could explain the different results obtained in the two studies. A previous trial with Streptococcus faecium strain SF68 resulted in clinical improvement in children with diarrhea associated with respiratory infection and treated with parenteral antibiotics (156). However, it had no effect in adults with diarrhea (157). Finally, the Bacillus clausii preparation had no effect. To our knowledge, ours is the first trial with this preparation. None of the preparations had a significant effect on secondary outcomes. This was probably due to the relatively low incidence of fever, vomiting and hospital admissions in our population of children with mild to moderate gastroenteritis. No side effects were recorded.

Diarrhea in developed countries is usually self-limiting and active treatment is not generally recommended. However, over-the-counter drugs or preparations are widely used to treat children with gastroenteritis, and are often self-prescribed. We did not consider the etiology of diarrhea. However, probiotics are generally prescribed without a specific etiology indication. All the children enrolled in our study were outpatients and microbiological investigations were performed only in a small number of subjects. Based on the findings of a recent large study conducted in Italy (33), it is reasonable to assume that most of the children were affected by viral gastroenteritis.

In conclusion, the efficacy of probiotic preparations for the treatment of childhood gastroenteritis is related to individual bacterial strains. We believe that probiotic preparations should be considered drugs and physicians should select preparations for which evidence of efficacy, in a given clinical condition, is supported by solid data.

Summary. Probiotics are increasingly used adjunctive to oral rehydration solution to treat childhood gastroenteritis. Numerous preparations are available, but clinical efficacy has not been proven for many of them. We aimed to investigate the efficacy of 5 probiotic preparations in the treatment of acute gastroenteritis in children. *Methods***.** We conducted a 12-month randomized controlled clinical trial in collaboration with family pediatricians. Children requiring an office visit for acute gastroenteritis were randomly assigned to one of the following treatment groups: 1. oral rehydration solution (control group); 2. Lactobacillus rhamnosus strain GG; 3. Saccharomyces boulardii; 4. Bacillus clausii; 5. mix of Lactobacillus delbrueckii var. bulgaricus + Streptococcus thermophilus + Lactobacillus acidophilus + Bifidobacterium bifidum; and 6. Enterococcus faecium SF68. Primary outcomes were diarrhea duration, and daily stool number and consistency. Secondary outcomes were vomiting and fever duration, and hospitalization rate. Safety and tolerability were also recorded. *Results.* 571 children were allocated to intervention. Median diarrhea duration was significantly shorter ($p<0.001$) in children receiving Lactobacillus GG (78 hours) and the mix of 4 bacterial strains (69 hours) than in children receiving oral rehydration solution alone (115 hours). One day after the first probiotic administration, the daily stool number was significantly lower ($p<0.001$) in children receiving Lactobacillus GG and in those receiving the probiotic mix than in the other groups. The remaining preparations did not affect primary outcomes. Secondary outcomes were similar in all groups. *Conclusions.* Not all commercially available probiotic preparations are effective in children with gastroenteritis. Pediatricians should choose bacterial preparations based on efficacy data.

Future implications. The concept of beneficial interaction (symbiosis) between the intestinal mucosa and the endogenous microflora is now firmly established. Any disturbances in endogenous (host control mechanisms that are crucial for homeostatic and symbiotic interaction) or exogenous (composition of the microflora during the colonization process or later in life) factors may cause acute or chronic disorders. It is therefore logical to develop new treatment strategies aimed at modifying the intestinal microflora. Those

modifications may enable re-equilibration of intestinal flora, for instance in response to a pathogen, or after antibiotics treatment. However, changes in the intestinal microflora may also be sensed by the intestinal mucosal immune system and give rise to specific or nonspecific changes in endogenous inflammatory and immune responses. Probiotics are an excellent tool with which to achieve controlled modification of the intestinal microflora. In all studies on probiotics it is important to define the specific clinical setting (prevention vs. treatment) and disease (infectious, immunoallergic, inflammatory, NEC) clearly. In this context, it seems crucial to analyze the effect of a specific strain on a specific indication, rather than to simplify the concept by considering probiotics as a whole and as a multipurpose response to a variety of disorders or diseases. At present, the knowledge that would enable selection of a specific strain for a specific condition remains fragmentary. Various bifidobacteria, lactobacilli, and saccharomyces may be promising probiotics in certain clinical contexts. The optimum dosages for specific probiotic interventions and the optimum durations of treatment have yet to be defined. Therefore, many well-designed comparative or placebo-controlled studies are required before clear recommendations can be formulated. Given the particular microbial nature of probiotics, various delivery systems may be envisaged, such as tablets or food additives, thus adding a completely different dimension to the use of probiotics. Finally, given the fact that probiotics are living microorganisms, particular quality standards are mandatory to ensure a safe and completely harmless approach.

Research Area 3.
Diagnosis and treatment of chronic diarrhea early in life

Genotype of children with congenital diarrhea

Results of this research were published on J Pediatr Gastroenterol Nutr 2010;50:360-66

Among different form of early onset chronic diarrhea the PhD project was focused on congenital chloride diarrhea, because of this condition could be considered a research model useful for other forms of early onset diarrhea (Table 17 a-d).

Congenital chloride diarrhea (CLD, OMIM 214700) is caused by mutations in the gene encoding the solute-linked carrier family 26 member A3 (SLC26A3) protein, which acts as a plasma membrane anion exchanger for Cl^- and HCO_3^- (3,10). The main clinical symptom is lifelong watery diarrhea with high Cl^- content and low pH, which causes dehydration and hypochloremic metabolic alkalosis (3,10). CLD may be fatal if not adequately treated. Long-term prognosis is generally favorable, but complications such as renal disease, hyperuricemia, inguinal hernias, spermatoceles and sub-fertility are possible. The clinical picture of CLD varies from individual to individual. The SLC26A3 gene maps on chromosome 7, in region q31, close to the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, and spans about 38 kb including 21 exons (3,10). In ethnic groups where the disease is frequent, there is a single mutation: in Finns, the p.V317del mutation affects up to 90% of CLD alleles; in Saudi Arabia and Kuwait, p.G187X is present in more than 90% of altered chromosomes; in Poland 50% of CLD alleles carry the I675-676ins mutation (official nomenclature: c.2022_2024dup – p.I675dup). Differently, a wide genetic heterogeneity was found in about 100 CLD patients from ethnic groups where the disease is sporadic (10). About 30 mutations have been identified so far and they involve a large number of exons and several introns of the SLC26A3 gene. In addition, various types of mutations have been reported, namely, point mutations (nonsense, frameshift and missense)

and small and large gene rearrangements. There are no reports on causing-disease mutations in promoter or enhancer regions.

Before our studies the mechanism by which these mutations undermine function was largely unknown. The C-terminal conserved domain called 'STAS' has various functions. This domain ensures the correct location of the SLC26A3 protein on the apical membrane of enterocytes. In addition, it interacts with the R-domain of the CFTR gene. Mutations in the STAS domain cause CLD by reducing the levels of the protein at the plasma membrane by at least two distinct mechanisms, both of which result in transporter miss-trafficking and cytosol retention. Mutations p.I675dup and p.G702TfsX10 cause the STAS domain to misfold so that the mutant transporters cannot reach the native state. In contrast, mutations p.Y526_527del and p.I544N probably disrupt important intra-molecular interactions that are critical for the formation of well folded, functional transporters. Moreover, these mutations may affect other intermolecular interactions critical for correct folding. These aspects may have important diagnostic and therapeutic implications.

STUDY DESIGN

Network for congenital diarrhea identification and treatment

Considering the low incidences of CLD we have founded an international network named European Consortium for the Study of Congenital Diarrheal Diseases that has been crucial for the enrolment of the patients with this extremely rare disease. The research involved the following centers: Dipartimento di Pediatria Università degli Studi di Napoli Federico II; Unità di Pediatria Università di Milano; Unità di Neonatologia Università di Cagliari; Pediatric Gastroenterology, Hepatology and Nutrition Hôpital Necker-Enfants Malades, Paris; Children's Hospital Medical Center, University of Bonn; Department of Pediatrics at UCLA School of Medicine, Los Angeles; James Whitcomb Riley Hospital for Children Indiana University School of Medicine, Indianapolis, IME; Division of Pediatric Gastroenterology, Rhode Island Hospital, Providence, RI; Section of Gastroenterology, University of Chicago Comer Children's Hospital, Chicago, IL, USA; Department of Pediatrics Medical University, Innsbruck, Austria; Department of Pediatrics Academic Medical Centre University of Amsterdam, Holland; Hospital for Children and Adolescents,

University of Helsinki, Finland; Department of Gastroenterology, Rigshospitalet, Copenhagen, Denmark; Riyadh Military Hospital Riyadh Kingdom of Saudi Arabia. From the September 2007 the European Consortium for the Study of Congenital Diarrheal Diseases has observed 34 children with CLD. We enrolled in this study 24 subjects. In 20 out of 24 enrolled subjects diagnosis was confirmed by molecular analysis, as described below.

Molecular analysis

In all children with clinical diagnosis of CLD a molecular analysis of whole coding region of SLC26A3 was performed as described (158). DNA was extracted from an EDTA blood sample using the Nucleon BACC2 kit (Amersham Biosciences, Piscataway, NJ, USA). We used primers previously described. The touchdown PCR protocol that enables all exons to be coamplified under the same PCR conditions is available on request. Sequencing analysis was performed on both strands with an automated procedure using the 3100 Genetic Analyzer (Applied Biosystem). All PCR fragments were sequenced using the same primers used for PCR amplification. We screened exons 12, 13, 14, 15 and 17 (where point mutations had been identified in patients 1, 3 and 6) in 100 unrelated healthy control subjects using D-HPLC and the WAVE Nucleic Acid Fragment Analysis System 3500 (Transgenomic, Omaha, NE, USA). PCR and DHPLC protocols are available on request. We used the Expand Long Template PCR System (Roche Molecular Biochemicals, Mannheim, Germany) to verify deletion extension in patient II. We used the forward primer of exon 17 and the reverse primer of exon 19, both known to be intact in exon-specific assays. The expected fragment is about 6300 bp. The PCR conditions are available on request. The walking analysis to identify the breakpoints was performed on both strands and directions using the automated sequencing system 3100 Genetic Analyzer, starting from exons 17 and 19. We aligned the sequences of the SLC26A3 proteins in *Homo sapiens* (NP_000102), *Pan Troglodytes* (XP_527858), *Rattus norvegicus* (NP_446207), *Mus musculus* (NP_067328), *Canis familiaris* (XP_540380), *Gallus gallus* (XP_415945), *Oryctolagus cuniculus* (AAK00897), *Xenopus laevis* (AAO44922), *Caenorhabditis elegans*

(CAE75275) and *Drosophila melanogaster* (NP_649024) using the MegAlign 4.00 software (DNASTAR Inc., Madison, WI, USA). This software is based on the Clustal V algorithm.

RESULTS

We identified 34 children with clinical diagnosis of CLD. Table 18 describes mutations of 24 out of 34 children with CLD that accept to participate in this study. We identify 12 new mutations on the gene SLC26A3. The scanning of the whole coding region of the SCL26A3 gene did not reveal any other gene variant. The DHPLC analysis of 100 control subjects (200 alleles) did not identify any of the mutations.

CONCLUSIVE REMARKS

Our study confirmed the genetic heterogeneity of sporadic CLD (159). Unlike other autosomal recessive disorders, in which a few mutations are identified in a high percentage of affected chromosomes, sporadic CLD is due to a large number of mutations spreading all over the gene (159). Among the mutations identified in the present study 12 are novel. These mutations involve different exons. Thus, molecular diagnosis in sporadic CLD patients should be based on scanning techniques of all coding regions of SCL26A3 gene, as the direct sequencing used in this study.

Several mutations identified in the present study clearly affect the synthesis of the protein. The large deletion of 1752 bp excludes the whole exon 18 (55 bp), and creates a frameshift (p.I670MfsX17). The novel deletion involves the nucleotides encoding “STAS-like” and “PDZ” domains thus, no residual function is associated to the truncated protein (160-162). There is only another report describing a large deletion of SLC26A3 gene in two Japanese siblings bearing a 3502-bp homozygote deletion involving exons 7 and 8 (163,164). In our cases, one of the breakpoints (c.2061+1546) lies within Alu sequence, as previously described (163,164). The G187X nonsense mutation gives rise to an early truncated protein with no residual activity. This mutation is present in most CLD patients from Saudi Arabia and Kuwait, one of the three ethnic groups where CLD is frequent due to founder effect (159) and to the high rate of consanguineous marriage. The novel c.1758delG, identified in homozygosis, affects a CG dinucleotide, creating a frameshift and an early stop codon

(p.L586FfsX4) that interrupts the protein at the variable loop of the "STAS-like" domain. The c.614delT gives rise to an early truncated protein at level of the 5th transmembrane helix (p.L205RfsX28), so none residual activity of the protein was present.

All missense mutations identified in this study are like to be disease-causing since: i) these mutations have not been identified in 200 normal alleles; ii) for all patients, each of the parents carried in heterozygosis one of the mutations identified in the proband; iii) no other mutations were identified in none of the patients, with the exception of patient 3 that had a complex CLD genotype (see below); iv) the involved aminoacids are highly conserved. Mutations p.Q495H and p.A547E lie within the mutation-bearing hotspots characteristic of most sporadic CLD cases (159). The mutation p.Q495H could prevent the correct folding of the last transmembrane domain of the protein, altering the charge distribution. Otherwise, the mutation could alter the sulphate transporter family domain (159). The mutation p.A547E also involves the first sheet of the "STAS-like". Glutammic acid is a strong alpha-sheet interrupter and probably also induces a strong charge distribution change. Indeed, mutations involving the "STAS-like" domain have been recently reported to be associated to the absence of any residual activity of the protein (160-162). One patient bears a very complex genotype. Novel mutation p.S654P and a putative gene variant i.e., p.T510M, are present both in homozygosis in one patient. Both the mutations involve highly conserved amino acids, and the first one involves an amino acid bearing to the "STAS-like", sheet structured, conserved loop. We suggest it could be the disease-causing mutation, since the Proline is a strong sheet interrupter and mutations affecting amino acids within the "STAS-like" domain have a high chance to be causative of the disease (160-162). Moreover, the p.S654P mutation involves a palindromic sequence (c.GACTTT**T**CAG -> c.GACTTT**C**CAG), which could be more susceptible to mutations. On the contrary, the p.T510M mutation lies in a tract of the protein lacking of a specific function, and could be a gene variant. The mutation p.S438P (patient n. 6), involves Serine 438, the first amino acid of the 11th transmembrane helix and Proline is a strong interrupter of helices. Otherwise, the mutation could alter the sulphate transporter family domain (159). Also in this case, the mutation falls in the hotspot region (159). The c.1234-11dupT, involves a poly T sequence. It is like that this variant does not affect protein synthesis, even if sequence contraction of

the poly T locus in Cystic Fibrosis (165) induces the exon-skipping, but not the 7T to 9T expansion. The second intronic variant is the c.1515-79delTGinsAAACTAACCAAA, a very complex variant which involves a palindromic sequence.

All the four alterations identified in one African patient are present in heterozygosis in both the parents. Familial anamnesis excluded consanguinity, furthermore, they bear to two different ethnic groups; i.e., the mother is from Central Zimbabwe and belongs to Gushungo, while father is from Northern Zimbabwe and belongs to Shamza. This is surprising, and opens a possibility that the SLC26A3 mutations described in this subject may be frequent among different ethnic groups in Southern Africa possibly due to some, still unknown, protective effects.

All the cases studied show the prenatal evidence of polyhydramnios typical features of the disease and prenatal evidence of polyhydramnios. This symptom should be recorded from the gynecologist and must alert pediatricians who will have in charge the newborn. The age at diagnosis is variable and this parameter is related to many variables such as knowledge of the disease, experience of the pediatricians, and severity of CLD symptoms. This point out to the crucial importance of molecular analysis for the earlier diagnosis of CLD. Correct diagnosis allows the people involved both family members and health care personnel, to be prepared to possible prematurity and lack of passing meconium such as to prevent diselectrolytemia and severe dehydration. At present treatment involves the replacement of continuous fluid electrolyte loses. Appropriate treatment from birth will have positive influence on the entire life, correctly treated patients are likely to develop normally and survive without complications (166). To our knowledge it is now possible to offer a more reliable pre- and postnatal diagnostics for sporadic cases of CLD, improving genetic counseling and clinical management.

Summary. Congenital Chloride Diarrhea (CLD) is an inherited disorder of intestinal electrolyte transport transmitted by autosomal recessive manner caused by a defect in SLC26A3/DRA gene that codifies for Cl^-/HCO_3^- exchanger. Patients affected by this disorder present clinically with intestinal Cl^- malabsorption that induce severe life-threatening chronic diarrhea with neonatal onset. Early identification of this disorder is

essential because an early introduction of appropriate therapy could be life-saving for these patients. Data on the prevalence and on molecular genetics of CLD are missing in many countries. Aims. To describe the genotype of children from different countries affected by CLD and to investigate genotype-phenotype correlations. *Methods*. Using automated direct sequencing (310 Abi Prism, PE, Transgenomic), we screened the whole coding region of SLC26A3 gene in 22 patients affected by CLD observed by the European Consortium for Congenital Diarrheal Diseases (ECoCoDiS). Main clinical characteristics of each child were recorded (nnumber of bowel movements per day; total fecal volume per day; stool consistency according the following score: 1: formed, 2: loose, 3: semi liquid; 4: liquid; urgency or incontinence; average doses of Cl- as mmol/kg in the substitution therapy). *Results*. Molecular analysis revealed 12 novel mutations. Majority of mutations were identified in STAS-like domain of the SLC26A3 gene. Not a clear correlation between mutations and clinical characteristics was found. *Conclusions*. Molecular analysis can contribute to rapid and specific diagnosis of CLD, and can be used to investigate the disease prevalence in different ethnic groups. We confirm the strong genetic heterogeneity of CLD in ethnic groups where the disease is sporadic, and conflicting genotype-phenotype correlations in this condition.

Future implications. The identification of disease genes is a step forward in the diagnostic approach to a patient in whom CCD is strongly suspected. However, it is conceivable that faster, less expensive molecular procedures will, in the near future, become available. This approach could spare the patient invasive procedures and limit complications associated with a delay in diagnosis. It is also possible that a more widespread use of efficient diagnostic tests may reveal a higher prevalence of the disorders classified within CDD. Furthermore, carrier and prenatal molecular diagnosis may help pediatricians to better manage the condition in the early stages of life. However, molecular diagnostics does not mean only identifying or excluding gene mutations; in some cases, “second level” approaches (including *in vitro* functional studies) are necessary to define the effect of a mutation and to confirm that a novel variant is indeed disease-causing. Clinical laboratories must be equipped for such studies. Nevertheless, proteomic studies may in the near future

predict the phenotype of congenital diarrhea, and guide physicians in the prescription of treatment procedures. Thus, close collaboration between clinical laboratory professionals and physicians may improve both diagnostics and research in the field of CCD, and may also lead to verify the efficacy of novel therapeutic approaches.

New therapeutic strategy for congenital diarrhea

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In patients with CLD supplementation therapy with NaCl/KCl is essential in reducing the risk of severe dehydration. Immediately after diagnosis, this therapy should be started and accurately monitored to ensure that the increasing body weight requirements are being met. Recently, the role of amylase-resistant starch has been increasingly recognized for the management of diarrheal diseases. On reaching the colon, amylase-resistant starch are fermented by resident bacteria into the short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate. As already shown, SCFAs have a great capacity for stimulating ion and water absorption; they provide energy and induce a trophic effect on both colonic and small bowel mucosa (158,167). The important regulatory role of SCFAs on fluid and electrolyte absorption has led to the hypothesis that butyrate treatment could reduce diarrhea in CLD patients. It has been demonstrated that butyrate could be effective in many cases of patients with CLD (158,166,167).

The mechanism underlying this therapeutic effect is unclear, but it could be related, at least in part, to stimulation of the Cl^-/butyrate exchanger activity. It is also possible that butyrate could reduce miss-trafficking or misfolding of the SLC26A3 protein. Alternatively, butyrate may enhance expression of the SLC26A3 gene. We investigated the therapeutic effect of butyrate in a series of patients with CLD. At the same time, studies evaluating effects of butyrate on DRA expression in cell lines from patients with CLD, were performed.

STUDY DESIGN

Clinical trial

We performed a multicenter (20 Center), open crossover trial including children with a diagnosis of CLD confirmed by molecular analysis of the SLC26A3. Enrolled children were

allocated into 2 different groups: Group 1 receiving only NaCl and KCl per os; Group 2 receiving salts plus butyrate for 1 week. After one week of treatment, a 1-week wash out period was performed. After this, group assignments were inverted for each patient for further 1 week. During the study period, parents were instructed to record daily in a special form data regarding fecal volume, number of bowel movements, stool consistency, presence of urgency and incontinence. At the end of each week the serum, urinary and fecal electrolyte concentrations, together with hemogasanalysis, rennin and aldosteron were determined.

We used as butyrate the product named SOBUTIR®, 1 g tablets, Promefarm, Milan, Italy. In subject with body weight < 40 Kg or 88 Lb, butyrate treatment was administered at 100 mg/kg/day, orally in 2 doses. In subject with body weight > 40 Kg or 88 Lb, butyrate treatment was administered at 4 g/day, orally in 2 doses.

The dosage of butyrate was established on the basis of previous report (158,166,167). Maximum dosage of butyrate was 4 g/day. During the entire length of the study, all CLD subjects were examined as outpatients and receive oral NaCl/KCl supplementation according to the serum and urinary values and continue a normal diet.

Primary outcome of the study was the reduction of fecal electrolytes loss. Secondary outcomes were number of bowel movements, stool consistency, fecal volume, presence of incontinence, urgency, and serum and fecal electrolyte balance, number of hospital admissions, and side events.

Enrolling 4 patients, the probability is 95% that the study detected a treatment difference at a two sided 5% significance level, if the true difference between the groups in fecal loss of sodium (from 80 ± 25 mmol/l to 25 ± 10 mmol/l) and chloride (from 145 ± 30 mmol/l to 90 ± 30 mmol/l) was 55 mmol/l.

Cloning and mutagenesis studies

Prediction studies on both missense mutations were conducted using the software MUpro1.1 and Protean 4.00. Furthermore, the SLC26A3 cDNA was cloned into an expression vector fused with GFP. Then, it was mutagenized to reproduce both missense mutations, transfected into NIH3T3 cells and observed by fluorescence microscope. We isolated W.T.

cDNA from a cDNA healthy intestinal library (Gene Pool™ Human cDNA-Colon; Invitrogen). cDNA was cloned into the pEGFP-C3 (Invitrogen) between XhoI and BamHI restriction sites, to obtain a fused protein to track for the localization by fluorescence microscopy. Fractions of the vector were mutagenized to replicate the selected mutations observed in patients. The vectors were used to transfect a NIH3T3 cell line (murine fibroblasts).

In brief we isolated, cloned and mutagenized the SLC26A3 cDNA. The cloning allowed us to yield SLC26A3 proteins tagged with EGFP to highlight them and to study the localization before and after the treatment of cells lines with rising concentrations of butyrate. To isolate, clone and mutagenize the SLC26A3 cDNA we use the following methods. The SLC26A3 cDNA was isolated from an intestinal cDNA library. To isolate the cDNA we modified the primers previously described by Silberg et al., 1995, using BamHI and XhoI restriction sites. The cDNA was amplified and cloned into the pEGFP-C3 vector. The plasmid was amplified transforming an E. coli strain. Then, we used the site-specific mutagenesis to obtain 6 SLC26A3 mutants (c.1181G>T, c.1312T>C, c.1484A>C, c.1529C>T, c.1640C>T, c.1960T>C). All plasmids were checked by direct sequencing to confirm the correct mutagenesis. We used these plasmids to transfect a NIH3T3 cell line (mouse fibroblasts) growing on slides. Every experiment was duplicated using the kit Attractene Tranfection reagent by Quiagen (Milano, Italy). Sixteen hours after transfection the slides were treated to highlight the presence of tagged proteins. Cytoskeleton was colored in red using phalloidine and nuclei were colored in blue with DAPI. The slides were fixed and treated with an anti-fading agent (Vectashield®, Vector Laboratories, USA). The slides were analyzed using a fluorescent microscope Leica DMI 4000B. Localization of DRA before and after the incubation of NIH3T3 cells with butyrate at rising concentrations was investigated.

To better understand the variations induced by mutations we used Protean 4.00 of Lasergene suite (DNASTAR Inc.) to predict structural variations and Mupro: http://ics.uci.edu/~baldig/mutation.htlm) to compute variations in Gibbs free energy.

Study of expression of DRA after butyrate stimulation

SLC26A3 expression was tested by quantitative RT-PCR on cultured nasal epithelial cells of 2 enrolled patients before and after butyrate addition.

RESULTS

Clinical trial

In 22 subjects diagnosis was confirmed by molecular analysis. The firts 4 patients were enrolled in the clinical trial. During butyrate therapy we observed a significant improvement of the incontinence (44 ± 36% *vs.* 19 ± 32%, $p<0.05$) and a reduction of fecal concentrations of sodium (83 ± 22 mmol/l *vs.* 24 ± 7 mmol/l, $p<0.05$) and chloride (147 ± 15 mmol/l *vs.* 119 ± 28 mmol/l, $p<0.05$). Not all subjects were clinical responders to butyrate therapy. A reduction in the number of evacuations were observed only in 2 out of 4 patients (3.0 ± 1.4 *vs.* 2.0 ± 0.6, $p<0.05$, Figure 9), such as an improvement in stool consistency were assessed in 3 out of 4 treated children (2.8±0.7 *vs.* 2.0± 0.3, $p<0.05$)(Figure 10). No difference was observed before and after butyrate therapy regarding daily fecal volume.

Cloning and mutagenesis studies

Patient, in which the therapy caused a significant improvement of the incontinence and of the stool pattern, is compound heterozygous for two missense mutations. These mutations were both associated to a correct folding and topogenesis of the protein on the membrane (Figure 11 and 12).

Mutation pS654P produce a DRA protein retained into the ER, pL205RfsX28 was the cause of truncated no-functioning protein, whenever the other investigated mutations determine a protein folded but with decrease conductance.

Studies of structural variations prediction and variations in Gibbs free energy were reported below: p.Q495H mutation: the glutamine, a polar neutral amino acid, should be involved in the 12th transmembran helix. This protein tract is philogenetically well conserved. Its substitution with histidine, a polar and basic amino acid, should greatly alter the charges

distribution into the channel. The MUpro analysis did not show a significant ΔΔG variation. These data suggest that p.Q495H protein could fold and arrive into membrane but its conductance could be altered. The microscope pictures, showing a spread signal in the cytoplasm, confirm these predictions. p.A547E mutation: the alanine is an apolar amino acid and it should be involved in the formation of the first b-sheet of STAS-like domain. This protein tract is philogenetically well conserved. Its substitution with glutamate, a polar acid amino acid, should greatly alter the charges distribution and should allow the extroversion of the amino acids from the idrophobic core. The MUpro analysis did not show a significant ΔΔG variation. Also in this case, protein could fold and arrive into membrane but the STAS-like domain could lose its structure abolishing the transport function. The microscope pictures, showing a spread signal in the cytoplasm, confirm that protein folds. p.T510M mutation: the threonine, a polar and neutral amino acid, is philogenetically highly conserved and is inside a highly conserved tract. Its substitution with a methionine, an apolar thioester, is inside a junction between two domains: the ST and STAS-like domains, but the analysis by Protean did not show a significant structural variation. Also the MUpro software did not show a significant ΔΔG variation. The microscope pictures did not show difference when compared with the wild type. Considering that this mutation was founded in a patient bearing the p.S654P mutation in homozygosis, it is probable that this variation is a polymorphism. p.S654P mutation: the serine, a polar and neutral amino acid, is involved in the formation of the STAS-like "conserved loop", a b-sheet. Amino acid and tract are both highly conserved. Its substitution with a Proline, an apolar aliphatic amino acid, could alter the STAS-like b-sheet. The Protean analysis confirms this hypothesis. The MUpro software showed a significant ΔΔG variation. These predictions are confirmed by the microscope picture that showed a pronuclear accumulation, probably at endoplasmic reticulum level. This mutant showed a folding problem. The p.S438P mutation: another highly conserved serine is substituted with a proline. The S438 is the first amino acid of the 11^{th} transmembrane helix. From literature data, proline is a strong helix interrupter and Protean showed a change in the hydrophobicity in the tract after the proline. The MUpro software did not show a significant ΔΔG variation. Also the microscope picture showed a spread green signal. In this case we could hypothesize that protein could fold forming a

shorter 11th helix: this allow the reaching of membrane but alter the conductance. p.S394I mutation: another well serine into a well conserved tract of the 9th transmembrane helix. In this case the serine is changed with an isoleucine. The Protean analysis showed a reduction in helix flexibility. The MUpro software did not show a significant ΔΔG variation. On the basis of predictions and of microscope pictures, we could hypothesize that protein could fold but its conduction is altered by a more unbending helix. Butyrate correct modifies the conductance of the protein produced by pQ495H and pA547E.

Study of expression of DRA after butyrate stimulation

The effect of butyrate on DRA/SLC26A3 expression obtained in epithelial nasal cell from the patients affected by CLD was reported in the Figures 13 and 14.

CONCLUSIVE REMARKS

Our research has made significant advances in the field of possible innovative therapeutic strategies for Congenital Chloride Diarrhea (CLD). Before this study, the therapeutic approach to children with CLD was based only on substitutive therapy with NaCl/KCl. This traditional therapeutic approach is able to limit the risk of severe dehydration but is ineffective to limit the severity of diarrhea and to improve the quality of life. Our studies represent a classical model of an epigenetic modulation of intestinal functions. Epigenetics is the study of inherited changes in phenotype (appearance) or gene expression caused by mechanisms other than changes in the underlying DNA sequence, hence the name *epi-* (Greek: *επί-* over, above) *–genetics*.

For the first time we have demonstrated the possibility to act efficiently on the expression and function of the gene of the CLD limiting severity of diarrhea and fecal ion losses, through the use of butyrate.

The efficacious collaboration with the others units involved in the research has provided the possibility to obtain new advances also on the pathophysiology of this condition, on the

mechanisms of action of butyrate, and it has pointed out the importance of particular genotypes in the clinical response to this therapeutic strategy.

We make light on the mechanism underlying the therapeutic effect of butyrate. Butyrate could be responsible of activation of Cl^-/butyrate exchanger activity (Figure 15). It is also possible that butyrate could reduce miss-trafficking or misfolding of the SLC26A3 protein (10). Alternatively, butyrate may enhance gene expression (Figure 15): the SLC26A3 gene contains a 290-bp region between residues -398 and -688 that is crucial for high-level transcriptional activation induced by butyrate. This might explain the variable response of CLD patients to butyrate. In fact, depending on the patient's genotype, mutations in the above-mentioned regulatory regions of the SLC26A3 gene could affect gene transcription rate. It is also conceivable that other channels are involved in the therapeutic effect of butyrate in CLD. SLC26A3, like other components of the SLC26 family, interacts with CFTR. The interaction between CFTR and these components is mediated by binding of the regulatory domain of CFTR to the STAS domain of SLC26. The interaction is enhanced by phosphorylation of the regulatory domain by PKA (10) and is modulated by PDZ-binding scaffold proteins. An important consequence of this interaction is that SLC26 anion exchange activity is enhanced when CFTR is activated by phosphorylation. Moreover, the two genes regulate each other, i.e. over expression of SLC26-A3 or -A6 causes up-regulation of CFTR and vice versa (8). In patch-clamp experiments, PKA-stimulated CFTR channel activity was six-fold higher in HEK293 cells co-expressing both SLC26 exchanger and CFTR than in HEK293 cells expressing CFTR alone. Mutations may impair the interactions between channels and thus reduce the effect of butyrate therapy. Interestingly, it has recently been demonstrated that butyrate can act by different mechanisms in *in vitro* models of cystic fibrosis (CF): i) it can increase the expression of the apical epithelial membrane of the CFTR; and ii*)* it can act as a "chaperone-like" molecule, as shown in the deltaF508del CFTR cell line (49, 50). Similar mechanisms could occur in CLD.

Summary. Congenital chloride diarrhea (CLD-OMIM 214700) is an extremely rare inherited intestinal electrolyte transport disorder transmitted by an autosomal recessive fashion due to mutations in the DRA/SLC26A3 gene. Clinical picture is characterized by

intestinal sodium and chloride malabsorption that lead to a persistent, life-long severe diarrhea associated with incontinence and urgency. Thus, the quality of life of these children is compromised. Unfortunately, oral substitution with NaCl and KCl replaces the intestinal loss of salts, but it not limits severity of the diarrhea. Sporadic cases treated successfully with Butyrate have been reported. The aims of the study were: 1) to evaluate the clinical efficacy of butyrate in children affected by CLD with different genotype; 2) to establish the mechanism of action of butyrate. *Methods*. Multicenter (20 Center), open crossover trial including children with a diagnosis of CLD confirmed by molecular analysis of the SLC26A3. Oral NaCl/KCl supplementation was administered in all patients. Enrolled subjects received oral Butyrate (100 mg/Kg/die as 2 doses) for 1 week. Butyrate therapeutic efficacy was assessed evaluating the daily stool electrolytes content, stool pattern (fecal volume, number of evacuations, stool consistency evaluated through a scoring system: normal = 1, loose = 2, liquid = 3), the presence of incontinence (expressed as number of evacuation associated with incontinence/total daily evacuations), and the electrolytes concentrations in serum, and urine. These parameters were compared with a baseline data recorded 1 week before butyrate therapy in all enrolled subjects. *Results*. Four patients were enrolled into the trial. During Butyrate therapy we observed a significant ($p<0.05$) improvement of the incontinence (44 ± 36% *vs.* 19 ± 32%) and a reduction of fecal concentrations of sodium (83 ± 22 mmol/l *vs.* 24 ± 7 mmol/l) and chloride (147 ± 15 mmol/l *vs.* 119 ± 28 mmol/l). Four different mutations were observed in the 4 analyzed patients. Our studies of mutagenesis and cloning demonstrated that only selected mutation may alter folding of DRA on the apical membrane of the enterocyte and that butyrate efficacy depend on the mutation causing diseases. *Conclusions*. Butyrate reduces the risk of severe dehydration reducing fecal loss of sodium and chloride, and improves quality of life limiting the incontinence. We hypothesize that the response to butyrate therapy could be variable depending on different genetic profile.

Future implications. Studies on the efficacy of the butyrate in congenital chloride diarrhea have make light on intestinal ions transport mechanisms and may open new therapeutic way for more common forms of diarrhea occurring in early life. During PhD we also developed a

new more palatable formulation of Butyrate. Amidic derivates of butyrate has been obtained by phenylalanine (Figure 16). This new butyric acids are less hygroscopic, easy to weight, stable in acid and alkaline conditions. These features are crucial to obtain a good absorption during intestinal transit. This formulation is odorless and tasteless thus more adaptable to pediatric use. This new formulation has been patented in Italy (RM 2008, A 000214) and in other countries (World Intellectual Property Organization n°WO 2009/130735). We tested in vitro this new formulation obtaining positive effects on ions intestinal transport (Figure 17). Clinical trial to test this new formulation has been planned.

Research Area 4.
Prevention of early onset diarrhea

Prevention of infectious diarrhea determined by gastric acidity inhibitors

Results of this research were published on Current Opinion in Gastroenterology 2010;26:31-35

Gastric acid secretion is a philogenetically old function firstly developed about 500 million years ago (168). Recent years have seen widespread use of potent acid- suppression in the management of many upper gastrointestinal disorders, also in pediatric patients. The efficacy of these drugs has continued to improve as more potent acid suppressants have been introduced, but concerns have been raised with the respect to the effects of gastric acidity inhibitors (GAI) on host defenses and thus, on the risk of infections. The concept of "*gastric microbicidal barrier*" was introduced in 1925, subsequently it has been repeatedly reported that the suppression of gastric acidity predisposes to infection by a variety of pathogens, but only recently the increased risk of infection induced by GAI has been investigated systematically, at clinical and laboratory level (168-170). The following is a summary of the recent clinical studies performed in adult, children and neonates exploring the possible association of GAI use with intestinal infections. Updated pathogenetic hypotheses have also been reviewed.

Evidences from children

Gastric juice consists of HCl and pepsin and can kill bacteria within 15 min when the pH is less than 3.0. If the pH is raised above 4.0, a state defined as hypochlorhydria, bacterial overgrowth and infections are more common (169). Different bacterial pathogens have been reported in the studies investigating the side effects of GAI use. Few studies have focused on parasitic or fungal infections, and no data are available on viral intestinal infections. The most investigated associations of selected pathogens with GAI use are reported in Table 20.

In this paragraph we will focus on more recent data on GAI-associated infections occurring during different ages of life. For systematic reviews see references 1-3.

A recent case-control study of risk factors for *Salmonella enteritidis* in the Netherlands showed an increased risk of gastroenteritis induced by these pathogens in children taking GAI (OR 3.6, range 1.9-6.9) (171). A prospective study performed in pediatric patients showing that the use of GAI is associated with an increased risk of acute gastroenteritis and community-acquired pneumonia in GERD-affected children has been recently published by our group (172). We obtained data in 186 subjects from 4 pediatric gastroenterology centers: 95 healthy controls and 91 GAI users (47 on ranitidine, 44 on omeprazole). The two groups were comparable for age, sex, weight, length and incidence of acute gastroenteritis and pneumonia in the 4 month prior to enrollment. Rate of subjects presenting acute gastroenteritis and community-acquired pneumonia was significantly increased in patients treated with GAI compared to healthy controls (acute gastroenteritis: 47% vs. 20%, p=0.001; pneumonia 12% vs. 2%, p=0.03) during the 4 month follow up period. In the GAI treated group, the rate of subjects presenting acute gastroenteritis (20% vs. 47%, p<0.0001) and community-acquired pneumonia (3% vs. 12%, p=0.02) was increased when comparing the 4 month before and after enrollment. No differences were observed between ranitidine and omeprazole users in acute gastroenteritis and pneumonia incidence in the previous 4 month and during the follow up period. On the contrary, in healthy controls, the incidence of acute gastroenteritis and pneumonia remained stable. It could be interesting to underline that we observed an increased incidence of intestinal and respiratory infections in otherwise healthy children taking GAI for GERD treatment. On the contrary the majority of the previous data showed that the patients most at risk for pneumonia were those with significant co-morbid illnesses such as diabetes or immunodeficiency, and this point out to the importance of gastric acidity suppression as a major risk factor for infections. The effect on infection susceptibility seems to be sustained even after the end of therapy. We observed a similar incidence of acute gastroenteritis and pneumonia during the use of GAI drugs and in the 2 months following the stop of their use, resembling that previous observed in adult patients (173). How much this effect can last remain to be defined in future studies. Gastroesophageal reflux disease is commonly clinically diagnosed in children with many

nonspecific symptoms and frequently the treatment is empiric. These results could leave pediatricians with some unanswered questions about how to handle GAI in selected patients with severe neurological impairment or chronic lung diseases. These subjects appear to be at increased risk for reflux and aspiration. Protecting these selected patients from aspiration pneumonia secondary to untreated reflux is probably more convenient that the risk of infection associated with GAI therapy. At the same time considering the high risk of inappropriate treatment in children, the pediatrician needs to consider kindly the increased risk of infection before prescribing a gastric acid blocker.

Evidences from newborn

Many drugs used in neonatal age are either not licensed for the use or are prescribed outside the terms of their product license (off label prescribing) (174). The use of these drugs in neonatal intensive care units (NICU) seems to be far greater than other pediatric settings. It has been recently reported that at least 30% of neonates received treatment with GAI at the moment of discharge from NICU (175). The European Parliament and the European Medicines Agency (EMEA), aiming to increase the information available on the use of medicinal products in pediatric population, indicates as priority safety studies on GAI use in children and in newborns. These observations are particular relevant for very low birth weight infants (VLBW) in which infections contribute significantly to morbidity and mortality. Recently, Soll et al. examined the relationship between postnatal steroid exposure and H_2RA therapy (beginning at 2 weeks of age) and late-onset sepsis in VLBW infants into a randomized, controlled trial (176). They observed that treatment with dexamethasone and H_2RA was associated with an increased risk of sepsis and meningitis). The increased risk of infection in infants treated with H_2RA before randomization to the study drug (steroid or placebo) was postulated to be the result of a change in small-bowel colonization after the development of hypochlorhydria. Similarly, in a prospective observational study, Beck-Sague et al. reported that 12 (36%) of 33 VLBW neonates who received H_2RA developed bloodstream infections, whereas only 30 (9%) of 343 of those not treated with H_2RA developed bacteremia (177). In logistic-regression analysis, the risk of a bloodstream infection was independently associated with lower birth weight, respiratory illness at the

time of admission, and receipt of H_2RA. They postulated that gastric acidity may be a protective mechanism against respiratory and gastrointestinal tract colonization by nosocomial pathogens and subsequent bacteremia. More recently it has been observed in a retrospective study an additional risk of infections and necrotizing enterocolitis (NEC) in newborn treated with GAI (175). Unfortunately, in VLBW infants, the diagnosis of GERD or of peptic disease is based on the evaluation of non-specific symptoms, and the empiric treatment represents frequently the first diagnostic test. In addition, there is no clear evidence of benefit of the use of H_2RA in many clinical conditions typical of neonatal age. These observations suggest the importance of a more careful use of GAI in these patients, in particular if other risk factors for severe infections are present.

In this light, we performed a prospective study involving VLBW infants observed in Neonatal Intensive Care Units (178). Rates of VLBW infants presenting infections were calculated for both GAI-exposed and GAI-unexposed patients, during 8 weeks of hospitalization after starting use of GAIs. Study population comprised 274 VLBW infants: 91 exposed to GAI (42 because treatment or prophylaxis of stress-induced peptic disease; 49 because of GERD) and 183 not exposed to GAI, similar for main clinical and demographic characteristics. Rates of patients presenting infections in GAIs-exposed and not-exposed were 34 out of 91 (37.4%) and 18 out of 183 (9.8%), respectively ($p<0.001$). GAIs users VLBW infants had OR 5.47 (95% CI 2,89-10) fold increased risk of infections compared with control subjects. Rate of subjects presenting sepsis (23 out of 91 vs. 16 out of 183), pneumonia (2 out of 91 vs. 1 out of 183) or urinary tract infections (7 out of 91 vs.1 out of 183)and acute gastroenteritis (2 out of 91 vs. None out of 183) was significantly increased in patients treated with GAIs compared to controls ($p<0.0001$) during observational study period (Table 21). In conclusion, gastric acidity-inhibitors therapy is associated with an increased risk of infections in VLBW infants. This effect seems to be sustained even after the end of therapy. These results may be attributable to many factors, including direct inhibitory effect of GA-inhibitors on leucocytes functions and qualitative and quantitative gastrointestinal microflora modification.

CONCLUSIVE REMARKS

The mechanism of the deleterious effects on the risk of infections in subjects receiving GAI is still not completely elucidated. A number of experimental and clinical evidences suggested a multifactorial pathogenesis (Figure 18). The preservation of gastric acid secretion during phylogenesis supports the biological importance of this highly energy consuming system developed to inactivate ingested microorganisms. Gastric acid is important in the killing of ingested acid sensitive organisms, in addition elevation in gastric pH may also have other deleterious effects on gastrointestinal (GI) host defense including delayed gastric empting , increased bacterial translocation, decreased gastric mucus viscosity, and changes in the normal microbial flora (169). Impaired gastric acid secretion has been shown to increase the colonization by several bacterial and parasitic agents. More recently, two experimental studies of *C. difficile* infection showed that in a mouse model, pretreatment with GAI before inoculation with *C. difficile* resulted in similar rates of infection, toxin production, and colon injury to a group of mice pre-treated with ampicillin. In addition spore germination was noted to be favored by elevated pH levels (pH = 6), and by the presence of potassium chloride and inorganic phosphate. As the proton pumps in the stomach exchange potassium for hydrogen ions, it is possible that their blockage, besides resulting in higher gastric pH levels, may also result in increased intraluminal potassium (169). Direct effects of GAI on several leukocyte functions have been demonstrated, including decreased adhesion to endothelial cells, reduced bactericidal killing of microbes and inhibition of neutrophils phagocitosis and phagosome acidification . This could be of particularly importance in elderly and neonatal age when immunity is still largely immature. A direct effect of histamine on intestinal immune response to selected pathogens has been demonstrated in animal model. To test whether inhibition of gastric acid was involved in the effects of H2 signaling on the mouse response to *Y. enterocolitica*, Handley *et al.* (179) compared the effects of cimetidine with the proton pump inhibitor omeprazole in the orally infected mouse model. The authors identified no effect of omeprazole under their conditions, suggesting that gastric acid production is not involved in the effects of cimetidine on *Y. enterocolitica* infection, but rather that the H_2 antagonism likely plays a role in regulating the innate immune response to infection at the level of the Payer's patches

(179). Increased production of histamine is important for controlling the infection, specifically through H_2receptor. This increase in histamine could stimulate a significant influx of mast cells or basophiles. After stimulation, the histamine, upon cellular release, would bind to cells expressing H_2. Activation of H_2 has been shown to have a variety of effects including altering the production of inflammatory cytokines and disrupting the Th1–Th2 balance during the immune response. The Th1–Th2 balance is known to be important for controlling such infection. A negative influence on the immune system could be the consequence also of the modification of quantitative and qualitative composition of intestinal microflora (179). A study of the numbers and type of bacteria in nasogastric tubes of patients receiving GAI demonstrated an increased numbers of bacteria including beta hemolytic *Streptococcus*, a known cause of community-acquired pneumonia (173). Considering the information on the cross-talk between intestinal microflora and immune system functions it is important that this aspect should be investigated in a more incisive manner in the future. Concomitant risk factors including pre-existence of chronic diseases, hospitalization, and antibiotic use could contribute to an increased risk of infections in patients taking GAI (Figure 18). Finally, it has been demonstrated that when PPI are prescribed to patients with *H.pylori* infection, the acid inhibition is more profound than in patients without *H.pylori* infection (180). Patients with *H.pylori* infection may therefore be at greater risk for infection.

Summary. Many studies and systematic reviews demonstrate an increased risk of bacterial infection in adults taking acid suppressors. Little evidence is derived from the early in the life period. The use of gastric acidity inhibitors has been associated with systemic infections and necrotizing enterocolitis in preterm infants. Reduced gastric acidity, delayed gastric emptying, increased gastric mucus viscosity, modification in microbiota, and impairment of neutrophils functions, are all conditions determined by gastric acidity blockers that potentially lead to an increased risk of gastrointestinal infections. A proper utilization of these drugs, particularly for patients at high risk, is imperative in order to reduce deleterious effects on infection risk and to optimize cost-effectiveness ratio.

Future implications. The GAI use accounts for significant cost expenditure in Western Countries including over-the-counter and prescription formulations (170,181). With the increasing use of GAI, the demonstration of an association with an increased risk of intestinal and extraintestinal infections, and increasing concerns of multiresistant pathogens, public and professional education is needed to stress the importance of appropriate use of anti-secretary drugs. Because no drug is without side-effects, physicians have to satisfy themselves that the benefits of treatment outweigh the potential risks. Further pharmacovigilance studies exploring all the possible variables influencing the impact of GAI therapy on the risk of infections are necessary, together with more basic and clinical investigations on the mechanisms of these GAI-related problems and on possible prophylactic measures to prevent them.

Primary prevention of food-induced diarrhea

Results of this research were published on Pediatr Allergy Immunol2010; 21:889-91 Web link

The prevalence of atopic manifestations has increased worldwide, especially in children (182-184). It is now recognized that early childhood events, including diet, are probably important in the development of atopic diseases. Various preventive measures have been proposed including definition of high-risk infants, maternal dietary restrictions during pregnancy, breastfeeding, dietary restriction while breastfeeding, the use of hypoallergenic formulas, and delays in the introduction of certain foods into the infant's diet (185). Family history is the best indicator for population screening (185). Current evidence does not support a major role for maternal dietary restrictions during pregnancy or lactation but there is evidence that breastfeeding for at least 4 months, compared with feeding formula made with intact cow's milk protein, prevents or delays the occurrence of atopic dermatitis, food allergy, and wheezing in early childhood (186). Data suggest for infants at high risk of atopy and who were not exclusively breastfed for 4–6 months that the onset of atopic disease may be delayed or prevented by hydrolyzed formulas compared with formula made with intact cow's milk protein, particularly for atopic dermatitis (18,186). The Committees on Nutrition and Section on Allergy and immunology of American Academy of Pediatrics (AAP) has recently published recommendations on early nutritional interventions on the development of atopic diseases in infants and children (18). Recommendations for primary prevention of atopy disorders should be applied early in the perinatal period to have a chance of success. The first care givers in contact with high-risk subjects are the neonatologists, but little is known about their awareness of and adherence to dietary management for the prevention of atopic diseases. Thus, we assessed adherence to the dietary management recommendations of the AAP for the prevention of atopic diseases in neonatal age through an audit study.

STUDY DESIGN

We invited by e-mail, the chiefs of 30 maternity units (MU) of the national public health system with more than 1500 live births/yr operating in South Italy to report the policy applied in their MU and to complete a questionnaire constituted by three simple items: (i) identification of high risk of developing allergy newborns, (ii) dietary restrictions for lactating women, (iii) use of hypoallergenic formula for bottle-fed high-risk infants.

RESULTS

Thirty MU were invited to participate representative of about 135,000 newborn/yr. Twenty-two MU returned the questionnaire. Results are reported in Table 22. Only 2/22 MU has a policy in complete agreement with the early nutritional intervention on development of atopic disease proposed by the AAP (18).

CONCLUSIVE REMARKS

Our study suggests scarce knowledge and adherence to dietary recommendations for primary prevention of atopic disease in neonatology clinical practice. These results could be influenced by a suboptimal awareness of atopic disorders by neonatologists and by the belief of little clinical benefit that these recommendations could obtain. However, considering the dramatic increase of incidence and prevalence of atopic disorders and the possibility to prevent these conditions by, at least in part, a nutritional approach, we believe that an increased awareness of the matter by neonatologists is important. This audit highlights the need for an awareness campaign to promote the use of primary prevention strategies for atopic disease among neonatologists.

Summary. The prevalence and severity of atopic manifestations in children are increasing in western countries in the last decades. Specific nutritional intervention may prevent or delay the onset of atopic diseases in infants at high risk of developing allergy. These nutritional interventions should be applied early in the perinatal period to have a chance of

success. Thus, we assessed adherence to the dietary management recommendations of the Committee on Nutrition and Section on Allergy and Immunology of the American Academy of Pediatrics (AAP) for the prevention of atopic diseases in neonatal age through an audit study. Questionnaire was administered to the chiefs of 30 maternity units (MU) with more than 1500 live births/yr to report the policy applied in their MU. Twenty-twoMU returned the questionnaire. Identification of high-risk newborns was routinely performed only in 7/22 MU (31.8%). High-risk newborns were identified by the presence of at least two or one first-degree relative (parent or sibling) with documented allergic disease by 18.2% and 45.5% of MU, respectively. Specific maternal dietary restrictions during lactation were adopted in 7/22 MU (31.8%).Extensively or partially hydrolyzed formula was prescribed for bottle-fed high-risk infants in 22.7% of MU. Only 2/22 MU have a policy in complete agreement with the nutritional intervention proposed by the AAP. Our studies suggest a poor adherence to dietary recommendations for primary prevention of atopic disease in neonatology clinical practice. Further efforts should be planned to improve the knowledge and the application of these preventive strategies.

Future implications. In recent years, much attention has been given to oral tolerance and gut flora. Oral tolerance mechanisms develop postnatal mainly in response to the gut flora and activation of specific Toll-like receptors on regulatory cells. The pivotal role of the luminal bacteria is highlighted by the impaired tolerance in germ-free mice, the different intestinal flora in populations that will later develop atopy, the immunomodulatory properties of specific probiotics, and the promising results of interventional studies. Allergic infants showed, even before the appearance of symptoms, a significant higher prevalence of *Clostridia*, coliforms, and *Staphylococcus aureus* versus lactobacilli and bifidobacteria (bifidum). Manipulation of the gut flora as early as in the first days or months of life may influence subsequent colonization and expression of regulatory cytokines through microenvironmental modification and competition. Specific probiotics, including LGG, may induce production of anti-inflammatory IL-10 and transforming growth factor, inducing a tolerogenic effect before sensitization occurs. According to two trials, using supplementation of LGG or *E. coli* in the perinatal period may reduce some non-IgE-

mediated allergic manifestations. Supplementation of a cow's milk-based formula with prebiotics has a bifidogenic effect, but a prospective beneficial effect on food allergy has up to now only been suggested with a specific prebiotics mixture.

CONCLUSIONS

During the PhD project, using a bench to bedside approach, several progresses in the field of diarrhea occurring in early life have been obtained. Epidemiology and etiology of diarrhea have been clarified, new therapeutic and preventive approaches have been developed for control of acute and chronic diarrhea occurring in early life. In addition, a novel model for the study of congenital diarrheal disease has been set. This model could be usefull to reduce the distance from in vitro to in vivo research on these rare conditions, and to identify new therapeutic targets for more common diarrheal disease occurring early in life.

The main future target was the intestinal ecosystem (i.e., epithelium, immunity, microflora, nutrients), a dynamic network that could be modulated by different therapeutic and nutritional approaches. Epigenetic modulation of gene expression in early life elicited by nutrients, in order to modify the natural course of the disease, represents the new research frontier.

Focusing on prompt identification and control of diarrheal diseases in early life, PhD project has outlined innovative approaches usefull to reduce immediate complications and influence body health in subsequent age of life.

Table 1. Etiology of diarrhea in early life

	Acute	Chronic	Overall
Number of patients	36	3	**39**
Unknown etiology	10	-	**10**
Cow's milk allergy	8	-	**8**
Infections	7	-	**7**
Rotavirus	4	-	
Salmonella parathyphi	1	-	
Shigella flexneri	1	-	
Enterotoxigenic E.coli	1	-	
Antibiotics-associated diarrhea	5	-	**5**
Neonatal withdrawal syndrome	2	-	**2**
Congenital diarrheal disorders	-	2	**2**
Glucose-galactose malabsorption	-	1	**1**
Congenital chloride diarrhea	-	1	**1**
Parenteral diarrhea ***Urinary tract infection***	1	-	**1**
Hirschsprung's disease	1	-	**1**
Cystic fibrosis	-	1	**1**
Immunodeficiency ***Adenosine deaminase deficiency***	1	-	**1**
Metabolic disorders ***Urea cycle defect***	**1**	**-**	**1**

Table 2. Main demographic and anamnestic data of the subjects with diarrhea in early life

	Acute	Chronic
Demographic data		
Number of patients	36	3
Demographic data		
Male, n (%)	15 (41.7)	1 (33.3)
Birth weight, g	2504 ± 778	2560 ± 664
Gestational age, weeks	36.4 ± 3.8	37.7 ± 1.5
Age at diarrhea onset, d	8.3 ± 6.6	5.7 ± 4.4
Age at hospitalization, d*	6.2 ± 8.5	19.7 ± 10.1
Anamnestic data		
Intrauterine growth restriction, n (%)	6 (16.7)	1 (33.3)
Polyhydramnios, n (%)*	1 (2.8)	2 (66.7)
Premature birth, n (%)	12 (33.3)	1 (33.3)
Familiarity for chronic diarrhea, n (%)*	1 (2.8)	2 (66.7)
Newborns at high risk of developing allergy, n (%)	12 (33.3)	-
Exclusive breast milk, n (%)	3 (8.33)	-

Data are expressed as mean ± Standard Deviation (SD) when not specified.
*$p<0.05$

Table 3. Symptoms associated with diarrhea occurring early in life

	Acute	Chronic
Gastrointestinal, n (%)		
Vomiting	18 (50.0)	1 (33.3)
Mucus in the stools	13 (36.1)	1 (33.3)
Blood in the stools	9 (25.0)	-
Abdominal distension	24 (66.7)	2 (66.7)
Systemic, n (%)		
Fever	4 (11.1)	-
*Hypovolemia**	1 (2.8)	1 (33.3)
Eczema	7 (19.4)	-
Seizures*	3 (8.3)	1 (33.3)

Data are expressed as mean ± Standard Deviation (SD) when not specified.
*$p<0.05$

Table 4. Severity of dehydration, modalities of rehydration and clinical outcomes of children with diarrhea occurring early in life

	Acute	Chronic
Number of patients	36	3
Dehydration degree		
*Severe dehydration, n (%)**	5 (13.9)	2 (66.7)
Rehydration modalities		
Exclusively oral rehydration, n (%)	18 (50.0)	1 (33.3)
Exclusively parenteral rehydration, n (%)	2 (5.6)	1 (33.3)
Oral plus parenteral rehydration, n (%)	16 (44.4)	1 (33.3)
Clinical outcomes		
*Duration of diarrhea, days**	5.7 ± 2.5	44.3 ± 6.7
Electrolyte abnormalities, n (%)	8 (22.2)	2 (66.7)
Deaths, n (%)	2 (5.5)	1 (33.3)

Data are expressed as mean ± Standard Deviation (SD) when not specified.
*$p<0.05$

Table 5. Subjects with intestinal failure in early life

Pt	GA (w)	BW (Kg)	Sex	Causes of intestinal failure	Age at the onset (d)	Follow-up length (m)
1	34	2.4	F	Abdominal wall defect	0	40
2	38	2.2	M	Abdominal wall defect	0	25
3	26	0.8	M	Congenital CMV infection	7	27
4	36	2.5	M	Congenital neuromuscular disease	5	33
5	34	2.1	F	Intestinal atresia	0	38
6	35	2.1	M	Intestinal atresia	0	41
7	34	1.9	F	Intestinal atresia	0	36
8	36	2.2	F	Intestinal atresia	0	43
9	35	2.1	M	Intestinal atresia	0	33
10	30	0.9	M	Intestinal atresia	0	42
11	37	2.5	F	Intestinal atresia	0	26
12	26	0.9	F	Intestinal atresia	0	40
13	26	0.9	M	Intestinal atresia	0	27
14	27	0.6	F	Intestinal obstruction	0	36
15	33	1.8	F	Intestinal obstruction	0	28
16	26	0.6	F	Meconium peritonitis	0	34
17	36	4.4	F	Megacystis Microcolon Hypoperistalsis syndrome	0	48
18	31	1.4	F	Necrotizing enterocolitis	12	48
19	27	0.9	F	Necrotizing enterocolitis	27	33
20	33	2.2	M	Necrotizing enterocolitis	10	50
21	28	0.6	F	Necrotizing enterocolitis	0	44
22	30	1.8	F	Necrotizing enterocolitis	0	34
23	30	1.3	M	Necrotizing enterocolitis	20	35

Table 5 (continued from page 79). Subjects with intestinal failure in early life

Pt	GA (w)	BW (Kg)	Sex	Causes of intestinal failure	Age at the onset (d)	Follow-up length (m)
24	30	1.0	F	Necrotizing enterocolitis	1	25
25	31	2.3	F	Necrotizing enterocolitis	10	28
26	40	3.0	F	Tufting enteropathy	10	30

Table 6. Natural history of subjects with intestinal failure occurring early in life

Cause	%	Successfully PN (%)	Duration of PN (d)	Death (%)
Congenital intestinal defects	42	91	50	9
Necrotizing enterocolitis	31	100	59	
Motility disorders	11	33	445	33*
Intestinal obstruction	8	100	60	
Ultrastructural defects	4	Waiting for IT*	920	
Meconium peritonitis	4	100	65	

**Due to complications of IT (intestinal transplantation)*

Table 7. Main features of subjects dependent from parenteral nutrition treated with 2 different nutritional strategy

	Group 1 (Total PN)	*Group 2 (PN + MEF)*
Male, n (%)	26 (53.1)	21 (43.8)
Birth weight, g (IQR)	1100 (415)	1095 (405)
Gestational age, weeks (IQR)	29 (3)	29 (3)
CRIB score	1 (2)	1 (2)
Age at the first episode of feeding intolerance, d (IQR)	6 (8)	7 (7)

Note. Data expressed as Median (IQR) when not specified.
Abbreviations. PN: parenteral nutrition MEF: minimal enteral feeding. IQR: interquartile range. CRIB: critical respiratory index for babies.

Table 8. Risk factors associated with NEC development in subjects dependent from parenteral nutrition treated with 2 different nutritional strategy

	Group 1 (Total PN)	*Group 2 (PN + MEF)*
Time to start enteral nutrition, h	11 (4)	8 (2)
Umbilical catheter, n (%)	38 (78)	38 (79)
Patent ductus arteriosus, n (%)	7 (14)	8 (17)
Intraventricular hemorrhage stage III-IV, n (%)	7 (14)	7 (15)
BM/total enteral feeding at day 14 of life	0.4 (0.3)	0.5 (0.3)
BM/total enteral feeding at discharge	0.3 (0.2)	0.3 (0.2)

Note. Data expressed as Median (IQR) when not specified.
Abbreviations. NEC: necrotizing enterocolitis. PN: parenteral nutrition. MEF: minimal enteral feeding. BM: breast milk. IQR: interquartile range.

Table 9. Time to reach full enteral feeding, occurrence of NEC and sepsis in subjects dependent from parenteral nutrition treated with 2 different nutritional strategy

	Group 1 (Total PN)	*Group 2 (PN + MEF)*	*p*
Patients with NEC, n (%)	1 (2)	1 (2.1)	0.747
Central vascular access duration, d	12 (5)	7 (4)	<0.001
Time to reach full enteral feeding, d	11 (5)	8 (5)	<.0001
Patients with ≥ 2 episodes of fed intolerance, n (%)	14 (28.6)	14 (29.2)	0.948
Patients with late onset sepsis, n (%)	17 (34.7)	8 (16.6)	0.047
Time to regained birth weight, d	12 (4)	9 (1.5)	<.0001
Death, n (%)	2 (4.1)	3 (6.3)	0.490

Note. Data expressed as Median (IQR) when not specified.
Abbreviations. NBE: nothing by enteral route. MEF: minimal enteral feeding. NEC: necrotizing enterocolitis. IQR: interquartile. range.

Table 10. Composition of the two oral rehydration solution used in a comparative study involving children with acute diarrhea occurring in early life

	Standard ORS	*Super ORS*
Commercial brand name	*Reidrax®*	*Prereid®*
Assigned group	*Group 1*	*Group 2*
Osmolarity (mOsm/L)	225	200
Na^+ (mmol/L)	60	50
K^+ (mmol/L)	20	20
Cl^- (mmol/L)	60	40
Glucose (mmol/L)	75	77
Citrate (mmol/L)	10	10
Zn^{2+} (mmol/L)	0	1
Fructooligosaccharides (g/L)	0	0.35
Xilooligosaccharides (g/L)	0	0.35

Note. Reidrax is a brand name of the EG SpA, Milan Italy. Prereid is a brand name of the Milte Italia SpA, Milan, Italy

Table 11. Comparative study on ORS involving children with acute diarrhea in early life: baseline main demographic and clinical characteristics

	Group 1	Group 2	*p*
N.	60	59	-
Age, m*	18.58 (15.5-21.6)	19.26 (15.9-22.6)	0.765
Body weight, kg*	10.68 (9.79-11.58)	11.25 (9.79-12.72)	0.474
Male, n (%)	34 (56.7)	36 (61.0)	0.630
Duration of symptoms before treatment, h*	9.0 (8.3-9.9)	10.0 (9.3-10.8)	0.083
Presence of vomiting, n (%)	14 (23.3)	22 (37.3)	0.098

*Mean (95% confidence interval) when not specified

Table 12. Comparative study on probiotics involving children with acute diarrhea in early life: microorganism load, administration, and main characteristics of preparations

Groups	Microorganisms	Strains	Dosage
2	*Lactobacillus casei*	*Rhamnosus* GG	6 x 10^9 CFU/dose (bid)
3	*Saccharomyces boulardii*	Sb It	5 x 10^9 live micro-organisms/dose (bid)
4	*Bacillus clausii*	*O/C84, N/R84, T84, SIN84*	10^9 CFU/dose (bid)
5	*L. delbrueckii var. bulgaricus* + *L. acidophilus* + *S. thermophilus* + *B. bifidum*	LMG-P17550 + LMG-P 17549 + LMG-P 17503 + LMG-P 17500	10^9 CFU+ 10^9 CFU+ 10^9 CFU+ 5 x 10^8 CFU/dose (bid)
6	*Enterococcus faecium*	SF 68	7.5 x 10^7 CFU/dose (bid)

Table 13. Comparative study on probiotics involving children with acute diarrhea: Baseline features

Groups	1 (n=92)	2 (n=100)	3 (n=91)	4 (n=100)	5 (n=97)	6 (n=91)
Male (%)	41 (44%)	60 (60%)	44 (48%)	49 (49%)	49 (50%)	39 (42%)
Median age in months (IQR)	17.0 (17.2)	20.0 (12.0)	18.0 (17.0)	19.0 (14.0)	16.5 (18.5)	15.0 (14.0)
Median weight in grams (IQR)	11925 (2805.0)	12100 (4072.5)	11450 (4700.0)	12300 (4000.0)	12700 (4625.0)	12234 (3150.0)
Feeding (%)						
Breast milk	13.5	11.7	13.3	10.0	13.4	17.9
Formula	55.8	55.0	55.6	60.2	46.2	43.6
Cow's milk	30.8	33.3	31.1	29.8	40.5	38.5
Median diarrhoea duration before treatment in hours (IQR)	9 (9.0)	10 (13.0)	11 (12.0)	10 (11.0)	9 (12.0)	10 (11.0)

IQR: Interquartile range.

Table 14. Comparative study on probiotics involving children with acute diarrhea: duration of diarrhoea (in hours) in the study groups

Groups	Treatment	Median hours	IQR	Estimated difference (95% CI)*	P value †
1	ORS alone	115	35	—	—
2	*Lactobacillus casei subsp. rhamnosus* GG	78	46	-32 (-41 to -24)	<0.001
3	*Saccharomyces boulardii*	103	43	-6 (-15 to 4)	0.262
4	*Bacillus clausii*	118	33	3 (-3 to 11)	0.311
5	*L. delbrueckii var. bulgaricus* + *L. acidophilus* + *S. thermophilus* + *B. bifidum*	69	50	-38 (-47 to -27)	<0.001
6	*Enterococcus faecium* SF 68	115	41	2 (-7 to 11)	0.670

IQR: Interquartile range.

* vs. ORS alone.

† Kruskal-Wallis H test with Dunn test for multiple comparisons. P *vs.* ORS alone

Table 15. Comparative study on probiotics involving children with acute diarrhea: daily stool outputs

Group	Treatment	Day						
		1	2	3	4	5	6	7
1	ORS alone	5 (2)	5 (3)	4 (2)	4 (2)	3 (2)	2 (1)	2 (2)
2	*Lactobacillus casei subsp. rhamnosus* GG **Estimated difference (95% CI)**	6 (2) —	4 (2)* -1 (-1 to 0)	3 (2) † -1 (-1 to 0)	3 (2)* -1(-1 to -1)	2 (1) † -1 (-1 to 0)	2 (1) —	2 (1) —
3	*Saccharomyces boulardii*	5(2)	5 (3)	4 (2.25)	4 (2)	3 (2)	2 (1)	2 (2)
4	*Bacillus clausii*	6 (2)	5 (3)	4 (2)	4 (2)	3 (2)	2 (1)	2 (1)
5	*L. delbrueckii var. bulgaricus* + *L. acidophilus* + *S. thermophilus* + *B. bifidum* **Estimated difference (95% CI)**	6 (2) —	4 (2)* -1 (-1 to 0)	3 (1.50)* -1 (-1 to 0)	3 (2)* -1(-1 to -1)	2 (1) † -1 (-1 to 0)	2 (1) —	2 (1) —
6	*Enterococcus faecium* SF 68	5 (2)	5 (2.75)	4 (2)	4 (2)	3 (2)	2 (1)	2 (1)

Day 1 is the day of the first probiotic administration.
Data are expressed as median (IQR, interquartile range).
*P < 0.001 or †P=0.001 vs. ORS alone at the same time point by Kruskal-Wallis H test with Dunn test for multiple comparisons.
The estimated differences are vs. ORS alone.

Table 16. Comparative study on probiotics involving children with acute diarrhea: daily stool consistency score

Groups	Treatment	Day						
		1	**2**	**3**	**4**	**5**	**6**	**7**
1	ORS alone	3 (0)	3 (1)	2 (1)	2 (2)	2 (1)	1 (0)	1 (0)
2	*Lactobacillus casei subsp. rhamnosus* GG 3 (0) **Estimated difference (95% CI)** —	3 (0) —	2 (0.50)* 0 (-1 to 0)	1 (1)* -1 (-1 to 0)	1 (1)* -1 (-1 to 0)	1 (0)* 0 (-1 to 0)	1 (0) —	1 (0) —
3	*Saccharomyces boulardii*	3 (0)	3 (1)	2 (1)	2 (1)	2 (1)	1 (0.25)	1 (0)
4	*Bacillus clausii*	3 (0)	3 (1)	2 (1)	2 (1)	2 (1)	1 (1)	1 (0)
5	*L. delbrueckii var bulgaricus* + *L. acidophilus* + *S. thermophilus* + *B. bifidum* 3 (0) **Estimated difference (95% CI)** —	3 (0) —	2 (1) † 0 (-1 to 0)	1 (1)* -1 (-1 to 0)	1 (1)* -1 (-1 to 0)	1 (0)* 0 (-1 to 0)	1 (0) —	1 (0) —
6	*Enterococcus faecium* SF 68	3 (0)	3 (1)	2 (1)	2 (1.75)	2 (1)	1 (0)	1 (0)

Day 1 is the day of the first probiotic administration.
Stool consistency score system (see ref. 15): 1, normal; 2, loose; 3, semi liquid; 4, liquid.
Data are expressed as median (IQR, interquartile range).
*P < 0.001 or †P=0.001 *vs.* ORS alone at the same time point by Kruskal-Wallis H test with Dunn test for multiple comparisons.
The estimated differences are vs. ORS alone.

Table 17a. Molecular basis of the main forms of congenital diarrheal diseases: defects of digestion, absorption and transport of nutrients and electrolytes

Disease	Gene	Location	Function
Disaccharidase deficiency			
Congenital lactase deficiency	LCT	2q21	Lactase-phlorizin hydrolase activity
Sucrase-isomaltase deficiency	EC 3.2.1.48	3q25-q26	Isomaltase-sucrase
Maltase-glucoamylase deficiency	MGAM	7q34	Maltase-glucoamylase activity
Ion and nutrient transport defects			
Glucose-galactose malabsorption	SGLT1	22q13.1	Na^+/glucose cotransporter
Fructose malabsorption	GLUT5	1p36	Fructose transporter
Fanconi-Bickel syndrome	GLUT2	3q26	Basolateral glucose transporter
Cystic fibrosis	CFTR	7q31.2	cAMP-dependent Cl^- channel
Acrodermatitis enteropatica	SLC39A4	8q24.3	Zn^{2+} transporter
Congenital chloride diarrhea	DRA	7q22-q31.1	Cl^-/base exchanger
Congenital sodium diarrhea	SPINT2*	19q13.1	Serine-protease inhibitor
Lysinuric protein intolerance	SLC7A7	14q11	Hydrolyzes endo-/exopeptidases AA basolateral transport
Congenital bile acid diarrhea	ABAT	13q3	Ileal Na^+/ bile salt transporter
Pancreatic insufficiency			
Enterokinase deficiency	PRSS7	21q21	Proenterokinase
Trypsinogen deficiency	PRSS1	7q35	Trypsinogen synthesis
Pancreatic lipase deficiency	PNLIP	10q26.1	Hydrolyzes triglycerides to fatty acids
Lipid trafficking			
Abetalipoproteinemia	MTP	4q22	Transfer lipids to apolipoprotein B
Hypobetalipoproteinemia	APOB	2p24	Apolipoprotein that forms chylomicrons
Chylomicron retention disease	SAR1B	5q31.1	Intracellular chylomicron trafficking

*This mutation has been associated to the syndromatic form of congenital sodium diarrhea.

Table 17b. Molecular basis of the main forms of congenital diarrheal diseases: defects of enterocyte differentiation and polarization

Disease	Gene	Location	Function
Microvillous inclusion disease	EpCAM	18q21	Intracellular protein trafficking
Congenital tufting enteropathy	Unknown	2p21	Cell-cell interaction
Syndromic diarrhea	Unknown	Unknown	Unknown

Table 17c. Molecular basis of the main forms of congenital diarrheal diseases: defects of enteroendocrine cells differentiation

Disease	Gene	Location	Function
Enteric anendocrinosis	NEUROG3	10q21.3	Enteroendocrine cell fate determination
Enteric dysendocrinosis	Unknown	Unknown	Enteroendocrine cell function
Proprotein convertase 1 deficiency	PCSK1	5q15-q21	Prohormone processing

Table 17d. Molecular basis of the main forms of congenital diarrheal diseases: defects of modulation of intestinal immune response.

Disease	Gene	Location	Function
IPEX (Immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome	FOXP3	Xp11.23-q13.3	Transcription factor
IPEX-like syndrome	Unknown	Unknown	Unknown
Immunodeficiency-associated autoimmune enteropathy	Unknown	Unknown	Unknown
Autoimmune polyglandular syndrome-1 (APS-1)	AIRE	21p22.3	Regulation gene transcription
Autoimmune enteropathy with colitis-generalized autoimmune gut disorder (GAGD)	Unknown	Unknown	Unknown

Table 18. Baseline demographic and clinical characteristics of children with Congenital Chloride Diarrhea

Number	22
Age at diagnosis, *month*	39 ± 42
Fecal volume, *ml/Kg/day*	42.3 ± 19.3
Evacuations, *n/day*	3.6 ± 1.8
Fecal Na concentration, *mmol/l*	83 ± 22
Fecal Cl concentration, *mmol/l*	147 ± 15

Data are expressed as Mean ± SD when not specified

Table 19. Molecular basis and protein functions of enrolled patients with congenital chloride diarrhea

Pts	Mutation	Nucleotide	Amino Acid	Exon	Protein Product	Hypothesis on DRA function
1	Missense	c.1960T>C	p.S654P	17	Unfolded protein	Protein retained in ER
	Missense	c.1529C>T	p.T510M	14	Folded protein	Decreased conductance
2	Missense	c.1312T>C	p.S438P	12	Folded protein	Decreased conductance
	Frameshift	c.614delT	p.L205RfsX28	6	Truncated	No function
3	Missense	c.1484A>C	p.Q495H	13	Folded protein	Decreased conductance
	Missense	c.1640C>A	p.A547E	15	Folded protein	Decreased conductance
4	Missense	c.1181G>T	p.S394I	10	Folded protein	Decreased conductance
	Missense	c.1181G>T	p.S394I	10	Folded protein	Decreased conductance
5	Nonsense	c.2132T>G	p.L711X	19	Unfolded protein	Protein retained in ER
6	Nonsense	c.559G>T	p.G187X	5	Truncated	No function
7	Nonsense	c.559G>T	p.G187X	5	Truncated	No function
8	Nonsense	c.559G>T	p.G187X	5	Truncated	No function
9	Nonsense	c.559G>T	p.G187X	5	Truncated	No function
10	Nonsense	c.559G>T	p.G187X	5	Truncated	No function
11	Nonsense	c.559G>T	p.G187X	5	Truncated	No function
12	Nonsense	c.559G>T	p.G187X	5	Truncated	No function
13	Nonsense	c.559G>T	p.G187X	5	Truncated	No function
14	Frameshift	c.614delT	p.L205RfsX28	6	Truncated	No function
	Missense	c.358G>A	p.G120S	4	Not determined	Not determined
15	Frameshift	c.1758delG	p.L586FfsX4	16	Truncated	No function
16	Exon Deletion	c.2008-151_2061+1546del	Not predictable	18	Truncated	No function

Table 19 (continued from page 97) . Molecular basis and protein functions of enrolled patients with congenital chloride diarrhea

Pts	Mutation	Nucleotide	Amino Acid	Exon	Protein Product	Hypothesis on DRA function
17	Abolished Splicing Acceptor site	c.1408-1G>C	Not predictable	13	Truncated	No function
18	Abolished Splicing Acceptor site	c.1312-1G>T	Not predictable	12	Truncated	No function
19	Frameshift	c.2022_2024dup	p. I675dup	18	Unfolded protein*	Protein retained in ER*
20	Missense	c.1522T>C	p.C508R	14	Not tested	Not tested
	Nonsense	c.1735C>T	p.R579X	16	Unfolded protein	Protein retained in ER
21	Exon Deletion	Unknown	Unknown	Unknown	Unfolded protein	Protein retained in ER
22	Macrodeletion	Unknown	Unknown	Unknown	Not predictable	Not predictable

Table 20. Pathogens possibly responsible for gastrointestinal infections in patients treated with gastric acidity inhibitors

Pathogen	Drugs°	Strength of evidences*
Bacteria		
Clostridium difficile	H_2RA / PPI	I
Non-typhoid Salmonella spp	H_2RA / PPI	I
Campylobacter jejuni	H_2RA / PPI	I
Brucella spp	H_2RA / PPI	Vb
Vibrio cholera	PPI	Vb
Parasites		
Giardia lamblia	H_2RA	Vb
Strongyloides stercoralis	H_2RA	Vb
Fungi		
Candida albicans.	H_2RA / PPI	Vb

°H_2RA: histamine 2 receptor antagonists. PPI: proton pump inhibitors
**Strength of evidences based on : Muir Gray JA. Evidences-based health care: how to make health policy and management decisions. London: Churchill Livingstone; 1997.*

Table 21. Intestinal and extraintestinal infections observed during treatment with gastric acidity inhibitors in early life

	Not -exposed to GAI (n=183)	Exposed to GAI (n=91)	*p*
Overall Infections (%)	18 (9.8)	34 (37.4)	<0.0001
Sepsis (%)	16 (8.7)	23 (25.3)	<0.0001
Pneumonia (%)	1 (0.5)	2 (2.2)	<0.0001
Urinary Tract Infections (%)	1 (0.5)	7 (7.7)	<0.0001
Acute Gastroenteritis (%)	0	2 (2.2)	<0.0001

Figure 1. Death due to diarrhea occurring in early life (data expressed per 100 000 children)

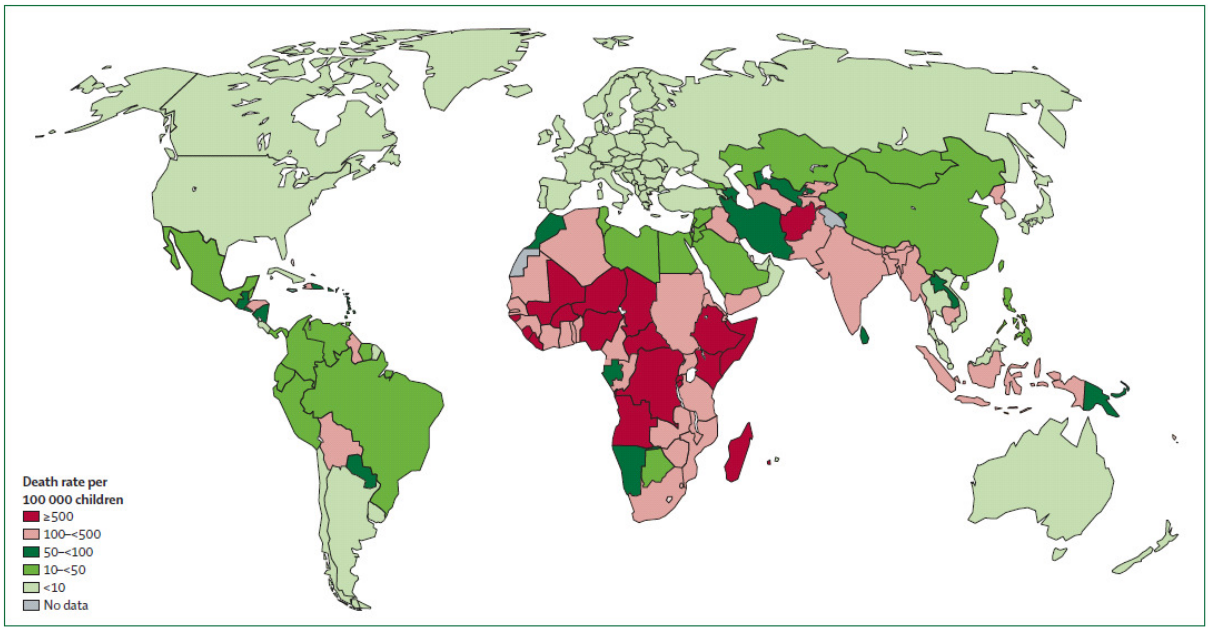


Figure 2. Mechanisms involved in the pathogenesis of chronic diarrhea occurring in early life

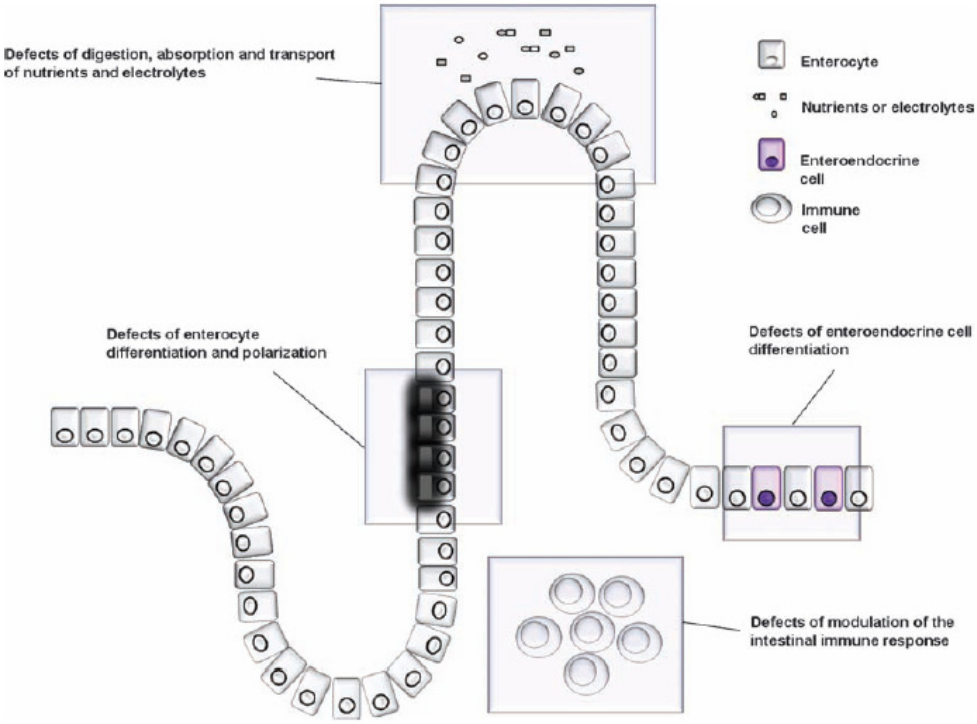


Figure 3. Interplay between the major components of the intestinal ecosystem and the expression of genetic program.

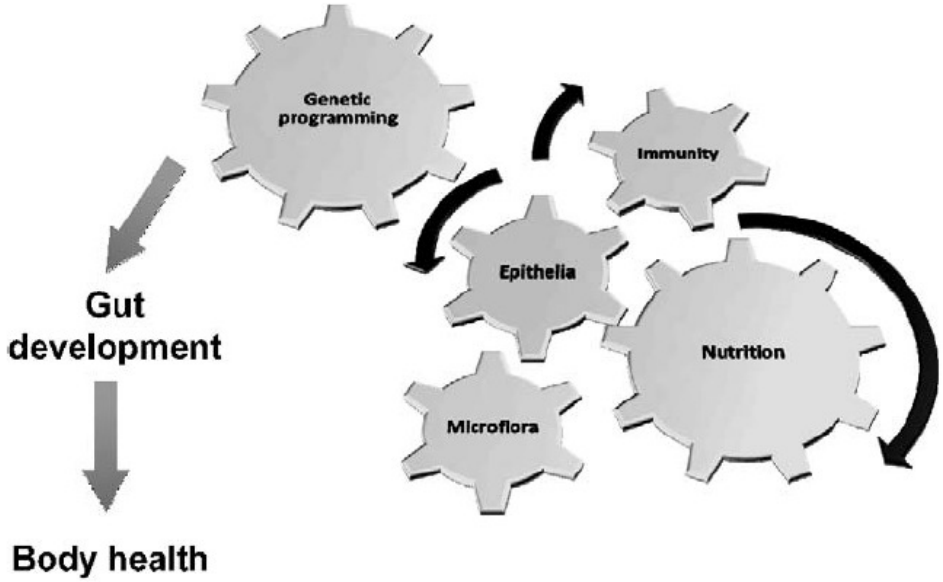


Figure 4. Main causes of intestinal failure occurring in early life

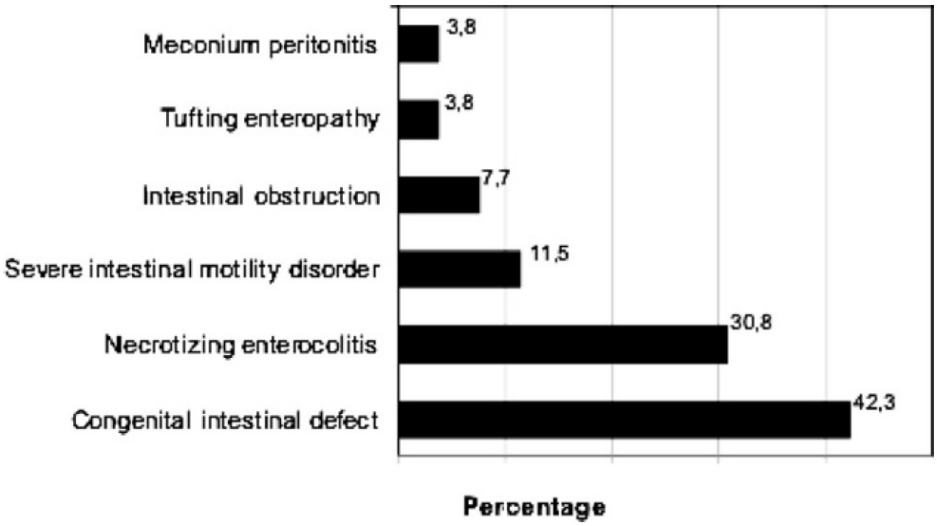
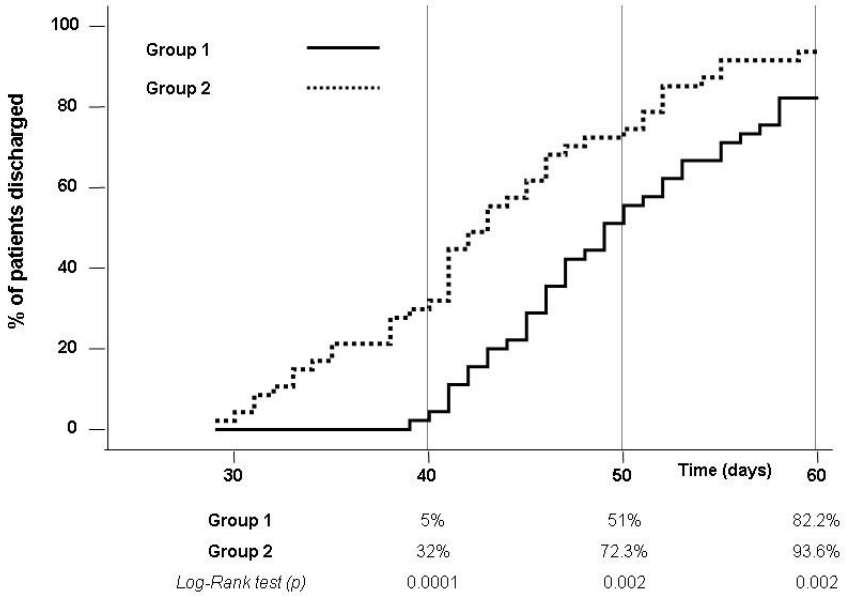


Figure 5. Duration of hospitalization in subjects dependent from parenteral nutrition treated with 2 different nutritional strategy in early life



Kaplan-Meyer analysis shows a significant difference between VLBW feeding intolerant infants administered with total parenteral nutrition (PN) (Group 1) and with PN plus minimal enteral feeding (MEF)(Group 2) at day 40, 50 and 60 of life in percentage of patients discharged from Neonatal Intensive Care Unit.

Figure 6. Comparative study on ORS involving children with acute diarrhea

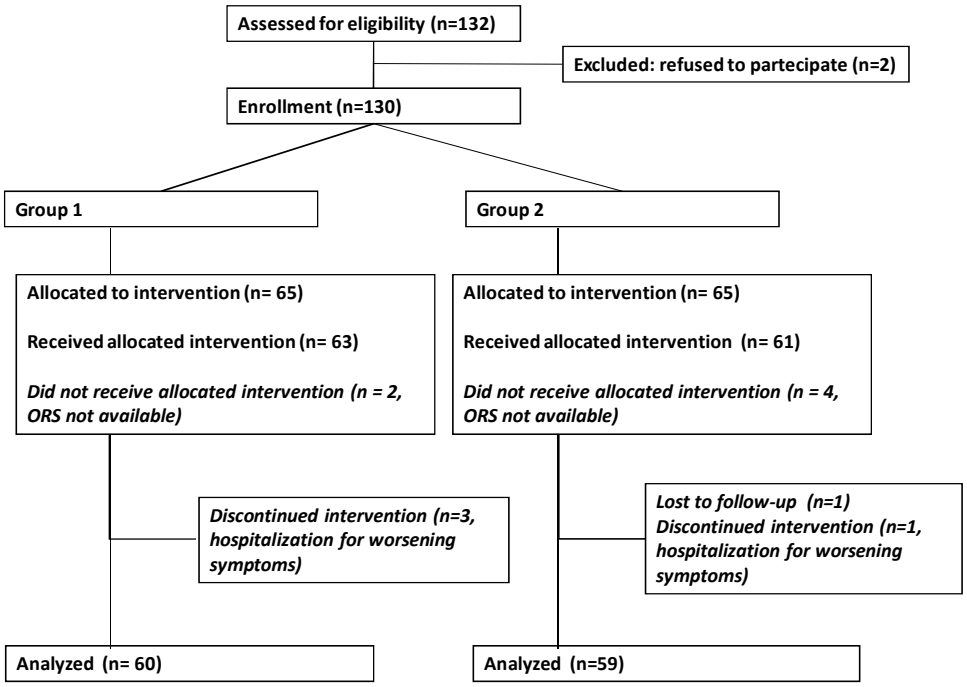


Figure 7. Comparative study on ORS involving children with acute diarrhea: probability of persistence of diarrhea in first 72 hours of treatment

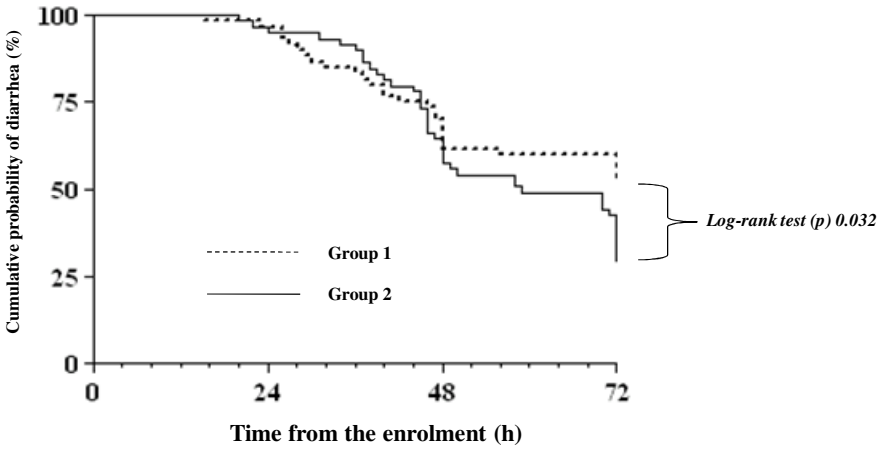
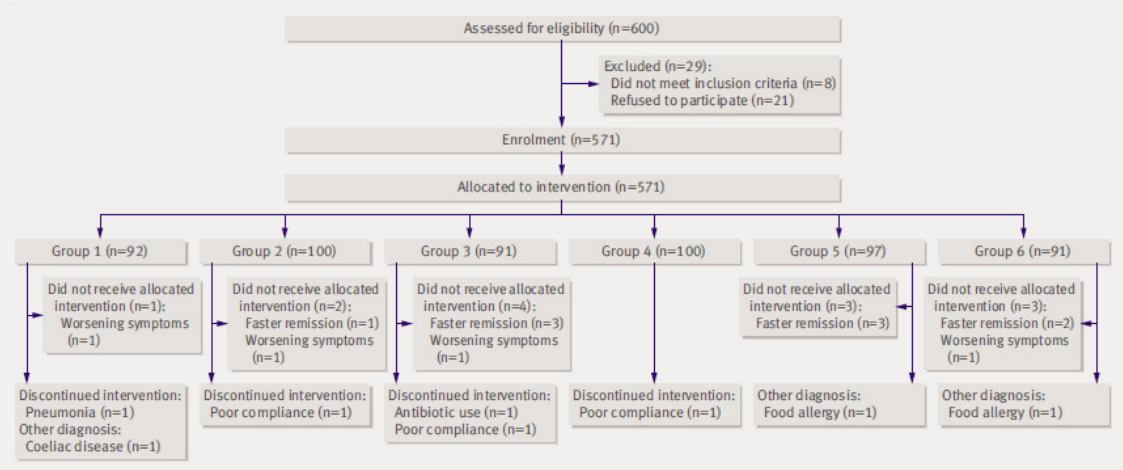
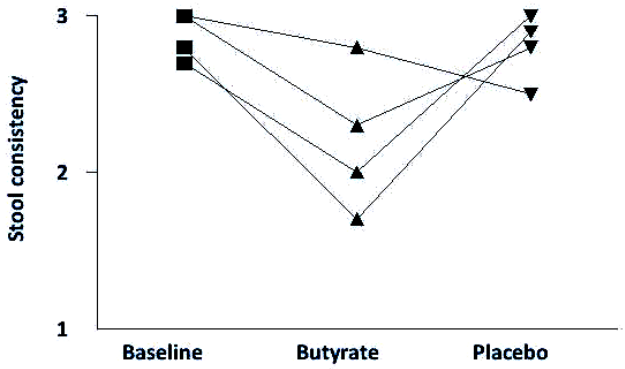


Figure 8. Comparative study on probiotics involving children with acute diarrhea: flow of patients



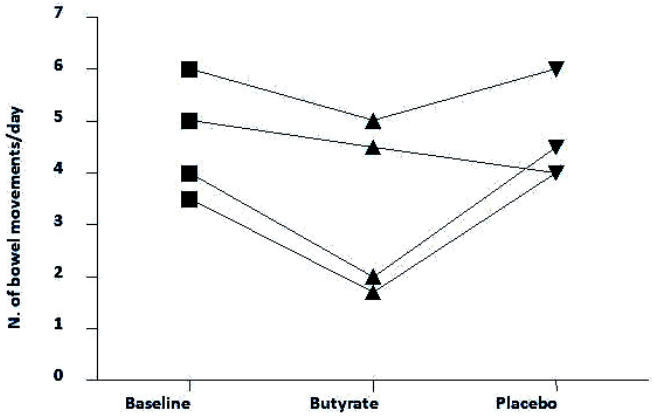
Flow of participants through trial of probiotic preparations for treatment of childhood acute diarrhoea

Figure 9. Efficacy of butyrate in children with congenital chloride diarrhea



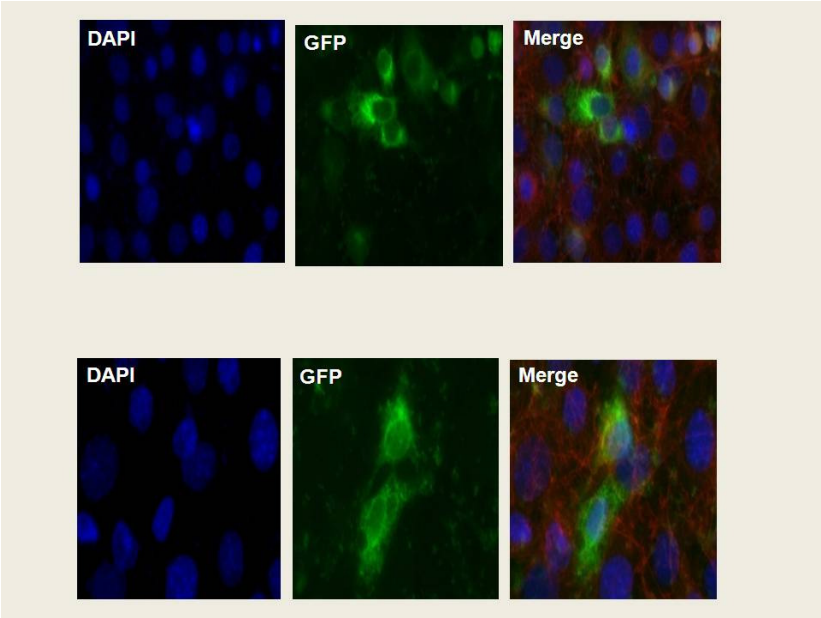
Stool consistency of children with congenital chloride diarrhea during a trial with oral butyrate

Figure 10. Efficacy of butyrate in children with congenital chloride diarrhea



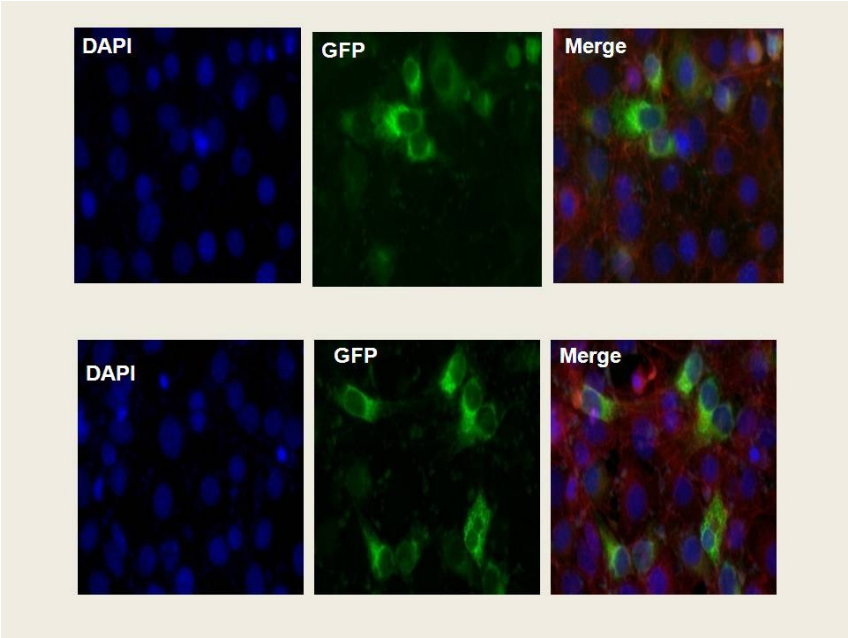
Number of daily bowel movements of children with congenital chloride diarrhea during a trial with oral butyrate

Figure 11. Fluorescence microscopy analysis of mutant gene of congenital chloride diarrhea



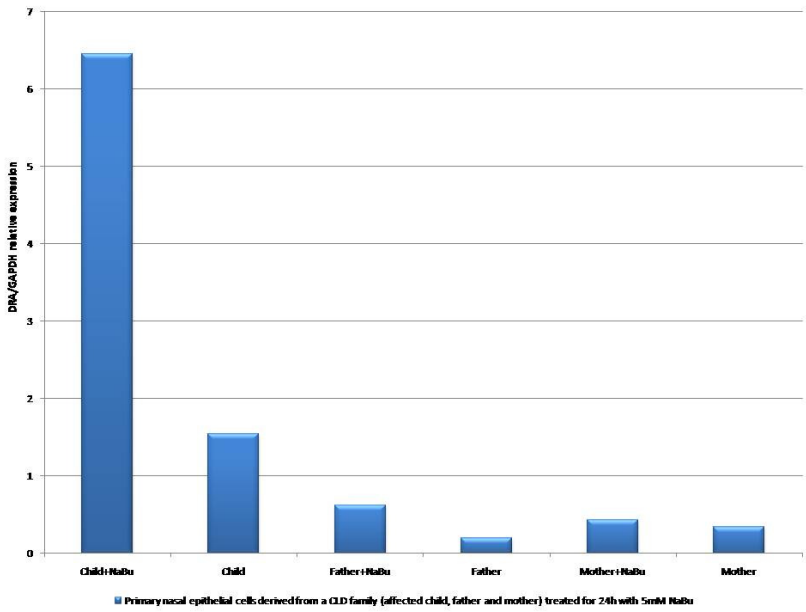
Fluorescence microscopy analysis of SLC26A3 p.Q495H mutant (bottom) in comparison to the wild-type (top). The first column showes the staining of the nucleus obtained with DAPI (blue); the second column shows the SLC26A3 protein fused with GFP (green); the protein is present in the cytoplasm and at membrane level. The third column shows the overimposition of the first two images with an addictional coloration in red (falloidine) that marks the cytoskeleton. This figure confirms the main localisation of the protein at membrane level. The comparison between the mutant (bottom) and the wild-type (top) protein shows that there are no significant variations of the cellular localization of the SLC26 A3 mutated protein.

Figure 12. Fluorescence microscopy analysis of mutant gene of congenital chloride diarrhea



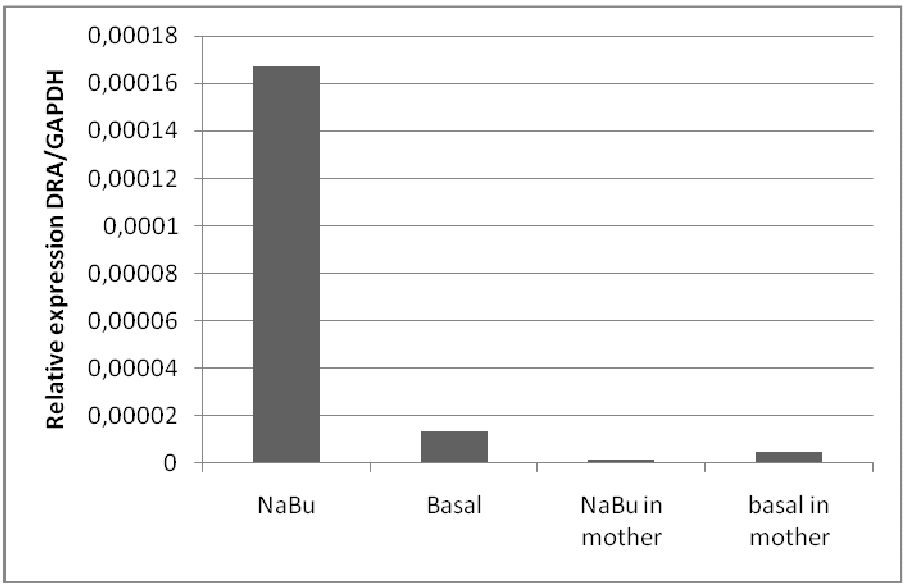
Fluorescence microscopy analysis of SLC26A3 p.A547E mutant (bottom) in comparison to the wild-type (top). The first column showes the staining of the nucleus obtained with DAPI (blue); the second column shows the SLC26A3 protein fused with GFP (green); the protein is present in the cytoplasm and at membrane level. The third column shows the overimposition of the first two images with an addictional coloration in red (falloidine) that marks the cytoskeleton. This figure confirms the main localisation of the protein at membrane level. The comparison between the mutant (bottom) and the wild-type (top) protein shows that there are no significant variations of the cellular localization of the SLC26 A3 mutated protein.

Figure 13. In vivo butyrate modulation of the gene of congenital chloride diarrhea



Effect of butyrate on DRA/SLC26A3 expression obtained in epithelial nasal cell of a children (missence mutation) with congenital chloride diarrhea and their parents

Figure 14. In vivo butyrate modulation of the gene of congenital chloride diarrhea



Effect of butyrate on DRA/SLC26A3 expression obtained in epithelial nasal cell of a children (mutation: exon deletion) with congenital chloride diarrhea and mother

Figure 15. Mechanisms of action of butyrate

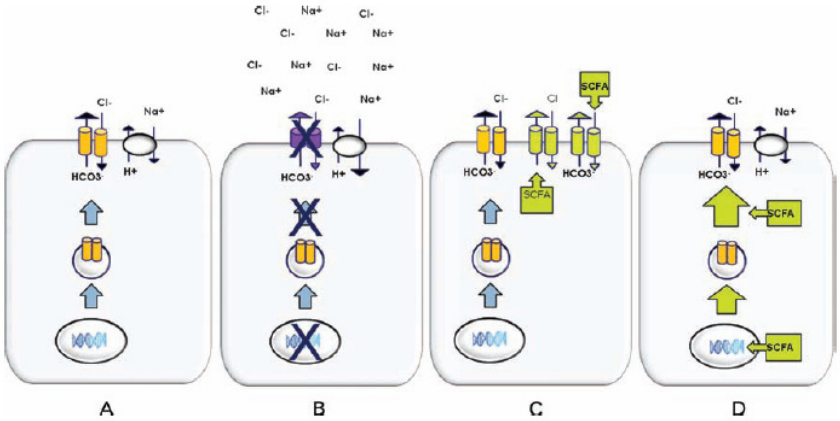


FIGURE 2. The rationale of the butyrate therapy in congenital chloride diarrhea. The most important process involved in intestinal absorption of Cl^- is the NaCl cotransporter that is mediated by 2 coupled exchangers, Na^+/H^+ and Cl^-/HCO_3^- (A). Congenital chloride diarrhea is caused by a defect in the Cl^-/HCO_3^- exchanger (SLC26A3 protein) that leads to chloride malabsorption with consequent watery diarrhea determined by an osmotic mechanism (B). Butyrate, a short-chain fatty acid (SCFA), could limit diarrhea in these patients by stimulating Cl^-/butyrate exchanger activity (C) and/or reducing the mistrafficking or misfolding of the SLC26A3 protein (D).

Figure 16. New butyrate formulation

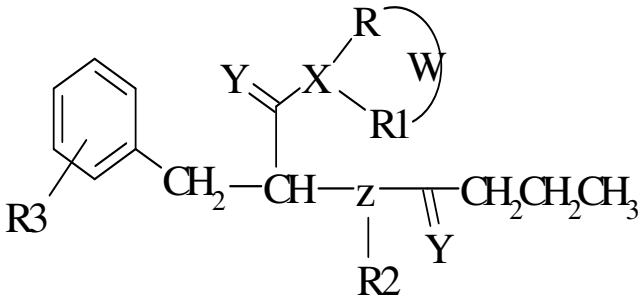
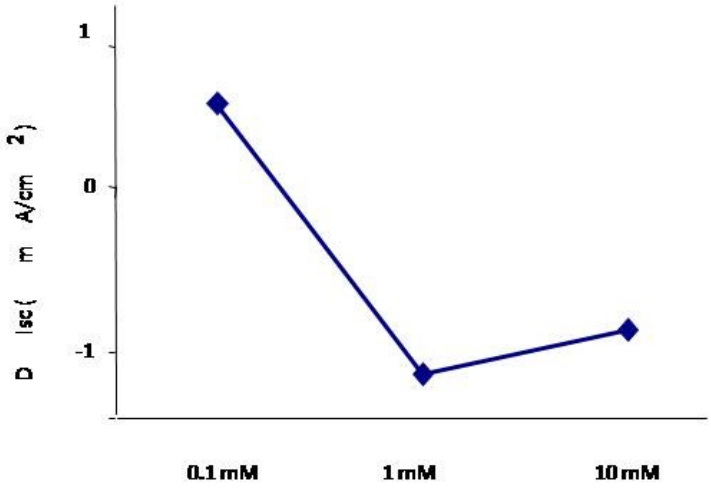


Figure 17. Effects of a new butyrate formulation on intestinal ions transport



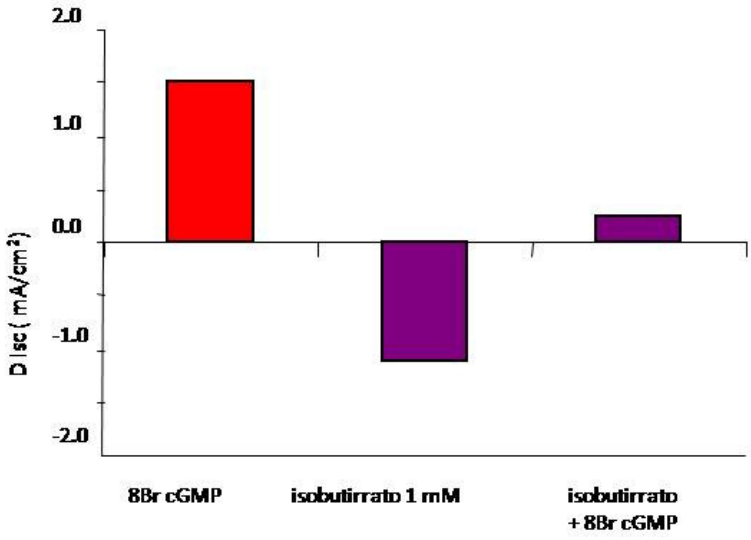
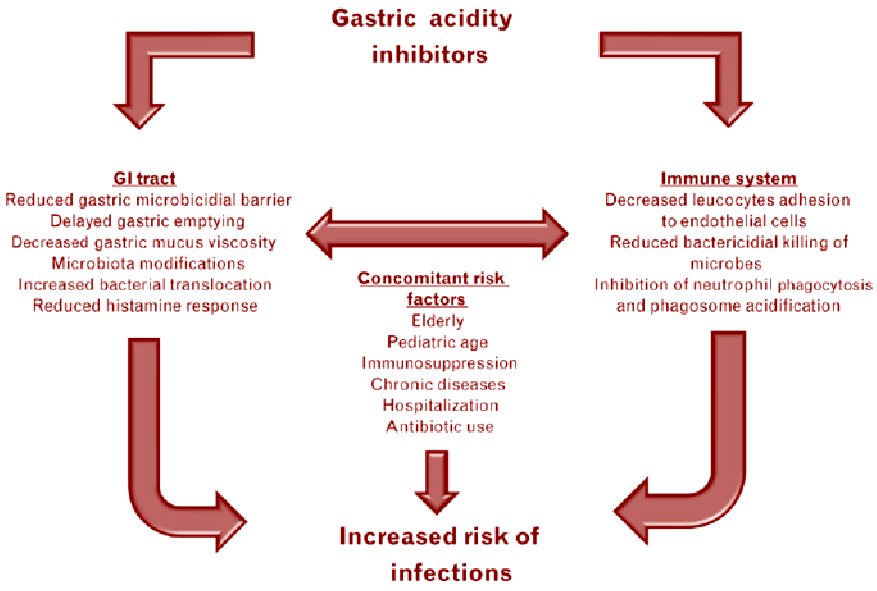


Figure 18. The multifactorial pathogenesis of the increased risk of gastrointestinal infections in patients treated with gastric acidity inhibitors



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