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Soluble plasma VE-cadherin concentrations are elevated in patients with STEC infection and haemolytic uraemic syndrome: a case–control study

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ABSTRACT

Objectives: To investigate whether the adherens junction protein vascular endothelial cadherin (VE-cadherin) is released during Shiga toxin 2 producing *Escherichia coli* (STEC) infection with haemolytic uraemic syndrome (HUS) and thus could be used to assist diagnosis.

Design: Using data from the large 2011 STEC outbreak in northern Europe, we determined VE-cadherin plasma concentrations in 356 patients distributed over three patient cohorts: patients with STEC infection accompanied by HUS (STEC-HUS), STEC patients without HUS (STEC) and control patients with diarrhoea but without STEC infection. We then looked for associations between VE-cadherin concentrations and disease severity defined by changes in lactate dehydrogenase, haemoglobin, creatinine, platelet count, haptoglobin and neurological symptoms.

Setting: This study was conducted at the University Medical Center Hamburg-Eppendorf, Germany.

Participants: 79 STEC-HUS patients, 77 STEC patients and 200 control patients were enrolled in the study.

Results: We analysed 864 specimens (207 STEC, 449 STEC-HUS and 208 controls) in total. At admission, VE-cadherin concentration tended to be lower in STEC-HUS patients compared to other patients. However, HUS patients later showed an increase in VE-cadherin concentrations with prolonged elevation beyond remission. This pattern clearly differs from that observed in non-HUS patients.

Conclusions: VE-cadherin concentrations are elevated in STEC-HUS patients and might be a biomarker reflecting endothelial damage in patients with HUS.

Strengths and limitations of this study

- This is the first study to investigate vascular endothelial cadherin (VE-cadherin) in the context of Shiga toxin 2 producing *Escherichia coli* (STEC) infection and haemolytic uraemic syndrome (HUS) complications in a large study cohort of 356 patients.
- The study was a longitudinal investigation over the disease time course and included follow-up data acquired 2 years after disease onset; several measurements repeated after 2 years demonstrated the robustness of our results.
- We compared VE-cadherin concentration levels between patient groups with regard to common clinical parameters such as lactate dehydrogenase, creatinine, haemoglobin, platelets, haptoglobin and C-reactive protein.
- Our data suggest that VE-cadherin concentration might help identify patients with severe neurological complications.
- Unfortunately, due to shorter hospitalisation, fewer measurement time points were available for the STEC and control patients than the STEC-HUS patients.

INTRODUCTION

In 2011 northern Europe experienced the largest ever outbreak caused by Shiga toxin 2 producing *Escherichia coli* (STEC). The infection was often complicated by haemolytic uraemic syndrome (HUS). The pathogen, STEC O104:H4, was identified as a Shiga toxin 2 producing *E. coli* strain combined with a virulent strain of enteroaggregative *E. coli*, producing an extended spectrum β lactamase.[1 2] The Robert Koch Institute in Germany reported 2987 cases of gastroenteritis caused by STEC O104:H4. HUS was also identified in 855 (~22%) of these patients, 53 of whom died. A further 83 cases of STEC O104:H4 were reported in several other EU countries; 54 of these patients also had HUS and two died. The population infected with STEC O104:H4 was characterised by a high incidence of HUS and an unusually high proportion of adults; in addition, 58% of all patients and up to 68% of HUS patients were female.[3 4]

STEC O157 is the classic strain associated with HUS. However, since 1988 an increasing

number of non-O157 strains belonging to the common pathogenic strains causing haemorrhagic enteritis with severe consequences such as HUS and neurological and myocardial damage, are being observed. One of these strains is STEC O104:H4.[5–7]

HUS was first defined by Gasser *et al*[8] as a triad of acute bilateral renal failure, thrombocytopenia and microangiopathic haemolytic anaemia. The common pathogenic mechanism of microangiopathic haemolytic anaemia is vascular endothelial damage.[9 10] This endothelial injury can be caused by leukocyte adhesion and can lead to thrombotic microangiopathy (TMA) through recruitment of platelets to the damaged endothelium. TMA as a feature of several clinical disorders including HUS, was first used to describe vessel lesions accompanied by fever, haemorrhagic manifestations, haemolytic anaemia and neurological disorders.[11]

The vascular injury occurring in HUS can be triggered by the Shiga toxin produced by STEC O104:H4. Here, Shiga toxin 2 (stx2) is primarily responsible for the endothelial cell damage via the proinflammatory cytokine pathway.[12 13] Likewise, sepsis or ischaemia can increase microvascular permeability and cause oedematous tissue damage, a process that is promoted by several endogenous inflammatory mediators such as activated leukocytes, TNF-α and cytokines. Among others, these mediators act through targeting an adherens junction protein called vascular endothelial cadherin (VE-cadherin).[14]

The human vascular endothelial cadherin (7B4 or cadherin-5) belongs to the large family of endothelial specific cadherin proteins. As a transmembrane protein it is localised in the intercellular junctions where it regulates the barrier function of the endothelium.[15–17] Soluble VE-cadherin can be released into the blood after increased proteolytic activity due to inflammation.[18] This proteolytic activity is mediated by metalloproteinase ADAM 10, increasing VE-cadherin proteolysis and thus leading to increased vascular permeability.[19]

HUS is characterised by inflammatory microangiopathy in the kidney and pathogenesis is associated with microvascular endothelial cell injury.[20] Currently, disease progression is monitored by measuring lactate dehydrogenase (LDH) activity, which has been shown to be elevated in similar diseases such as thrombotic thrombocytopenic purpura.[21] However, increased LDH activity in plasma is associated with cell damage in a variety of tissues and occurs in several clinical conditions.

Since VE-cadherin is of major importance for vascular integrity, we hypothesised that VE-cadherin concentrations in plasma might reflect the endothelial damage associated with HUS. Therefore, we investigated the association between VE-cadherin plasma levels and disease severity at patient admission and over time. The large number of patients and the high rate of HUS during the outbreak in 2011 allowed us evaluate this association in an uniquely large cohort. Our results indicate that the time course of VE-cadherin concentrations in STEC-HUS patients differs significantly from the time course in patients without HUS.

METHODS

Patients

In this study, three patient cohorts (STEC-HUS, STEC and CTRL) were investigated during the outbreak which lasted from the middle of May until the end of July 2011 and mainly occurred in northern Germany.[2] Diarrhoea was the common symptom for all patients in the examined groups. The STEC-HUS cohort consisted of patients with STEC infection and HUS. The STEC cohort included patients with STEC infection but without HUS. The control (CTRL) patients included patients with corresponding symptoms such as diarrhoea but not STEC infection. The clinical characteristics of the three groups are shown in table 1. All samples used in the study were acquired for routine blood analysis; ELISA testing was carried out after routine analysis was completed using leftover material.

Ethics statement

Approval for the study was granted by the Ethics Committee of Hamburg under votes PV4447 and WF-037/11.

Microbiology

Stool was analysed for microbiological proof of STEC (Shiga toxin producing *E. coli*, O104:H4) for all patients with diarrhoea or bloody diarrhoea attending the University Medical Center Hamburg-Eppendorf during the outbreak. The stool samples were plated on sorbitol MacConkey agar (Oxoid) and ESBL agar (Biomerieux)

Table 1 Clinical characteristics of the patients included in the study

Patient group	Clinical characteristics
CTRL	Diarrhoea, faecal blood, abdominal pain
STEC	Bloody diarrhoea, abdominal pain, microbiological proof of STEC O104:H4, vomiting, leucocytosis and elevated LDH
STEC-HUS	Bloody diarrhoea, abdominal pain, microbiological proof of STEC O104:H4, vomiting, leucocytosis, elevated LDH and creatinine, low haemoglobin and platelet count, acute renal failure and neurological symptoms

The listed characteristics describe the range of symptoms observed within the groups but were not mandatorily observed for each patient.
CTRL, control; HUS, haemolytic uraemic syndrome; LDH, lactate dehydrogenase; STEC, Shiga toxin 2 producing *Escherichia coli*.

and incubated at 36°C for up to 48 h. A 10 µL loop of bacteria from the lawn of grown colonies was suspended in 500 µL of TE buffer and incubated at 95°C for a further 10 min. The cleared supernatant was used as a template for stx2-specific polymerase chain reaction (PCR) assay. All stool samples were also tested for the presence of *Salmonella* spp and *Campylobacter* spp according to standard microbiological culture techniques.

Identification of HUS patients

STEC-HUS patients were defined by thrombocytopenia below the common reference range ($>150\times10^9$/L), decreased haemoglobin, and elevated LDH and creatinine using the 95th percentiles of the age- and gender-defined reference range for each parameter.

Specimen collection

For VE-cadherin detection, we used NH4-heparin plasma specimens collected immediately after routine analysis was completed. All specimens were kept frozen at −80°C until testing. Specimens were slowly thawed and centrifuged again for 5 min at 3000 rpm. All samples were diluted 50-fold using calibrator diluent (buffered protein base). Samples with values above the highest standard were further diluted. The first sample of each patient was collected after microbiological proof that STEC O104:H4 was present.

VE-cadherin ELISA

For the quantitative determination of VE-cadherin concentrations in heparin plasma, we used the solid phase enzyme-linked immunoassay Quantikine (R&D Systems, Minneapolis USA). Analysis was performed according to the manufacturer's instructions. To validate assay linearity, we used heparin plasma samples identified by procalcitonin concentrations above 10 µg/L and made dilution series using calibrator diluent, including four dilutions steps of 1:25, 1:50, 1:75 and 1:100. We calculated the linearity measures for three independent samples (y-intercept: −0.18 to 0.07; R^2: 0.74 to 0.93). The standard curve with SDs and the protein dilution series are shown in online supplementary figures S1 and S2. We determined intra-assay and inter-assay coefficients of variation of between 3.2% and 6.8% and between 6.0% and 8.4%, respectively (see online supplementary table S1). The minimal detectable doses (MDD) ranged from 0.75 to 1.34 ng/mL with a mean MDD value of 1.19 ng/mL. Recovery confirmation of the ELISA test was performed with recombinant human VE-cadherin Fc chimera provided by R&D Systems. Between 50% and 80% of recombinant VE-cadherin was recovered (see online supplementary figure S3).

Haematology and clinical chemistry

Haematology parameters were analysed on an ADVIA 2120i system (Siemens, Germany) in EDTA blood according to the manufacturer's instructions. Creatinine was determined by the bichromatic rate technique, a modification of the Jaffé reaction. LDH was measured by an enzymatic reaction, according to the IFCC method. Haptoglobin and CRP were determined by immunochemical reaction. Clinical chemistry parameters were measured by Dimension Vista systems (Siemens) in NH4-heparin plasma according to the manufacturer's instructions. Procalcitonin (PCT) was measured in serum with the Elecsys (BRAHMS) immunoassay on cobas (Roche) analysers.

Statistical analysis and follow-up

Statistical analysis was carried out using the R statistical platform (V.2.15.2). VE-cadherin concentrations measured in the STEC-HUS group were compared to those of the STEC group and the control group. Significance was determined using an unequal variance-based t test. To investigate VE-cadherin concentrations during the time course of infection, we defined five time points reflecting admission, disease climax, start of remission, advanced recovery and 2-year follow-up. The 'at admission' time point constitutes the time point after admission and receipt of microbiological results. Disease climax was determined by identifying the peak of LDH activity. To determine the start of remission, we identified the first sample that no longer showed the triad of elevated creatinine, decreased platelet counts and decreased haemoglobin. We defined the time point of advanced recovery as the last sample acquired before the patient was discharged. The time points were calculated individually for each patient. Reference ranges are always referred to normal age- and gender-dependant reference ranges for the analysed clinical chemistry and haematology values.

To investigate the VE-cadherin time courses, we plotted the concentrations and included information about dialysis, plasmapheresis and antibody therapy (eculizumab).

To exclude technical variance as the source of VE-cadherin concentration changes, 2 years after the initial measurements we validated the measurements for nine STEC-HUS patients (59 samples in total) using one assay for all specimens from each patient. The correlation of the initial and the validation measurements (r^2=0.6992) was determined and the time courses compared (see online supplementary figure S4).

Finally, we checked the records of all included STEC-HUS patients for remarks on disease severity and classified the patients into three groups: those with no neurological symptoms, those with moderate symptoms such as dizziness or double vision, and those with severe symptoms such as seizures or status epilepticus. We determined the association between disease severity and observed increase in VE-cadherin plasma concentrations from admission to remission.

RESULTS

A total of 356 patients (338 adults and 18 children) were enrolled in the study. The STEC cohort consisted of 77 patients (47 females, 30 males) with a median age of

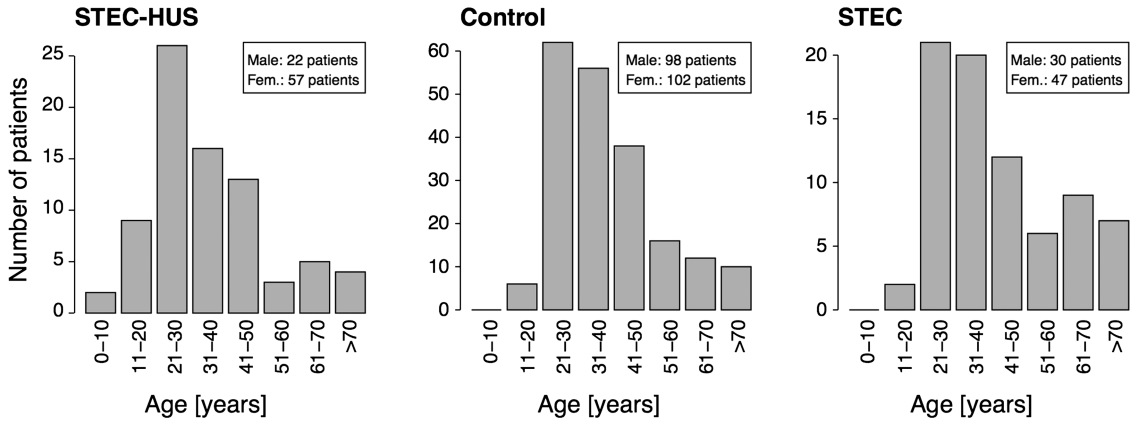

Figure 1 Age distribution of 356 patients (338 adults and 18 children). The Shiga toxin 2 producing *Escherichia coli*- haemolytic uraemic syndrome (STEC-HUS) group included 79 patients with a median age of 31 years, the STEC group included 77 patients with a median age of 38.5 years and the control group included 200 patients with a median age of 36.5 years.

38.5 years (40.0 years for females and 37.0 years for males). The STEC-HUS cohort consisted of 79 patients (57 females, 22 males) with a median age of 31 years (32.0 years for females and 29.5 years for males). The control group consisted of 200 patients (102 females, 98 males) with a median age of 36.5 years (38.5 years for females and 34.0 years for males). The age distribution of the three populations is shown in figure 1. We analysed 864 specimens (207 STEC, 449 STEC-HUS and 208 CTRL). Two years after the outbreak, we collected specimens from 20 STEC and 16 STEC-HUS study patients in order to perform follow-up measurements.

STEC O104:H4 infection was diagnosed by stool analysis at patient admission. The onset of HUS was marked by haemolytic anaemia (low haemoglobin and haptoglobin), thrombocytopenia and renal impairment (high creatinine levels). In addition, high plasma LDH activity indicated severe cell damage. STEC and control patients exhibited normal plasma LDH activity, normal creatinine and haemoglobin plasma concentrations as well as platelet counts within reference ranges (figure 2). Patients with HUS at admission had lower VE-cadherin concentrations compared with the STEC or control populations.

The means of different diagnostic parameters in STEC patients with HUS at admission are shown in figure 3. We observed a significant increase over time in VE-cadherin plasma concentrations in these patients. Compared to other diagnostic parameters, VE-cadherin concentrations remained elevated over an extended period of time and mean levels were still increased at remission (p=0.0000125). Even at advanced recovery (around 3 months after the onset of disease) VE-cadherin concentrations remained slightly raised.

The prolonged elevation of VE-cadherin plasma concentrations contrasts with the time courses of LDH, creatinine, CRP and haptoglobin (figure 3). Creatinine showed a good correlation with LDH activity over time

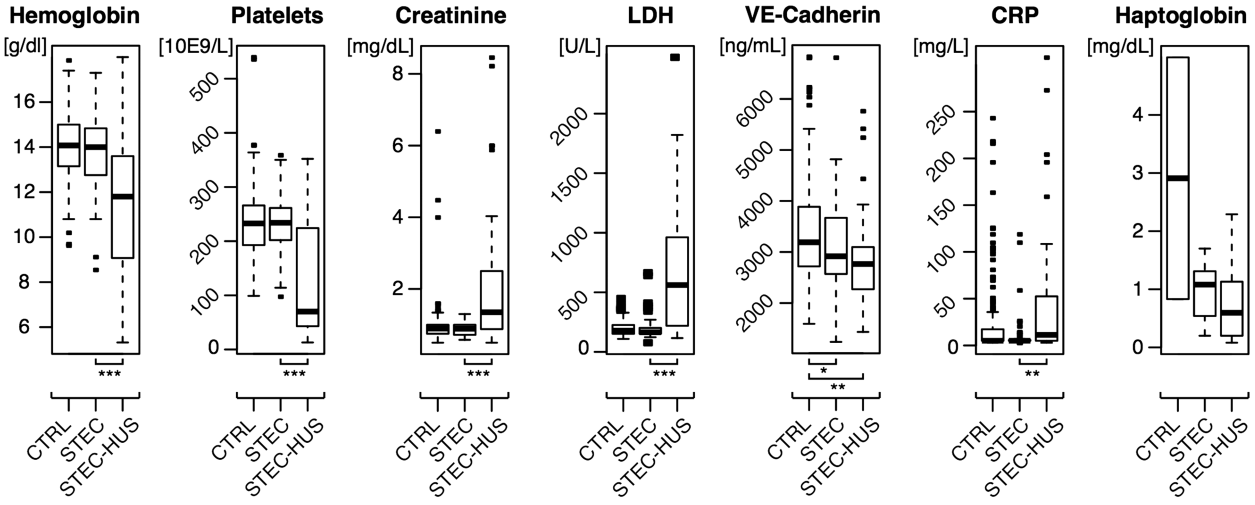

Figure 2 Comparison between the Shiga toxin 2 producing *Escherichia coli*-haemolytic uraemic syndrome (STEC-HUS), STEC and control (CTRL) groups at the time point 'first measurement after admission' for VE-cadherin concentrations and the important clinical parameters of lactate dehydrogenase (LDH), creatinine, platelet counts, C-reactive protein (CRP), haemoglobin and haptoglobin. Asterisks denote a significant difference between concentrations (*p<0.05, **p<0.01, ***p<0.001).

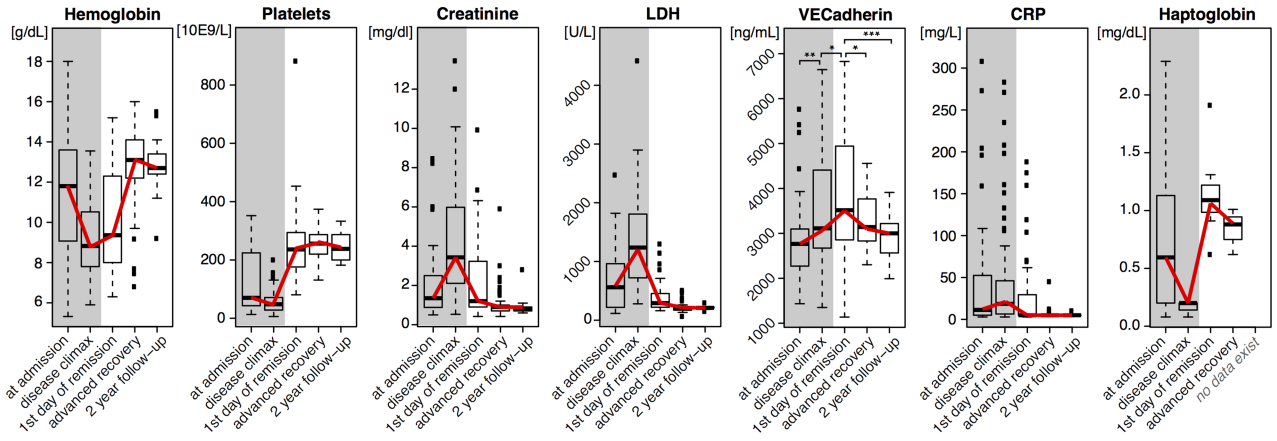

Figure 3 Comparison between VE-cadherin concentrations and the important haemolytic uraemic syndrome (HUS) parameters of lactate dehydrogenase (LDH), creatinine and platelet counts, C-reactive protein (CRP) and haptoglobin at the different time points: first measurement after admission, disease climax, first day of remission, advanced recovery and 2-year follow-up measurement for the Shiga toxin 2 producing *Escherichia coli*-HUS group. Asterisks denote a significant difference between VE-cadherin concentrations (*p<0.05, **p<0.01, ***p<0.001).

but exhibited a prolonged decrease before reaching the reference range. A correlation between creatinine and VE-cadherin plasma concentrations was not observed at remission (see online supplementary figure S5). The CRP time course was similar to that of LDH, with a quick decrease to the reference range (<5 mg/L). The few cases with high CRP levels also had elevated PCT levels, indicating potentially severe progression of the infection. Haptoglobin concentrations showed a significant decrease at disease climax and a quick recovery to the reference range at remission.

Representative graphs of the development of VE-cadherin concentrations in individual STEC-HUS patients and non-HUS STEC patients are shown in figure 4. Creatinine and CRP concentrations and LDH activity are also given for comparison. The times of plasmapheresis, haemodialysis and treatment with the monoclonal antibody eculizumab are listed for STEC-HUS patients. In the STEC-HUS patients, the increase in VE-cadherin concentrations lasts much longer than the increases in creatinine, CRP and LDH. There is no indication that plasmapheresis, haemodialysis or antibody treatment affects the course of VE-cadherin concentrations. Interestingly, the VE-cadherin concentrations exhibit a much higher dynamic in the STEC-HUS group than in the STEC group. In the STEC patients, the time course of concentration remains quite constant. In contrast, in the STEC-HUS group the initial concentrations are lower but the maximum concentrations are much higher (~1000–6000 ng/mL) in comparison with the non-HUS STEC group (~2000–4000 ng/mL). STEC-HUS patients with severe neurological symptoms tended to exhibit a stronger increase in VE-cadherin concentration over time than patients with no neurological symptoms (p=0.0631; see online supplementary figure S6).

The time courses for STEC patients have fewer measurements as they had milder illness with shorter hospitalisation. These time courses (figure 4) were confirmed by additional measurements (see online supplementary figure S7).

DISCUSSION

In 2011 Germany experienced a severe outbreak of STEC infection.[2] STEC is one of the main pathogenic *E. coli* strains causing haemorrhagic enteritis in developed countries.[20] We assume that more outbreaks may occur in the future due to (a) the increasing number of STEC strains since 1988[7] and (b) the potentially increasing speed of infectious diseases spread due to globalisation. This and the high proportion of patients developing HUS (22%)[4] during the German outbreak and the high mortality during the acute phase of the illness require improved diagnostic tools to detect complications and monitor disease progression.

HUS is defined as a triad of acute bilateral renal failure, thrombocytopenia and microangiopathic haemolytic anaemia[8] associated with endothelial cell damage.[9 10] Currently, disease activity can be monitored by measuring LDH activity. LDH is found in the cytosol of cells and even minor tissue damage results in increased LDH activity. Different LDH isoenzymes are found in different tissues: LDH-1 and LDH-2 are found in cardiac muscle, erythrocytes and kidney; LDH-3 in platelets, lung and spleen; and LDH-4 and LDH-5 in liver and skeletal muscle. Because of its wide distribution, LDH is elevated in a variety of clinical conditions including haemolytic anaemia and kidney disorders.[22] To date there is no parameter for specific monitoring of vascular endothelial damage, so we therefore considered whether a vascular endothelial membrane bound molecule such as VE-cadherin could reflect this damage.

In order to monitor endothelial damage, we looked for a suitable biomarker in human plasma which should be

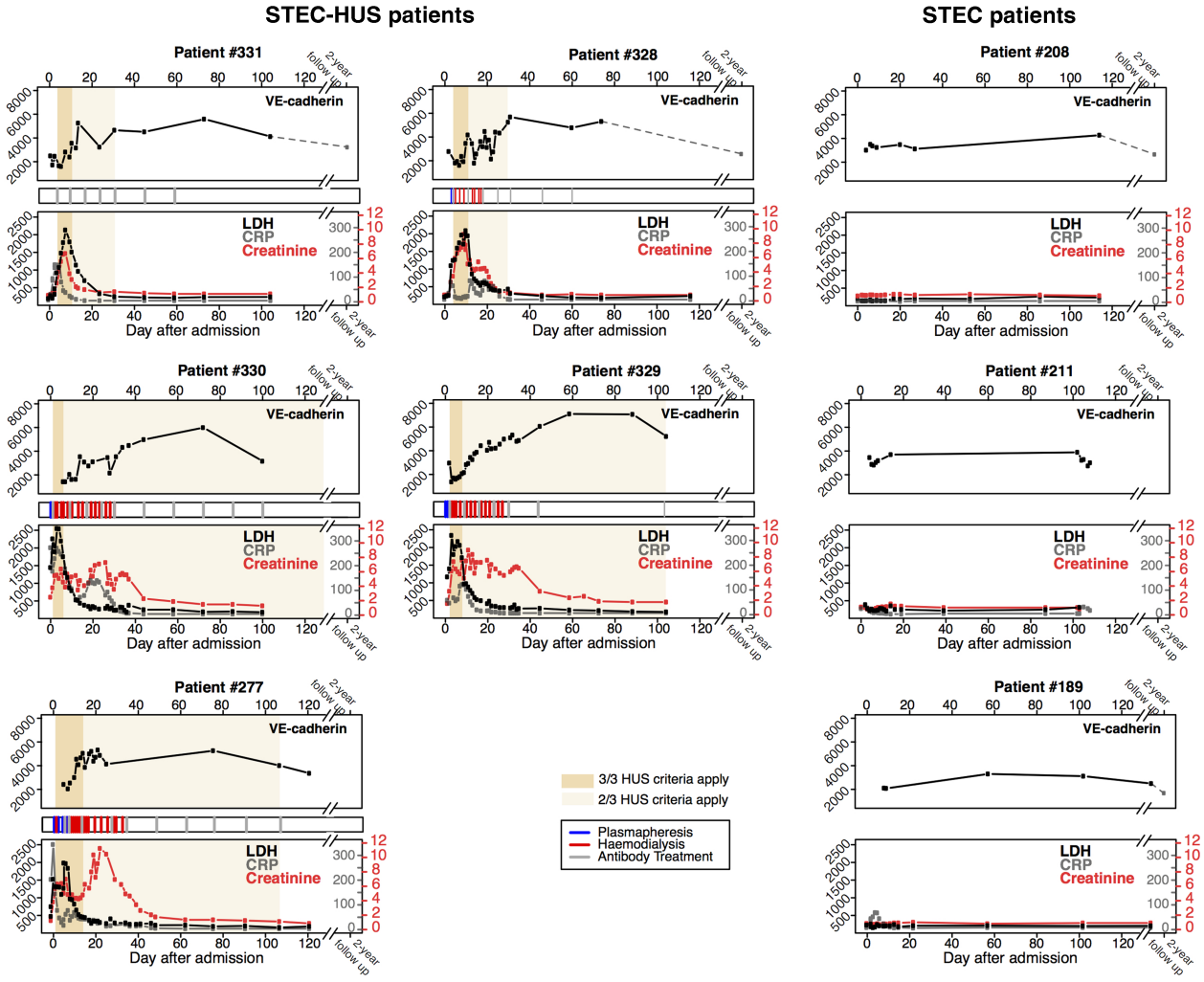

Figure 4 Representative time courses for VE-cadherin concentrations and the clinical parameters lactate dehydrogenase (LDH), creatinine and C-reactive protein (CRP) of five Shiga toxin 2 producing *Escherichia coli*-haemolytic uraemic syndrome (STEC-HUS) and three STEC patients. The VE-cadherin data have been validated by a second measurement.

specifically expressed by endothelial cells. VE-cadherin appears to fulfil this criterion. This adhesion molecule is a major component of endothelial adherens junctions, and can be quantified in plasma. To evaluate the diagnostic value of VE-cadherin, we measured VE-cadherin concentrations by ELISA in the plasma of 156 patients with STEC infection who developed (50.6%) or did not develop (49.4%) HUS complications (figure 1). In addition, we included 200 control patients who presented with suspicious symptoms such as bloody diarrhoea but without microbiological evidence of STEC infection. In order to investigate the time course of disease, we analysed specimens at different time points: patient admission, disease climax, remission, recovery and follow-up. We also measured LDH activity, haemoglobin and creatinine concentrations, number of platelets, CRP and haptoglobin concentrations.

Our results, derived from more than 800 measurements, show that HUS-STEC patients in comparison to STEC patients and control patients tended to exhibit lower VE-cadherin concentrations at admission (figure 2) but clearly higher concentrations at later time points. Compared to LDH and creatinine, it took longer for the VE-cadherin concentration to recover (figure 3). The pattern of low VE-cadherin concentration at admission and the steady increase up to 70 days after admission was only observed in STEC-HUS patients and not in non-HUS STEC patients (figure 4). Our study is based on STEC-HUS patients and does not include other TMAs. Further studies are needed to show the relevance of VE-cadherin in non-STEC-associated HUS.

VE-cadherin is localised selectively in the intercellular junctions of endothelial cells[17] as well in the corticomedullary region of the kidney.[23] Brodsky *et al*[24] described the vulnerability of renal microvascular endothelium to ischaemic injury. Kidney endothelial cells, similarly to several other renal cell types, have receptors for stx2 and are damaged by the toxin in vitro.[25] Zhang *et al*[26] and Ebihara *et al*[27] described the association between severe sepsis and soluble VE-cadherin concentrations in serum.

The initial plasma concentration of VE-cadherin observed in the control group (figure 2) indicates that

there must be some turnover of VE-cadherin at the adherens junctions. In STEC patients, this turnover appears not to be affected since VE-cadherin concentrations remain in a similar range. In STEC-HUS patients, however, VE-cadherin concentrations seem to deviate from this normal steady state. The pattern observed most probably reflects the fact that Shiga toxin 2 enters the circulation.

It is interesting to note that, in STEC-HUS patients, VE-cadherin concentrations reached peak levels when creatinine concentrations were already within or close to normal range. This finding indicates that the VE-cadherin concentrations in plasma appear not to be significantly affected by renal function. The initially low concentrations of VE-cadherin may reflect the acute endothelial damage by Shiga toxin 2, which may lead to disruption of cell–cell contacts. In endothelial monolayers, the disruption of cell–cell contacts is associated with a loss of VE-cadherin expression.[28] It has been reported that downregulation of VE-cadherin in atherosclerotic lesions is accompanied by increased entry of immunocompetent cells into the intimal matrix.[29] This indicates that inflammatory conditions lower endothelial expression of VE-cadherin. Interestingly, stx2 induced chemokines and cytokines in cultured endothelial cells at sub-inhibitory concentrations. This finding indicates that stx2 induces a general and multifaceted inflammatory reaction in host endothelial cells.[30] Although the initial elevation of median CRP concentrations was relatively mild, higher concentrations were observed in a number of patients. Taken together, the relatively low VE-cadherin concentration in STEC-HUS patients at admission may be due to a combination of toxic effects and inflammatory conditions.

In contrast to the STEC patients and control patients, VE-cadherin concentrations increased steadily in STEC-HUS patients and reached a maximum at remission, by which time all the other diagnostic parameters had returned to their normal ranges. This time course of VE-cadherin concentrations, which differs significantly from the patterns observed in STEC and control patients, indicates that VE-cadherin in the plasma of STEC-HUS patients reflects a unique pathophysiological condition. This condition likely extends over the time course during which VE-cadherin concentrations deviate from those in STEC and control patients.

VE-cadherin is released during membrane damage or degradation of endothelial cells and can be detected in plasma.[26 31] As information is limited on the long-term effects of Shiga toxins on endothelial cells in vivo, any explanation for the time course of VE-cadherin concentrations is speculative. Repair of endothelial damage and reconstitution of adherens junctions may contribute to elevated VE-cadherin concentrations. An additional or alternative explanation may be related to HUS-associated TMA, which mainly affects the kidney and the brain. It is not known if or how the resorption and dissolution of thrombi affect VE-cadherin levels in plasma. However, since this process likely extends over a longer time period, this may be a reasonable explanation.

Significantly, a high proportion of the STEC-HUS patients developed neurological complications during the course of their disease. Patient recovery from neurological symptoms took much longer than normalisation of kidney function as reflected by plasma creatinine levels. In each of these patients, the typical patterns of increasing concentrations of initially relatively low VE-cadherin levels were observed. Patients with severe neurological complications had a tendency to show a stronger increase in VE-cadherin concentrations from admission to remission than those with no symptoms ($p=0.0631$) (see online supplementary figure S6).

In conclusion, VE-cadherin is elevated in patients with HUS and may be a biomarker which reflects the '*restitutio ad integrum*'. This may be particularly relevant in patients with neurological complications.

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Contributors JD collected the samples, performed the experiments and wrote the paper. BO assisted in statistical analysis and wrote the paper. MN collected the samples and assisted in parameter measurement. GW-E assisted in experimental analysis. HR performed the microbiological analysis. TM provided the neurological data and assisted in their analysis. CW and TS designed and supervised the research study and revised the manuscript.

Competing interests None.

Ethics approval This study was approved by the Ethics Committee of Hamburg under vote PV4447.

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