Pathogenesis of hemolytic uremic syndrome associated with Shiga toxin-producing Escherichia coli infections: mechanisms of toxin delivery from the gut to the target organ endothelia

Summary

Hemolytic uremic syndrome (HUS) although included in the list of the "rare diseases", represents the main cause of acute renal failure in childhood in industrialized countries. Most cases arise as a complication of intestinal infections with Shiga toxin (Stx)-producing Escherichia coli (STEC), often presenting with bloody diarrhea. The main steps in the pathogenesis of STEC-associated HUS include the colonization of the gut by the bacteria, the release of Stx in the intestinal lumen, their absorption into the blood circulation and delivery to the renal and cerebral endothelia endowed with specific toxin receptors.

The toxin-induced endothelial injury is the primary pathogenetic event in HUS and occurs when Stx have made their journey from the gut to the kidney. This incubation period, extending 2-3 days after the onset of diarrhea, might represent a window of therapeutic opportunity for implementing strategies of morbidity and mortality reduction. Two different hypothesis have been proposed to explain the mode of delivery of Stx in the blood stream: transport of free Stx in the plasma or shuttling by PMN. The clarification of this crucial point might allow the development of evidence based therapeutic strategies in the treatment of HUS. The first aim of this project will be the early enrollment of children with STEC-associated bloody diarrhea through a network connecting pediatricians, the Center for HUS control in Milan and the Regione Lombardia. This will allow the early application of oral and intravenous overhydration protocols, since hemoconcentration is associated with more severe HUS. The kinetics of Stx in the feces and blood of these patients will be studied by a daily sampling until recovery. A quantitative evaluation of the Stx present in the samples will be performed and the possible relationships between the amounts and the kinetics of Stx in the feces and the blood (plasma or PMN) of patients and their clinical features and outcome (development and severity of HUS) will be established. Experimental models of endothelial intoxication with PMN-bound Stx or free Stx will be developed to test the effects of Stx on protein synthesis, cell viability and release of proinflammatory cytokines. Understanding the mechanisms of Stx delivery from the gut to the kidney, in particular if the toxins circulate free in the blood or bound to PMN, could have an important impact on the therapeutic strategies for preventing the onset of HUS or attenuate its

clinical expression (plasmaexchange, prevention of PMN/endothelium interactions with yet available drugs: selectin inhibitors, integrin inhibitors, chemoattractant receptor inhibitors). The results of the proposed study might help to change the current standard of HUS management from purely supportive treatment to curative strategies.

Collaborations

- Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico (IRCCS-MI), Centro per la Cura e lo Studio della Sindrome Emolitico Uremica (Gianluigi Ardissino) Preliminary diagnosis of STEC infection, clinical evaluation and management of index cases. Investigation on the role of overhydration and/or Stx blood purification in preventing or mitigating HUS.

- Istituto Superiore di Sanità (ISS), Dip. Sanità Pubblica Veterinaria e Sicurezza Alimentare (Alfredo Caprioli); laboratory diagnosis of STEC infection by microbiologic and serologic methods; studies on risk factors for HUS and data elaboration.

- Università di Bologna (UNIBO), Facoltà di Medicina, Dip Patologia Sperimentale (Maurizio Brigotti) Detection and quantitative evaluation of Stx in the different blood fractions of STEC infected patients; development of an experimental model of Stx transfer from PMN to endothelium reproducing the physiological leukocyte/endothelia interactions.

- Regione Lombardia, Direzione Generale Sanità, Struttura Profilassi Malattie Infettive e Igiene Alimenti e Nutrizione (Anna Pavan) Coordination of the surveillance system for cases of bloody diarrhea.

- Progetto ALICE ONLUS, Associazione per la lotta alla SEU (Paolo Chiandotto) The Association of HUS patients and parents (www.progettoalice-seu.org) will support the project by

disseminating information and by helping to find the commitment of parents to be involved in the research project.

The partners IRCCS-MI, ISS and UNIBO have documented experience in the field of HUS and STEC infections and a long history of collaboration. The partners' experience covers public health, clinical management of and clinical research on HUS, laboratory diagnosis of STEC infections, experimental pathology in the field of Stx. This combination will represent an important factor for a successful outcome of the project.

Rational purpose, and specific impacts on the subject

HUS and STEC infection

The hemolytic uremic syndrome (HUS) is the most common cause of acute renal failure in early childhood, and is characterized by thrombocytopenia and microangiopathic hemolytic anemia (Trompeter et al. 1983). Most cases arise as a complication of intestinal infections with Shiga toxin-producing Escherichia coli (STEC) (Griffin and Tauxe, 1991; Tozzi et al. 2003). However, there are no specific antimicrobial therapies for HUS nor vaccines to prevent it. STEC are zoonotic pathogens that represent a major public health concern, because they can cause potentially fatal and often epidemic food- or waterborne illness (Caprioli et al. 2005) with a clinical spectrum that includes diarrhea, hemorrhagic colitis, and the HUS (Griffin and Tauxe, 1991). STEC produce two main types of bipartite toxins, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) that are capable to bind to glycolipid receptors (globotriaosylceramide, Gb3) present on the surface of target cells through their B subunits (Paton and Paton, 1998). After endocytosis, the A subunit of both toxins damages ribosomes and DNA (Brigotti et al. 2002), thus arresting protein synthesis and triggering the formation of apurinic sites in the nucleus. Target cells showed a broad spectrum of responses including the production of proinflammatory cytokines involved in HUS pathogenesis (Brigotti et al. 2007) and the triggering of the apoptotic program (Nakao and Takeda, 2000). There is no doubt that the major portion of the histopathological lesions observed in HUS is the consequence of the interaction of these toxins with the

endothelial lining of intestine, brain and kidney (Paton and Paton, 1998).

Pathogenesis of STEC-associated HUS

The initial symptoms of STEC infection, typically abdominal cramps and non-bloody diarrhea, manifest after an incubation period of 3-5 days (Griffin and Tauxe, 1991). There is no evidence that they are related to the action of Stx, rather they are probably caused by the characteristic "attaching and effacing" mechanism of adhesion (Nataro and Kaper, 1998) of STEC serogroups mainly associated with HUS, such as E. coli O157 and E. coli O26 (Tozzi et al. 2003). Approximately one-third of patients develop hemorrhagic colitis (Griffin and Tauxe, 1991), which probably results from Stx interaction with endothelial cells lining the microvasculature of the gut lamina propria (Richardson et al. 1992). HUS occurs in approximately one-tenth to one-fourth of cases (Karmali, 1989; Nataro and Kaper, 1998) and manifests about 1 week after the onset of diarrhea (Tarr et al. 2005). Histologically, HUS is characterized by widespread thrombotic microvascular lesions in the renal glomeruli, the gastrointestinal tract, and other organs, such as the brain (Richardson et al. 1988). Diagnostic studies of the detection of Stx and STEC in stool samples from patients indicate that STEC bacterial counts and Stx titers in stool are probably already at their peak when the initial gastrointestinal symptoms appear, and the levels decrease thereafter (Karmali et al. 1985; Tarr, 1995). Thus, Stx translocation from the gut to the circulation must occur at some point between the incubation period, when Stx is being actively produced, to about 1 week after the start of the illness, when HUS, the end result of toxin action, becomes clinically evident. While the mechanisms of endocytosis and intracellular sorting of the toxins are well known (Sandvig and van Deurs, 2005; Schuller et al. 2004), as well as the transcellular process that allows Stx to cross polarized intestinal epithelial cells (Acheson et al. 1996), the journey of the toxins from the gut to the kidney has been the object of great scrutiny and stimulated intense debates.

Potential mechanisms of Stx carriage in the blood

Stx have been found to bind to circulating polymorphonuclear leukocytes (PMN), which might

represent their carriers to renal endothelial cells since PMN do not internalize the toxic ligand (te Loo et al. 2000). Stx become bound to circulating neutrophils through an unknown receptor exhibiting 100-fold lower affinity than the Gb3 receptor present on target endothelial cells (te Loo et al. 2000). This difference in affinity is likely the molecular basis of Stx transfer from blood to the endothelial lining of kidney. Stx bound to circulating PMN can be detected by specific antibodies and flow cytometric techniques (Tazzari et al. 2004; te Loo et al. 2000) and this opportunity has been exploited for diagnosis of STEC infection in HUS patients (Te Loo et al. 2001; Tazzari et al. 2004). In a recent study, we evaluated the kinetic of Stx during the course of natural disease by detecting their presence in the feces and in the circulating PMN of children with HUS (Brigotti et al. 2006). We observed a positive relationship between the amounts of toxins present in the intestinal lumen and in the blood stream. We also showed that the toxins were detectable on the PMN several days after the onset of prodromal diarrhea and for a median period of 5 days after they were no longer detectable in stools. However, the interaction between Stx and PMN and their role in the pathogenesis of HUS is not generally accepted. Most of the criticisms have been centered on the weak affinity of the binding (Kd=10-8 M) that was defined by some authors as non-specific and of little biological significance. In fact, some experts in the field failed to appreciate the relevance of binding of a toxin to a low affinity receptor, rather than to the presumably equally accessible higher affinity receptors in renal endothelium. However, to the best of our knowledge, free Stx have never been found in the plasma of patients suffering from HUS (Karmali et al., 1985; Caprioli et al., 1992), even though some authors have hypothesized a rapid uptake of free plasmatic Stx during the incubation time that precedes HUS.

Intervention strategies to prevent or mitigate HUS in children with STEC diarrhea

Unfortunately, there are no experimental animal models of HUS in which tests can be made to determine the journey of the toxins in the different body compartments and the efficacy of treatment resulting in toxin trapping (Karmali, 2004). Observations from studies of the natural history and pathogenesis of STEC infections suggest that a narrow window of therapeutic opportunity probably exists during the incubation period and might perhaps extend to a period of 2-3 days after the onset of diarrhea. Several evidence indicate that hemoconcentration is associated with more severe HUS, both at short term, as incidence of central nervous system involvement and need of renal replacement treatment, and at long-term, as incidence of renal sequele (Beth et al. 1997; Tarr 2005; Ardissino et al. 2008). This provides to the window period between the onset of diarrhea and the onset of thrombotic microangiopathy a unique role for implementing stategies of morbidity and mortality reduction by means of appropriate rehydration. Furthermore, the possible use of plasmaexchange for Stx blood purification requires the demonstration that the toxins are actually present and removable and the identification of patients that will eventually benefit from the procedure. For the correct application of these clinical procedures (overhydration and plasmaexchange) and for increasing the likelihood of success, it is of paramount importance that cases of STEC infection are diagnosed before the development of overt HUS and that Stx delivery to the target organs is

investigated early enough during the course of the disease.

The project has two main objectives:

1. Pathogenesis of HUS: a better understanding of the crucial point represented by the mechanisms of Stx delivery from the gut to the kidney before the onset of overt HUS. This objective should be obtained by:

- investigating the kinetic of Stx in the feces and blood (free plasmatic toxins and PMN-bound toxins) of patients with STEC-associated bloody diarrhea enrolled in the study in the early stage of the infection.

- evaluating the clinical outcome with respect to the amount of Stx present in the feces and the blood of patients during the course of the infection.

- comparing the kinetics of Stx in the blood and feces in patients who: i) did not develop HUS; ii) did develop mild HUS; iii) did develop severe HUS (ie with CNS involvement).

- developing an experimental model of endothelial intoxication with PMN-bound Stx to evaluate the passage of the toxic ligand to the endothelial cells and the effects of such a passage on protein synthesis, on cell viability and on the release of proinflammatory cytokines involved in HUS pathogenesis.

- comparing the latter with conventional models of endothelial intoxication by free Stx

2. Prevention of HUS in STEC-associated diarrhea: evaluation of the efficacy of overhydration and plasmaexchange in preventing or mitigating HUS in children with STEC-associated bloody diarrhea. This objective should be obtained by:

-early identification and enrollment in the study of children with STEC-associated bloody diarrhea

-early referral of indentified cases to the Center for HUS control in Milan and early application of vigorous oral and intravenous overhydration protocol with saline solution.

-if Stx is identified as unbound to PMN in patients at high risk of developing severe HUS (with CNS involvement) these will be eventually addressed to detoxification procedure by means of plasmaexchange

Impact of the project results on the subject

Understanding the mechanisms of Stx delivery from the gut to the kidney, in particular if the toxins circulate free in the blood or bound to PMN, could have an important impact on the therapeutic strategies for preventing the onset of HUS or attenuate its clinical expression (plasmaexchange, prevention of PMN/endothelium interactions with yet available drugs: selectin inhibitors, integrin inhibitors, chemoattractant receptor inhibitors).

The results of the proposed study, in case the working hypothesis is proven, is likely to change the current standard of HUS management from purely supportive treatment to curative strategies.

Originality of the project

The project aims at producing data on the pathogenesis of HUS that are not presently available in the literature. The original issues that will be addressed are detailed below.

1. The kinetics of Stx in the body compartments of patients with STEC infection

Our group has already investigated the presence of Stx in the feces and in the circulating PMN of children with STEC infection (Brigotti et al. 2006). However, the patients had already developed overt HUS and the study was conducted on samples not collected on a systematical basis (the first feces and blood specimens were collected as soon as possible after hospital admission). In this project, we plan to study the kinetics of Stx in the feces and blood of patients with STEC infection in an early stage, e.g. before the onset of HUS, and by a regular daily collection of samples in all enrolled patients with bloody diarrhea until recovery. We will make a quantitative evaluation of the Stx present in the samples and will investigate the possible relationships between the amounts and the kinetics of Stx in the feces and the blood of patients and their clinical features and outcome (development and severity of HUS).

2. The mechanisms of Stx carriage in the blood

We will investigate the presence of Stx in the plasma and PMN fractions of the blood samples collected from cases of STEC infection, to definitely clarify this issue, which still represents an important matter of controversy.

Development of an experimental model of Stx transfer from PMN to endothelium

The project objectives include the development of experimental models to investigate: i) the binding of Stx to PMN in vitro and the effects on their activation status (degranulation, production of reactive oxygen species); ii) the mechanism of toxin transfer from PMN to endothelial cells during transmigration; iii) the response of these cells to intoxication (protein synthesis, cell viability and release of proinflammatory cytokines involved in HUS pathogenesis). The model will consist of PMN, loaded in vitro with Stx, transmigrating through a confluent monolayer of human endothelial cells in a two-chambers transmigration device, with the presence of chemokines (IL-8) in the lower chamber. The originality of such a model is the reproduction in vitro of the physiological mode of interaction of circulating PMN with endothelia, which mainly occur during leukocyte transmigration in the presence of chemoattractants.

3. Prevention of HUS or attenuation of its clinical expression in children with STEC-associated diarrhea:

Most studies regarding HUS are focused on the stage of overt disease when no therapeutic intervention has proven to be efficacious, except for supportive treatment. The proposed study will identify cases before the development of HUS and will therefore provide the opportunity to test the efficacy of overhydration and/or plasmaexchange in reducing HUS-related mortality or morbidity. This is based on the working hypothesis that hemoconcentration is a major risk factor for CNS involvement and on the possibility of implementing strategies of Stx blood purification.

Development strategy of the project, methodology, preliminary data and bibliographic references

The project will combine studies performed on patients with STEC infection with the development of experimental models of endothelial intoxication with free Stx or with transmigrating PMN loaded with Stx. The clinical studies will be conducted on patients with STEC-associated bloody diarrhea enrolled in an early as possible stage of the infection. This will provide the opportunity to test the efficacy of overhydration and/or plasmaexchange in reducing HUS-related mortality or morbidity. The clinical outcome of these patients will be evaluated with respect to the amount of Stx present in blood fractions and feces during the course of the infection. The experimental models mimicking the toxin-induced renal endothelial injury will consist of i) a two-chamber transmigration device in which PMN, loaded in vitro with different amounts of Stx, transmigrate through confluent monolayers of endothelial cells; ii) endothelial cells treated with free Stx added to plasmatic fractions.

The project activities will be articulated in workpackages (WP) and tasks, with specific objectives. The ethical approval of the study protocol and the informed consensus form for the patients' parents will be requested to the Ethical Committee of IRCCS-MI.

WP1.Clinical, diagnostic and epidemiologic studies

Objectives

-To enroll patients with STEC infection in the study before the development of HUS

-To evaluate risk factors for STEC infections and for development of HUS in Italy

-To evaluate clinical strategies for preventing the onset of HUS or attenuate its clinical expression

-To evaluate the feasibility and efficacy of early plasmaexchange for unbound Stx blood purification in patients at risk for severe HUS

Task 1.1 Establishment of the surveillance system for cases of bloody diarrhea

The study will be conducted in the Lombardia Region (9 millions inhabitants) and will involve children (0-14 yrs) with frank blood in the stools and diarrhea (at least 3 loose stools per 24h). Family pediatricians will be asked to refer index cases to the nearest hospital (pediatric or emergency unit). A HUS regional network involving 1 pediatrician for each of the 63 pediatric units of the region has been developed with the official support of the Regional Health authority. The members of the HUS Network will meet regularly twice a year to organize the procedures and analyze and share the results of the network activity. Family pediatricians will be informed on the project and will be asked to refer their patients with bloody diarrhea to the nearest hospital.

Task 1.2 Enrollment and evaluation of patients

Based on the available data from the regional infectious diseases surveillance system, it can be estimated that between 600 and 1,200 patients with bloody diarrhea are expected in 1 year. One tenth of them might be caused by STEC infection and 12-25 will eventually turn into HUS. Once referred to the hospital, cases will be clinically evaluated and a stool sample will be sent to the Center for HUS in Milan to be tested for the presence of free fecal Stx by a commercial immunoassay test. The result of the test is expected within 24 hours from patient referral. Stool samples will also be tested for other pathogens. Patients negative at the free fecal Stx assay will be managed locally according to usual clinic protocols. Patients positive for fecal Stx will be invited to refer to the Center for HUS, where they will be admitted for the procedures described below.

Task 1.3 Clinical evaluation and management of patients with STEC infection

Since admission to the Center for HUS control patients will be evaluated as to hydration status and addressed to a rehydration protocol towards restoration of fluid losses and to obtain a moderate overhydration (Tarr 2005; Ardissino et al. 2008). Suspect of HUS onset will based on the following parameters: hemoglobinuria and/or proteinuria, serum LDH, aptoglobin level. HUS, diagnosed according to Trompeter (1983) will be scored as follows: Class I: no need of RRT no CNS involvement; Class II: need of RRT no CNS involvement; Class III:CNS involvement regardless of RRT need. Starting from admission and every 24 h blood and stool samples will be collected for detection of Stx. Those patients who will develop HUS will receive standard supportive treatment according to individual needs (blood transfusion, dialysis, antihypertension treatment, dietary restriction). If there is evidence of free Stx in the blood and this is correlated both to the risk of developing HUS and/or severity of HUS itself, selected patients might be addressed to procedures of Stx blood purification, such as plasmaexchange with a substitution of 100% of plasma volume with a standard solution for as many days as necessary to maintain a low Stx concentration.

Task 1.4 Evidence of STEC infection

After the early detection of fecal Stx by the commercial kit, diagnosis of STEC infection will be completed as follows. For STEC isolation, feces will be streaked onto MacConkey plates and colony sweeps tested for Stx production by the Vero cells assay and for the presence of stx genes by PCR amplification (Tozzi et al.2003).The presence of free Stx will be assessed by the Vero cell assay (Caprioli et al.1992). Serum samples will be tested for antibodies to the LPS of five major STEC serogroups (O157, O26, O103, O111,O145) by ELISA and immunoblotting

(Caprioli et al.1994).

Task 1.5 Epidemiological studies

Information on patients will be collected by a questionnaire administered to their parents. A matched case control study for the identification of risk factors associated to STEC infection will be carried out. Evaluation of excess risk of STEC-associated bloody diarrhea will provide useful information for the estimation of the burden of STEC infection in pediatric population.

WP2. Studies on the pathogenesis of STEC infection and the development of HUS

Objectives

-To investigate the kinetics of Stx in the blood and feces of patients with STEC infection.

-To clarify if Stx circulates free in the blood or bound to PMN.

-To evaluate the clinical outcome with respect to the amount and the kinetics of Stx in the feces and the blood of patients.

Task 2.1 Evaluation of the kinetics of Stx in the organism of patients

The quantitative evaluation of free Stx in stools will be performed by the Vero cell cytotoxicity assay as described previously (Caprioli et al. 1995). Briefly, doubling dilutions of fecal filtrates

will be inoculated into Vero cell and the titer of the cytotoxic activity will be expressed as the reciprocal of the highest dilution inducing cytopathic effect. Stx will be identified by neutralization tests. Blood samples will be examined for the presence of Stx on either the PMN or the plasma fractions. Stx bound on PMN will be detected by flow cytometry as previously described (Tazzari et al., 2004). The assay has been validated by comparing control subjects and HUS patients in a blinded fashion (Tazzari et al., 2004) and by challenging Stx-positive PMN with a negative control antibody (Brigotti et al., 2006). The mean channel value of fluorescence (MCV) will be used as objective parameter for the quantitative determination of Stx bound to PMN (Tazzari et al., 2004). Plasma from patients will be tested for the presence of free Stx by a protein synthesis inhibition assay conducted on human umbilical endothelial cells (HUVEC) or on Vero cells (Brigotti et al.2002).

To establish relationships between the main clinical features of the patients and the amounts of Stx present in their feces and blood we will daily record clinical and laboratory findings: platelet and neutrophil counts, hemoglobin and serum creatinine concentrations, need for dialysis and/or transfusions, and neurological complications. The clinical data recorded will be matched with the measured parameters of fecal and plasmatic or PMN-bound blood toxins in order to find the critical breakpoint before the onset of HUS.

WP3. Development of experimental models

Objectives

-To study the effect of Stx binding on PMN function (activation, degranulation)

-To study the transfer of Stx from PMN to endothelial cells and to investigate the endothelial cellular response that follows intoxication

-To study if PMN loaded with different amounts of Stx can elicit different responses in endothelia

Task 3.1. Binding of Stx to PMN and related effects

Highly purified endotoxin-free PMN will be isolated from different healthy donors (Brigotti et al.2008). The binding of Stx to PMN will be performed with native and radiolabeled Stx to obtain Stx saturation levels comparable with those previously observed in HUS patients (Tazzari et al.2004; Brigotti et al.2006). The occurrence of degranulation will be revealed by assessing the presence of specific degranulation markers on PMN surface by flow cytometry. Oxidative burst as a marker of activation will be measured by flow cytometry by using dihydrorhodamine 123.

Task 3.2. Development of a model of PMN transmigration through an endothelial cell monolayer

A two-chambers transmigration device in which HUVEC are seeded in the upper chamber to form a confluent monolayer will be deployed. PMN will be treated with Stx as described in Task 3.1 to obtain amounts of bound Stx comparable with those observed in the PMN from HUS patients, and will be stimulated to transmigrate through the monolayer by the presence of IL-8 in the lower chamber. The effect of the transmigration of PMN carrying different amounts of Stx on the protein synthesis of the endothelial cells will be evaluated as described in Task 2.1. The production of proinflammatory cytokines (IL-8, MCP-1) will be evaluated by ELISA (Brigotti et al.2007) in the overnight culture supernatants of HUVEC after PMN transmigration. Necrosis or apoptosis in intoxicated cells will be evaluated by fluorescence microscopy after staining with Hoechst 33342 and propidium iodide. All the results will be compared with those obtained with the same model in the absence of chemoattractant and in cultured HUVEC treated with free Stx added to plasmatic fractions from healthy donors.

Structure and equipment available for research and collaborations

Partner IRCCS-MI is the regional reference centre for HUS (Centro per la Cura e lo Studio della Sindrome Emolitico Uremica) where all children with HUS are referred to and, together with the Lombardia Regional Health Office is responsible for the survelliance system of HUS over the entire region. A Regional Network for HUS involving all 63 Pediatric Units of the region has

been recently developed, to improve the management of HUS cases. The Department of Pediatrics has a capability of 50 inpatients and is equipped for dialysis (peritoneal dialysis, hemodyalisis, hemofiltration) in preterm and term neonates, infants and children. The Unit is the only one in the region equipped for plasmaexchange in children. IRCCS-MI has many scientific collaborations, including those with the Division of Pediatric Nephrology, Center for Pediatric and Adolescent Medicine, University of Heidelberg,Germany and the Molecular Biology Laboratory at the University of Iowa, US.

Partner ISS is the National Reference Laboratory for E.coli and the equipment available involves both classical microbiology and molecular biology. It includes: class 2 microbiology laboratories and three class 3 biohazard laminar flow cabinets. Molecular biology equipment includes conventional and real-time PCR machines for the detection of virulence genes, a PFGE apparatus and a shared nucleotide sequencer for molecular typing (MLVA),facilities for cell cultures to be used for cytotoxicity assays. The laboratory is accredited according to ISO/IEC 17025, and participates in several international research and surveillance activities, such as:

-The Food and Waterborne Diseases and Zoonoses (FWD) Surveillance Network, of the European Centre for Disease Control (ECDC), which is constituted by the reference laboratories in EU Member States.

-The EU VI FP Network of Excellence "MED-VET-NET, the European Network of Excellence for research on the prevention and control of zoonoses

-The Pathogenic E. coli Network (PEN), an EU Coordination Action on the pathogenesis of E.coli infections.

Partner UNIBO has facilities for biochemistry, molecular biology and cell biology. Equipment for toxin purification includes an FPLC apparatus and conventional chromatography tools. Molecular biology facilities include protein and nucleic acid electrophoresis and Western blot apparatuses, microcentrifuges, thermal cyclers for PCR, thermoblock, and a fume hood. The laboratory has cell culture facilities, including a biohazard vertical laminar flow hoods, two horizontal laminar flow hoods, one CO2 incubator, phase-contrast microscope, video camera, and general purpose microscopes, The laboratory has also access to shared resources including a flow cytometer (two lasers and CXP-dedicated program), a fluorescence microscope, an H.P.L.C. apparatus, an U.V. transilluminator with digital camera, anELISA

reader, centrifuges and ultra-centrifuges, a betacounter, a gamma-counter. The laboratory participates in the surveillance activities of Italian Registry of HUS. The collaboration of partner UNIBO include several Nephrology pediatric center (Milan, Rome and Naples), the Department of Molecular Medicine of the University of Parma (Italy), the Institute of Pharmacology of the University of Urbino (Italy) and the Transfusion Center, S. Orsola Hospital, Bologna (Italy).

Partner Regione Lombardia coordinates the official surveillance system of infectious diseases in the area of the study and has developed a web based reporting system for this purpose. The partner will assure the cooperation of the public health system and will coordinate the surveillance system for cases of bloody diarrhea.

Relevance of the project for the National Health Service (on basis of rapid trasferibility on assistance)

So far, the standard management of HUS has been based on supportive treatment (hemotransfusion, fluid restriction, renal replacement therapy, antihypertensive, etc). Understanding the mechanisms of Stx delivery to the target organ endothelia before the onset of HUS could provide clues for the development of new treatment options.

According to previous observations (Beth et al. 1997; Tarr 2005; Ardissino et al. 2008) and to our working hypotheses, fluid restriction cannot be appropriate for HUS patients and overhydration is expected to prevent or mitigate the severity of the disease with a significant reduction in costs arising from the management of HUS patients (intensive care, acute dialysis, chronic dialysis, renal trasplantation).

Another possible benefit of the study is an increased awareness of family doctors towards STEC infections, and in particular STEC-associated bloody diarrhea. In Italy, although in northern regions such as Lombardia the incidence of HUS is comparable to that reported in northern Europe countries (Tozzi et al, 2003), STEC infections are rarely diagnosed, due to the lack of awareness by both pediatricians and clinical microbiologists. A change in the attitudes through 'ad hoc' guidelines could have a significant impact on HUS-related mortality and morbidity.

Finally, the project will contribute to the consolidation of the surveillance system for intestinal infections in Lombardia, the most populated Italian region, and the data collected on the incidence of STEC-associated bloody diarrhea will be useful for the evaluation of the burden of STEC-associated diseases in Italy.

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