Particulate Shiga Toxin 2 in Blood is Associated to the Development of Hemolytic Uremic Syndrome in Children

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Abstract

Hemolytic uremic syndrome (HUS), the leading cause of acute renal failure in children (<3 years), is mainly related to Shiga toxins (Stx)-producing *Escherichia coli* (STEC) infections. STEC are confined to the gut resulting in hemorrhagic colitis, whereas Stx are delivered in blood to target kidney and brain, with unclear mechanisms, triggering HUS in 5-15% of infected children. Stx were found on circulating cells, free in sera (soluble Stx) or in blood cell-derived microvesicles (particulate Stx), whereby the relationship between these forms of circulating toxins is unclear. Here, we have examined 2846 children with bloody diarrhea and found evidence of STEC infection in 5%. Twenty patients were enrolled to study the natural course of STEC infections before the onset of HUS. In patients, Stx were found associated to circulating cells and/or free and functionally active in sera. In most children Stx were bound to neutrophils when high amounts of toxins were found in feces. Time-course analysis showed that Stx increased transiently in patients' sera while the decrease of toxin amount on leukocytes was observed. Notably, patients who recovered (85%) displayed different settings than those who developed HUS (15%). The distinctive feature of the latter group was the presence in blood of particulate Stx2 (Stx2 sedimented at *g*-forces corresponding to 1 μm-microvesicles) the day before diagnosis of HUS, during the release phase of toxins from circulating cells. This observation strongly suggests the involvement of blood cell-derived particulate Stx2 in the transition from hemorrhagic colitis to HUS.

Introduction

Most cases of hemolytic uremic syndrome (HUS) are the consequence of intestinal infections by Shiga toxins (Stx)-producing *Escherichia coli* (STEC)[1-3]. These bacteria enter human body mainly through contaminated food and water, and then intimately adhere to the intestinal mucosa, hence altering adsorption functions and causing watery diarrhea[1-3]. STEC multiply in the intestine, where they are confined, and release powerful AB5 toxins (Stx1 and/or Stx2) which reach the lamina propria and damage the endothelial lining of the gut microvasculature, thus causing bloody diarrhea (BD)[1-3]. Within a short time (5-7 days), 5-15% of the infected patients develop HUS due to the presence of Stx in blood and to their action on the endothelial cells of the microvasculature of kidney, brain and few other organs[1-3]. Damage/dysfunction of endothelial cells in these body compartments triggers the formation of microthrombi which consume platelets, reduce renal flow and mechanically damage red cells. As a consequence, the HUS triad arises: fall in platelet count, acute renal injury and red cell lysis with anemia[1-3]. Thrombotic microangiopathy may also occur in other organs such as brain. Accordingly, some patients with HUS experience neurological symptoms (severe headache, lethargy, seizures)[3,4]. Moreover, a cytotoxic action of Stx towards erythroblasts has been reported which might contribute to the fall in red cell count[5].

Sensitive endothelial cells express a glycolipid receptor (globotriaosylceramide, Gb3Cer) targeted by the B-chain pentamer of Stx[4,6]. After toxin internalization, the A-subunit removes adenine residues from rRNA in ribosomes and from nuclear DNA, producing irreversible inhibition of protein synthesis[4,7,8]. In humans, Stx A-chain is recognized by circulating cells through engagement of Toll-like receptor 4 (TLR4)[9], as in the case of the bacterial lipopolysaccharide (LPS)[10]. In blood, Stx bind to monocytes and platelets which express both the

B and A-chains specific receptors (Gb3Cer and TLR4, respectively) and to neutrophils which in humans express only TLR4[11,12]. These multiple interactions with blood components allow the formation of leukocyte/platelet aggregates and the release of microvesicles bearing Stx, tissue factor and activated complement components[13-15]. Microvesicles derived from leukocytes and platelets have been suggested to be involved in the transport of the toxins to target cells and in their intoxication/dysfunction since they have been found in the blood of patients during HUS[13,15]. Different studies on STEC-infected patients have demonstrated the presence of Stx on circulating cells or in sera[16-24]. Most of them have been focused on patients with HUS[16-20,22,23], or the studies have been mostly dedicated to a specific blood subset. It should be noted that very few groups were able to detect Stx in blood[21,24], only when STEC-infected patients were studied before the onset of HUS. Nevertheless, the relationship between the fleeting appearance of Stx in sera and the development of HUS remained puzzling[21]. Moreover, negative (HuSAP, human serum amyloid P component) or positive (soluble TLR4) modulating factors which bind to Stx2 preventing or favoring its toxic activity have been described[25-28]. Therefore, it is important to study the journey of Stx in the blood of STEC-infected patients in the days preceding the onset of HUS, considering their presence in serum and in different blood components, and the relationship with the clinical features emerging in children during the observation. This would shed some light (i) on the reason why only a small percentage of STEC-infected patients develop HUS after the intestinal phase, and (ii) on the trigger allowing the transition from BD to HUS in humans.

Here we show the time-course of Stx in the blood of 20 STEC-infected children and the emergence of blood cell-derived particulate Stx2 during the development of HUS.

Materials and Methods

Case definition

In Northern Italy, a screening program for the detection of Stx in the stools of children (<18 years) with acute BD is ongoing since May 2010, with 56 pediatric units covering a population of ~2 million. The operative definition of BD is as follows: acute diarrhea with visible blood in at least 1 bowel movement, either seen by health professionals or reported by caregivers. HUS was defined as the concomitant presence of low platelet count (<150x10^9/L), evidence of non-immune-mediated hemolysis (Coombs negative), hemoglobin concentration reduction and increased serum LDH level (>330 U/ml) or haptoglobin <LNL or schistocyte >1% and acute renal damage (serum creatinine or urinary proteins/urinary creatinine ratio above the upper limit for age) in the setting of Stx-positive BD.

Enrollment assay

Enrollment test for Stx gene identification in feces was performed as follow. Stools aliquots were inoculated onto MacConkey broth, after 18-24 hours DNA from bacteria was extracted and used for identifying *stx1, stx2* and *eae* genes by PCR-based reverse dot blot. This diagnostic approach includes the amplification of the target genes by PCR and their visualization through hybridization with specific probes immobilized on a membrane strip. The hybridized amplicons are then revealed through labeling with alkaline phosphatase and a colorimetric reaction (GenoType EHEC—Arnika).

Patients' enrollment and presentation

Between 2010 and 2015, 2846 pediatric patients presenting with BD were tested for STEC infection using rapid screening assays (enrollment assay). Among the 155 positive children, 20 consecutive

patients were enrolled according to specific inclusion or exclusion criteria (Supplementary Scheme S1). The enrolled patients were hospitalized (Center for HUS, Milan) and subjected to complete diagnostic procedures and kinetic studies. Patients were not related, their median age was 24 months (range: 6-188), 50% was female. Samples from patients and healthy donors were taken with the informed written consent of their parents and of the subjects if over 15 years. The study was approved by the Ethics Committee of the Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Milan, Italy (May 18th, 2010). All the records of the 20 enrolled patients were reviewed to collect detailed information. Patients received generous intravenous fluid infusions (+10% working weight) since this treatment is known to improve short and long-term disease outcome in patients with STEC-induced HUS[29]. Suspicion of HUS onset was monitored through daily urine analysis for the appearance of hemoglobinuria. Children with hematuria did undergo blood tests to check platelet count, serum LDH, haptoglobin levels and creatinine levels to evaluate renal function. Patients who developed HUS during the study have received standard supportive treatment according to individual needs (blood transfusion, dialysis, antihypertension treatment, dietary restriction). Starting from admission and every 24 h, blood, serum and stool samples were collected for chronological studies and for laboratory diagnosis of STEC infection (see below). Blood was obtained by venipuncture and serum by centrifuging at 1250*g* for 10 min.

Evidence of STEC infection

A loopful of each stool sample was inoculated in 10 ml tryptic soy broth and incubated at 37°C for 18 h. An aliquot of overnight culture (1 ml) was treated with the InstaGene Matrix (Bio-rad Laboratories, Hercules, CA, USA) to extract the DNA, as indicated by the supplier. The DNA was diluted 1:10 and subjected to real-time PCR for the identification of the Stx-coding genes (*stx1* and

stx2)[30] and the intimin-coding gene (*eae*)[31]. PCR-positive cultures were streaked onto MacConkey agar plates and the isolated colonies were singularly transferred onto nutrient agar and tested for the presence of *stx* and *eae* genes by End point PCR amplification[32,33]. The *stx*- and/or *eae*-positive isolated strains were subjected to serogroup determination by slide agglutination using O antisera against different STEC serogroups (O26, O103, O111, O145, O157; Statens Serum Institut, Copenhagen, Denmark). Fecal extracts of the stool specimens were also examined for the presence of Stx by the Vero cell cytotoxicity assay[18].

Detection of Stx on patients' leukocytes

Stx bound to neutrophils were detected by indirect flow cytometric analysis in the presence of monoclonal antibodies to Stx1 and Stx2 (Stx1-13C4, Stx2-BB12 Toxin Technology,Sarasota, FL, USA)[34]. The mean channel value of fluorescence (MCV) was chosen as an objective parameter to measure the extent of binding of Stx to neutrophils. The assay was validated by comparing control subjects and HUS patients in a blinded manner[34] and by challenging Stx-positive neutrophils with a negative control antibody[16].

Detection of functional Stx in patients' sera by Raji assay

Human Gb3Cer-expressing Raji cells were used for the detection of the translation inhibition induced by Stx in patients' blood as described in Arfilli et al.[24]. Protein synthesis was measured, after incubation (3-20 h) with 50 µl of sera from STEC-infected patients, as the rate of incorporation of [^{3}H]leucine into proteins during 60 min incubation in complete medium[24]. Stx-dependent translation inhibition was confirmed by the addition of monoclonal antibodies to Stx1 and Stx2 that abolished or strongly reduced the induced effects. As additional control, ribosomes from Raji

cells treated with purified Stx2[35] were isolated and assayed (luminometric test) for their ability to accomplish protein synthesis[36]. The inhibitions of protein synthesis obtained in Stx2-treated samples (Raji cells or isolated ribosomes) were very similar at 3 h (62% or 67%; respectively) confirming the specific detection of Stx-induced injuries in cells. At 20 h, the inhibition obtained in whole cells (87%) is higher than that measured with isolated ribosomes (62%) indicating the presence of secondary effects following ribosomal impairment.

Detection of the amount of Stx in patients' sera by ELISA

Stx1 and Stx2 present in patients' sera were quantified by a specific improved ELISA as previously reported[37]. The determinations were performed on untreated serum or on fractionated serum prepared as follow. Serum samples from patients (100 µl) were centrifuged at 21000*g* for 40 min at 21 °C, then the upper serum fraction (50 µl) was withdrawn (21000*g* upper fraction). Particulate Stx present in the remaining volume was carefully resuspended in the residual serum by pipetting and, finally, 50 µl of the suspension were withdrawn (21000*g* lower fraction). This procedure avoided loss of particulate Stx since pellets were not visible after centrifugation. In both lower and upper fractions, the concentrations of Stx were evaluated by ELISA. Assuming that the 21000*g* lower fraction contains particulate Stx and an amount of soluble Stx equal to that present in the 21000*g* upper fraction, particulate Stx concentration was calculated by subtracting the 21000*g* upper fraction concentration from the 21000*g* lower fraction concentration and by dividing by 2 the obtained result. The latter operation was necessary since particulate Stx sedimented in the 50 µl-lower fraction derived from 100 µl serum. The above-mentioned assumption was validated by performing control experiments: Purified Stx2[35] was added to pooled sera from healthy donors to the final concentration of 5 ng/ml and samples were incubated (in duplicate) 10 min at 37°C. The

ratio of the concentrations of Stx2 detected by ELISA in the 21000*g* lower fraction and 21000*g* upper fraction (21000*g* lower fraction/21000*g* upper fraction), obtained by centrifuging Stx2-containing serum at 21000g for 40 min at 21 °C, was 0.98 ± 0.03 (mean ± SD), i.e. lower 21000*g* fraction and upper 21000*g* fraction contained the same amount of Stx2. This demonstrates that free Stx2 or the complex HuSAP/Stx2 did not sediment at 21000*g*. In contrast, Stx2 associated to microparticles would have been expected to sediment at 21000*g*, hence causing an enrichment of the lower 21000*g* fraction and giving a ratio value higher than 1. Consistently, calculation of the amount of particulate Stx2 in these Stx2-spiked cell-free serum samples gave undetectable amount. The use of patients' plasma as source of vesicular Stx would have been preferable to achieve a setting resembling in vivo circulation. We have used patients' sera to this purpose since the sample collection (blood, sera and feces) in our study (started on May 2010) was planned well before the publication of the papers (2015-2017) suggesting the role of microvesicles in HUS[13,38]. Hence, we have detected particulate Stx in frozen serum samples that have been already collected from patients.

Detection of cell-free TLR4 in patients' sera by ELISA

Cell-free TLR4 present in patients' sera was quantified by a specific ELISA (SEA753Hu, Cloud-Clone Corp, Houston, TX, USA) according to manufacturer's instructions. The determinations and calculations were performed on untreated serum and 21000*g* serum fractions as described above.

Statistics

Statistical analysis was performed with GraphPad Prism 6 software. Differences in continuous variables (means, SD) were tested with a t-test after controlling the normality of their distribution. Differences in proportions were assessed through the Chi-square text or Fisher exact test, correlation between variables by using the Pearson correlation coefficient. Significance, for two group comparisons, was calculated by two-tailed and/or one-tailed unpaired Mann-Whitney t-test. In paired analysis one-tailed paired t-test was applied. $p < 0.05$ was considered statistically significant.

Results

Detection of Stx in blood fractions

The enrolled STEC-infected patients with BD (n=20) had no evidence of HUS at presentation, thereby the time-course of serum or neutrophil-bound Stx was studied by daily sampling. Stx were found associated to neutrophils in 11 out of 20 STEC-infected patients (55%) by indirect flow cytometric analysis; as in previous studies on patients with HUS[16,17]. By means of a radioactive assay (Raji cell assay)[24] which measures the inhibition of translation in human Gb3Cer-expressing cells (Raji cells) challenged with patients' sera for 3 and 20 h, detectable amounts of Stx were found in 75% or 100% of STEC-infected children, respectively.

Fig. 1A shows the maximum amount of Stx on neutrophils and the division of patients in two groups; i.e. N+pts (patients with Stx bound to neutrophil) and N-pts (patients without Stx bound to neutrophils). The presence of functional active Stx in sera measured by the Raji cell assay (highest translation inhibition, 3 h) (**Fig. 1B**) revealed a continuous distribution in N+pts (7-51%

inhibition), whereas N-pts split in two subgroups characterized either by high amounts of functionally active Stx (30-72% inhibition), or by their absence (≤ 5% inhibition). All the patients became positive to Raji cell assay at 20 h (**Fig. 1C**). It is worth noting that Stx activity in patients' sera increased over time, particularly in N+pts (**Fig. 1B, C**). No significant differences in sampling time with respect to the onset of intestinal symptoms were observed (**Fig. 1D**), nor the distribution of the toxin types significantly differed with Stx2 (N+pts=6/11; N-pts=4/9, *P*=1.0 Fisher's exact test) prevailing over Stx1 (N+pts=3/11; N-pts=1/9) or Stx1/Stx2 (N+pts=2/11; N-pts=4/9).

Relationship between blood Stx and the presence of STEC/Stx in feces

Blood tests for Stx detection (Raji cell assay and ELISA) were positive for all patients whereas in feces the detection rate was lower (**Table 1**).. Fecal specimens of patients were analyzed for the presence of STEC or STEC virulence genes (RT-PCR for *eae, stx1, stx2*). Most N+pts were positive, whereas few positive specimens were observed among N-pts (**Table 1**). Consistently, STEC strains were isolated in 87.5% of the analyzed N+pts (n=8) with respect to 25% of the examined N-pts (n=4). As this observation could reflect a higher amount of STEC in the intestine of N+pts, the amount of Stx in patients' fecal extracts was analyzed in Vero cells (toxin-induced cytopathic effects). Free fecal toxin was found in 75% of the N+pts (n=8, titer 1:40 to 1:640), while only 25% of N-pts were positive (n=8, titer 1:20). Moreover, a positive correlation between the amount of Stx in feces and on neutrophil membrane was observed (**Fig. 2A**), as in HUS patients[16]. TLR4 is present also on the membrane of human monocytes, which also possess Gb3Cer receptors. Indeed, the presence of monocyte with bound Stx was constantly found in the analyzed N+pts (n=5) accompanying neutrophil membrane positivity for Stx (**Supplementary**

Fig. S1). Consistently, the analyzed N-pts (n=4) displayed only one patient with monocyte engagement by Stx. Despite the different intestinal toxin amounts, the duration of bloody or watery diarrhea was similar in the two groups (**Fig. 2B, C**).

It has been shown that TLR4 is released by activated cells in truncated or full forms as decoy receptor, which dampens LPS-induced responses[39-41]. In vitro, the formation of Stx2/cell-free TLR4 complexes allows the toxin to escape the negative regulation imposed by HuSAP[28]. We found cell-free TLR4 by ELISA (**Fig. 3A**) in sera from STEC-infected patients with amounts significantly higher than healthy donors (**Fig. 3B**), even though no significant differences were observed between N+ and N-pts (**Fig. 3C**) or HUS and non-HUS patients **(Fig. 3D)**. TLR4 on patients' circulating cells could be engaged both by LPS and Stx released by STEC, moreover the cell-free form of serum TLR4 could be soluble or microvesicle-associated (**Fig. 3A**).

Transition from BD to HUS and time-course of Stx in blood

The synoptic panels depicted in **Supplementary Fig. S2** compare the time-course of Stx/TLR4 in blood with the emergence of renal symptoms in patients (overview in **Supplementary Fig. S1**). Proteinuria/hematuria were observed as evidence of renal involvement, without overt HUS, in several patients belonging to N+group (7/11) or N-group (4/9)N+pts;. Higher values of proteinuria were recorded in HUS patients (before HUS diagnosis) with respect to non-HUS patients (**Fig. 2D**). Most patients (n=17) fully recovered after the intestinal phase and did not show abnormal values of laboratory findings related to HUS, while three patients developed HUS (**Supplementary Fig. S2A**, pts. 9-11). Although evidence of STEC infection in HUS patients did not significantly differ compared to non-HUS patients (**Supplementary Table S1**), children who developed HUS showed common features: they belonged to N+group, were positive to RT-PCR

for *stx2* gene and in their feces STEC strains were isolated (O26, O127, O157) as well as Stx2 was detected by Vero cell assay (**Supplementary Fig. S2A**). In addition to HUS patients, many other children in N+group harbored Stx on neutrophil membrane (**Supplementary Fig. S2A**, black), thereby the mere presence of Stx on neutrophils (or other circulating cells) apparently is not the only requirement for the transition from BD to HUS. Moreover, the appearance of high amounts of fully active free Stx2 in sera, as in N-pts (**Supplementary Fig. S2B**, green), is not a condition sufficient to induce renal impairment. We also analyzed the N+pts to search for the differences between those progressing to HUS and those who recovered. In the days preceding the onset of HUS, a progressive decrease in the amount of Stx2 on neutrophils was observed (**Supplementary Fig. S2A**), even though the same decrease appeared also in different non-HUS patients. Hence, the detachment of Stx from circulating cells is not sufficient, in and of itself, to ensure development of sequelae, although it may be the first step in the process of delivering toxins to target endothelial cells. It should be noted that 9 out of 11 N+pts, including all the patients infected by STEC that release only the Stx2 type (**Supplementary Fig. S2A**, pts 6-11), and the 3 HUS cases (pts 9-11) showed a functional Stx peak emerging in serum during the decreasing phase of Stx on neutrophils and this was accompanied by a cell-free TLR4 peak. The time course of functional Stx coincided with the cell-free TLR4 when N+pts were chronologically aligned (day 0) based on the first symptom (**Fig. 4A, C**) or of the zenith of the peak of Stx on neutrophils (**Fig. 4B, D**). Clearly, two discrete peaks for both functional serum Stx and cell-free TLR4 appeared, the first ones overlap with the neutrophil peak, whereas the second ones followed the neutrophil zenith and have similar medians and distributions.

Determination and time course of cell-free Stx amount in patients' sera

A recently improved ELISA[37] allowed the detection of Stx1 and, in particular, of Stx2 (**Supplementary Fig. S3**) despite the presence of HuSAP, the inhibitory Stx2-specific binding factor present in human serum[25-27]. This ELISA (**Fig. 5A**) was conceived for the detection of Stx as virulence factors, therefore capture antibodies for Stx1 and Stx2 were chosen that recognize the B-pentamer of the toxins. This means that microvesicle-associated or free toxins capable of intoxicating target cells because of the exposure of their B-chains did not escape detection. ELISA revealed the presence of Stx in blood (**Supplementary Fig. S3**, gray Stx1, orange Stx2), hence confirming the presence of the toxins in STEC-infected patients (Stx1 0.60-13.95 ng/ml, Stx2 2.23-6.02 ng/ml).

The amount of Stx2 (**Fig. 5B**) or Stx1 (**Fig. 5C**) did not differ by comparing N+ with N-pts. However, HUS patients had higher amounts of serum Stx2 than other STEC-infected patients (**Fig. 5D**) or those in N+ group who recovered (**Fig. 5E**). In spite of this, no linear correlation was found between functional Stx2 and its absolute amount in patients' sera (**Fig. 6A**). These findings exclude a direct amount/function relationship for Stx circulating in patients' blood and might be explained by the interaction of Stx2 with factors (HuSAP, TLR4) which modulate toxic activity or by its association to extracellular vesicles. The correlation improves by analyzing N+pts (**Fig. 6C**), while worsening in N-group (**Fig. 6B**); this was likely due to differences in modulating factors or in the nature of microvesicles.

Particulate Stx in the blood of patients

We measured the amount of Stx by ELISA after centrifugation of patients' sera at 21000*g*, referring to the lower fraction as particulate Stx. The applied *g*-force allows the sedimentation of the 1 μm-microvesicles suggested to be involved in HUS[13-15]. The centrifuged layer did not

contain protein aggregates bearing Stx, as assessed in control experiments (see Materials and Methods). Particulate Stx were found in both N+ and N-groups (**Fig. 7A**), its presence in N-pts might be due to the release of microvesicles from monocytes or platelets. Strikingly, the percentages of particulate toxin on the total toxin burden were significantly higher for Stx2 than Stx1, which showed barely detectable values (**Fig. 7B**). This finding might contribute to explain the clinical observation that infections with Stx2-producing strains is more likely to progress to HUS. Most importantly, the amounts of particulate Stx2 significantly increased over time in patients infected by STEC that produce only the Stx2 type (**Fig. 7C**). It is worth to note that those patients developing HUS showed higher amounts of particulate Stx2 which significantly increased over time (**Fig. 7D**). The correlations between amount of Stx2 and toxic activity found in patients' sera (Raji assay) improve by plotting particulate Stx2 levels rather than total Stx2 levels (**Fig. 6D** *vs* **Fig. 6A**), particularly in N+pts (**Fig. 6F** *vs* **Fig. 6C**), but not in N-group (**Fig. 6E** *vs* **Fig. 6B**). The strongest linear correlations with particulate Stx2 were obtained in HUS patients, as indicated by Pearson correlation coefficients at both incubation times (**Fig. 6G**). These findings strongly suggest that a specific form of Stx2, identified as particulate Stx2, may be responsible for the toxic effects on target cells.

Particulate Stx2 in the transition from BD to HUS

Fig. 8 shows the time course of the clinical features (haemoglobin, serum LDH and serum creatinine concentrations, platelet counts and proteinuria) of the three patients who developed HUS during the study. The vertical line across the panel (**Fig. 8)** indicates the day in which the criteria for HUS diagnosis were fulfilled. The day preceding the diagnosis of HUS, a peak constituted of particulate Stx2 appeared which, however, was not observed in the remaining

N+pts, nor in N-pts (**Fig. 8**, **Supplementary Fig. S3**). At the same time, we also found particulate TLR4 (**Supplementary Fig. S3**). We calculated the particulate Stx2/particulate TLR4 ratios which were close to stoichiometry (ratio = 1.2-1.9) in the two patients with the severer form of HUS (**Fig. 8**, pts 9 and 10), whereas a lower value (ratio = 0.1) was recorded in the child with a milder form of HUS (**Fig. 8**, pt 11). Therefore, blood particulate Stx2 appeared in STEC-infected patients during transition from hemorrhagic colitis to HUS.

Discussion

Our study is focused on the transition from BD to HUS caused by STEC. During the observation, the set of clinical data collected from each patient was combined with the time course analysis of Stx in feces and blood to find the critical breakpoint before the onset of HUS. The rate of Stx detection was higher in blood than in feces according to the choice of patients enrolled in our study, i.e. STEC-infected patients during the precocious toxemic phase. Among the circulating cells that bind Stx, the neutrophil was chosen as witness since its role in the syndrome is well documented[16,17,23,42,43].

By combining the blood chronological sequences of (i) Stx on neutrophils (and monocytes), (ii) the toxic activity of free Stx, (iii) the amount of Stx, and (iv) the amount of decoy TLR4 we tried to clarify a very puzzling scenario. It is worth to note that the simple presence of Stx on neutrophil or of free toxin is not sufficient to trigger HUS, even though the three patients who had developed HUS during the study belonged to N+group. In this group, the amount of Stx on neutrophils was progressively reducing during the study. Although leukocyte-endothelial (or other) cell interactions followed by toxin transfer could explain this phenomenon, we have also found that functionally active Stx clearly appeared in patients' blood corresponding to the

decrease of Stx amount on neutrophils (and monocytes). This peak was temporally convoyed by an almost overlapping peak of cell-free TLR4. Based on this quantitative and temporal analyses, we hypothesize that Stx2 bound to neutrophils during the prodromal intestinal phase would gradually detach from the leukocyte hence appearing in serum as a complex with TLR4 during the phase preceding the renal involvement in STEC-infected patients. Neutrophils could be one of the actors involved, since Stx could also bind to monocytes (**Supplementary Fig. S1**) and to platelets (here not analyzed) then detaching from them. Moreover, soluble or microvesicle-associated complexes between Stx2 and TLR4 would allow the toxin to escape the negative regulating effect induced by HuSAP[28]. Experimental evidence has been provided in recent years that Stx-harboring extracellular vesicles are shed from platelets, monocytes and neutrophils in patients with HUS and are experimentally obtained by challenging human blood with Stx2[13-15]. The size of the involved vesicles has been determined (1 μm) as well as their composition which reflects the releasing mechanism based on budding from the cell plasma membrane. Therefore, non-internalized receptor-bound Stx have been found on vesicle surface whereas toxin molecules already internalized have been entrapped within them as a toxic cargo.

In our study, the Stx2 type was found associated to the development of HUS in three patients. No correlations were found between amount and toxic activity of Stx found in patients' sera, unless we consider particulate Stx2 (sedimented as ~1 μm-microvesicles) appeared in patients developing HUS the day before the diagnosis. Our experimental settings (ELISA with capture antibody to Stx2 B-chain) allowed the capture of microvesicles exposing the B-pentamer which would be able to transfer Stx2 to target cells or to allow the docking of the pathogenic factor-containing microvesicles to renal target cells. In this regard, Stx2 should be considered a pathogenic factor involved in the onset of HUS, but also a leading factor for the shuttling of other

pathogenic factors associated to or entrapped in microvesicles (Stx within vesicles, tissue factor and activated complement components) to target organs. The uptake process is assisted by the presence of complementary molecules on the surfaces of recipient cells and microvesicles[38].

In the pathogenesis of HUS, the site of the culprit lesion is the Gb3Cer-expressing endothelial lining of target organs. Therefore, any triggering or contributing factor should have been directed on the Gb3Cer of target organs. Microvesicles containing or harboring pathogenic factors involved in HUS can hit the target only if they are avidly captured by the Gb3Cer receptor of sensitive organs allowing the discharge of such a pathogenic load. For this purpose the presence of Stx on microvesicles membrane and the exposition of the B pentamer as in the case of Stx bound to microvesicle surface through TLR4 via A-chain is mandatory. This would be unavoidable for neutrophil-derived Gb3Cer-free microvesicles, being also possible for monocyte- and platelet-derived vesicles endowed with Gb3Cer and TLR4. Therefore, it will be of paramount importance the direct identification in patients of the subset of microvesicles responsible for triggering HUS and of the circulating cell(s) involved in their release. Understanding of this crucial point in HUS pathogenesis would allow planning therapeutic interventions aimed at reducing the chance for STEC-infected children to develop HUS. A promising tailored therapy should prevent the binding of Stx2 to the impeached blood cell(s) (or to target cells), therefore impeding the yielding of a host of predictable pathogenic consequence culminating in HUS. Recently, glycovesicles decorated with new oligosaccharides inhibiting the binding of Stx to Gb3Cer have been developed. This approach could be directed to capture free and particulate Stx hence reducing the toxic burden circulating in STEC-infected patients[44].

Conflicts of interest

None declared

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Table 1. Evidence of STEC infection in 20 patients with BD

	No. of cases		
	All (n = 20)	**N+pts[a] (n=11)*** **	**N-pts[b] (n=9)**
Feces[c,d]			
RT-PCR for *eae, stx1, stx2* genes	12/20	9/11*	3/9
Vero cell assay for Stx	8/16	6/8*	2/8
STEC isolation	8/12	7/8*	1/4
Blood			
ELISA assay for Stx	20/20	11/11	9/9
Raji cell assay for Stx	20/20	11/11	9/9
LPS-antibodies	2/8	2/7	0/1

[a]N+pts: patients with Stx bound to neutrophils

[b]N-pts: patients with no detectable amount of Stx bound to neutrophils

[c]Stool samples were also tested for *Salmonella*, *Shigella*, and *Campylobacter* through standard microbiological techniques

[d]The toxin subtype detected in feces by the enrollment assay (PCR-based reverse dot blots for *stx1* and *stx2* genes) was always confirmed at least by one of the assays shown in the table.

Chi-square test: *$p < 0.05$; ***$P<0.0001$

Figure legends

Fig. 1 Stx in the blood of STEC-infected patients. (Panel A) Determination by indirect flow cytometric analysis of Stx bound to neutrophils (inset) in patients' blood samples obtained after the onset of intestinal symptoms; the maximum value obtained in each patient was shown and expressed as MCV (cut-off value = 0.3). See also Materials and Methods. (Panels B-C) Determination by Raji cell assay of the toxic activity of Stx present in patients' serum samples (inset) collected after the onset of intestinal symptoms. Time elapsing from the first intestinal symptom to the first determination was similar for serum and neutrophils (medians = 4 days). Data are expressed as the maximal percentage inhibition of cell protein synthesis obtained after 3 h (Panel B) or 20 h (Panel C) challenge with the first collected serum sample and with the serum sample which gave the maximal inhibition. Inhibition of protein synthesis was calculated with respect to control run with the same amount of pooled sera from healthy donors. Stx-dependent translation inhibition was confirmed by the addition of monoclonal antibodies to Stx1 and Stx2 that abolished or strongly reduced the induced effects. Additional controls were performed as described in Materials and Methods[35,36]. . (Panel D) No difference in sampling times was observed between N+ and N-pts. One-tailed paired t-test was performed when time-courses were analyzed in Panels B, C. Two-tailed unpaired Mann-Whitney t-test was performed to analyze the other data in Panels A-D. *** $p < 0.0001$, ** $p < 0.001$, otherwise exact p values is shown.

Fig. 2 Stx and symptoms in STEC-infected patients. (Panel A) Positive correlation between the amount of Stx present on patients' neutrophils (maximum values) and the amount of Stx found in fecal extracts (maximal dilution titers in Vero cell assay positive patients). Duration of intestinal symptoms in STEC-infected children: (Panel B) BD and (Panel C) watery diarrhea. (Panel D)

The maximum value of pathologic proteinuria according to age observed in HUS and non-HUS patients is shown and expressed as urinary protein/urinary creatinine (mg/mg). For each HUS patient, the highest value before the diagnosis of HUS is shown. Correlation for data in Panel A was calculated by Pearson correlation coefficient. Two-tailed unpaired Mann-Whitney t-test was performed in Panel B, C, D. * $p < 0.05$

Fig. 3 Cell-free TLR4 in the blood of STEC-infected patients. (Panel A) ELISA for the detection of the decoy form of TLR4 (cell-free TLR4) released in patients' sera by neutrophils, monocyte or platelets (depicted in the panel) in soluble or particulate form (blue circles). Cell-free TLR4 in sera from STEC-infected children was detected by ELISA (maximal amount) (Panel B); no significant differences were observed between N+ and N-pts (Panel C) or HUS and non-HUS patients (Panel D). Two-tailed unpaired Mann-Whitney t-test was performed. ** $p < 0.001$.

Fig. 4 Relationship between Stx and cell-free TLR4 in patients' blood. Patients were chronologically aligned (Panel A, C) starting from the first recorded symptom (diarrhea or BD) or (Panel B, D) on the basis of the zenith of Stx peak on neutrophils (time 0). The relationships between cell-free TLR4 (ELISA), Stx on neutrophil membrane (indirect flow cytometric analysis) or functional toxic cell-free Stx in serum (translation inhibition in Raji cells treated with patients' sera) were studied. In the latter case, the data obtained after 3 h-challenge have been preferred as by this time only direct effects on ribosomes are measured (Additional controls in Materials and Methods). Two-tailed unpaired Mann-Whitney t-test was performed. *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.01$, otherwise exact p values are shown.

Fig. 5 Determination of total amount of Stx in patients' sera by ELISA. The ELISA is shown in Panel A; neutrophils, monocyte or platelets are depicted, blue circles represent microvesicles. The capture antibodies for Stx1 and Stx2 recognize B-chains. The maximum amount of Stx2 (Panel B) or Stx1 (Panel C) found in patients' sera by ELISA did not differ in N+ and N-pts. (Panels D-E) HUS patients differ in the maximum amount of Stx2 with respect to other STEC-infected patients. Two-tailed unpaired Mann-Whitney t-test was peformed. * $p < 0.01$, otherwise exact p values are shown.

Fig. 6 Correlation between amount and functional activity of Stx in patients' sera. Correlation between the maximum amount of Stx2 found in patients' sera by ELISA and the toxic activity measured as inhibition of protein synthesis in Raji cells challenged with patients' sera for 3h (blue) or 20 h (red). Correlation was measured by linear regression between translation inhibition (%) and (Panels A-C) concentrations of Stx2 (ng/ml) or (Panel**s** D, E, F, G) particulate Stx2 (ng/ml) and assessed by using the Pearson correlation coefficient.

Fig. 7 Determination of particulate Stx in patients' sera by ELISA. To obtain particulate Stx, patients' sera (100 µl) were centrifuged at 21000*g*, the *g*-force sedimenting 1 µm-microvesicles, (21000*g* lower fraction). Additional controls in Material and Methods. The maximum amount of particulate Stx did not significantly differ between N+ and N-pts (Panel A), whereas particulate Stx2 is present in higher percentage (Panel B) and increased over time in patients (Panel C) and, in particular, in N+pts developing HUS (Panels D). One-tailed paired t-test was performed when time-courses were analyzed in Panel C, D. Two-tailed unpaired Mann-Whitney t-test was performed to analyze the other data in Panels A, B, D. * $p < 0.01$, otherwise exact p values are shown.

Fig. 8 Transition from hemorrhagic colitis to HUS and particulate Stx2 in patients' sera. (Panels A-C) Laboratory findings and clinical features (hemoglobin, serum LDH, platelet counts, serum creatinine and proteinuria) of three patients developing HUS during the study (patients 9, 10, 11 as in Supplementary Figs. S1, S2, S3) are shown, as well as particulate Stx2 in blood measured by ELISA. Arrows indicate red cell transfusions (Hb graphs) or dialytic treatment (creatinine graphs). The vertical lines across the panels indicate the day of HUS diagnosis.