UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Institut für Klinische Chemie und Laboratoriumsmedizin

Prof. Dr. Dr. Thomas Renné

The Diagnostic Value of Circulating DNA and DNase1 for Hemolytic Uremic Syndrome

Dissertation

zur Erlangung des Grades eines Doktors der Medizin an der Medizinischen Fakultät der Universität Hamburg.

vorgelegt von:

Elodie Christina Meral Janas
aus Heidelberg

Hamburg 2024

(wird von der Medizinischen Fakultät ausgefüllt)

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am: 08.01.2025

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg.

Prüfungsausschuss, der/die Vorsitzende: Prof. Dr. Ulf Panzer

Prüfungsausschuss, zweite/r Gutachter/in: Prof. Dr. Dr. Thomas Renné

Table of content

1 WORKING HYPOTHESIS AND QUESTION 4
2 INTRODUCTION 5
2.1 Clinical picture of thrombotic microangiopathies and hemolytic uremic syndrome 5
2.2 Epidemiology of hemolytic uremic syndrome 6
2.3 Causes of hemolytic uremic syndrome 7
2.4 Therapy of hemolytic uremic syndrome 8
2.5 Pathophysiology of hemolytic uremic syndrome 9
2.6 The role of neutrophils and plasma DNA 9
2.7 The role of plasma DNase1 11
2.8 Aims 12
3 MATERIAL AND METHODS 13
3.1 Material 13
3.2 Ethics vote 14
3.3 Blood samples 14
3.4 Preparation of STEC biobank for high throughput-screening 15
3.5 Experiments 17
3.6 Statistical analysis 19
4 RESULTS 20
4.1 Organization of STEC biobank 20
4.2 Comparison of diagnosis groups 25
4.3 Analysis of HUS patients 32
4.4 Correlation of parameters 44
5 DISCUSSION 49
6 ABSTRACT / ZUSAMMENFASSUNG 59
7 ABBREVEATIONS 62
8 REFERENCES 64
9 ACKNOWLEDGEMENTS 77
10 CURRICULUM VITAE 78
11 EIDESSTATTLICHE ERKLÄRUNG 79

1 WORKING HYPOTHESIS AND QUESTION

NETs are released from neutrophils as an innate response to an infection with STEC. The formation of NETs (NETosis) can stimulate hypercoagulability and damage tissue (Fuchs et al., 2012a). It is hypothesized that NETs are persisting in HUS patients due to a plasma DNase1 deficiency, whether initially or due to depletion. If DNases were inhibited, mutated or showed impaired activity during a STEC infection, this would lead to high amounts of procoagulant free extracellular DNA, increased activation of coagulation and tissue injury. Thus, impaired plasma DNase1 levels could promote the development of HUS in patients with STEC infection. The DNA degradation by DNase1 or the inhibition of NETosis may prevent or treat the disease. I look for the mechanisms which lead to the development of HUS only in a selected group of EHEC patients and in others not. Do all EHEC patients show elevated DNA levels or only those who developed HUS? Therefore, I aim to answer the question if DNA degradation is reduced as a consequence of impaired DNase1 activity, if the DNase1 is stable during the disease, comparable to healthy controls or if the DNase1 changes during disease. Furthermore, I want to investigate the origins of plasma DNA and DNase1 and if both parameters recover from acute disease state to remission. I want to clarify if extracellular DNA and DNase1 correlate with common diagnostic parameters or if they could be used as diagnostic parameters themselves.

2 INTRODUCTION

2.1 Clinical picture of thrombotic microangiopathies and hemolytic uremic syndrome

EHEC bacteria form a certain strain of E. coli bacteria which cause infections with non-bloody watery diarrhea, feeling of sickness and stomach pains (Ruggenenti et al., 2001). A few cases of EHEC infections have a more serious and threatening course of disease and are associated with the development of hemorrhagic colitis, thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) (Gonzalez Garcia, 2002). HUS is a life-threatening disease characterized by acute TMA in terminal arterioles and capillaries. TMAs show thrombocytopenia and microangiopathic hemolytic anemia. The capillary microangiopathy can be found throughout most organs and causes organ injury (Moake, 2002). HUS initially presents with diarrhea and abdominal cramps, followed by microangiopathic organ manifestation (George and Nester, 2014). The TMA in HUS predominantly affects kidneys and brain. The clinical picture of severe acute renal failure and/or serious neurological deficits can be observed in HUS patients (Trachtman et al., 2012) as well as myocardial sequelae (Rohde et al., 2011).

HUS can be classified into a diarrhea-associated typical and a non-diarrheal atypical form. The atypical HUS can be sporadic or familial and has a poorer outcome (Tarr et al., 2005). It is caused by disorders of the complement regulation due to hereditary reasons or autoimmune dysfunction. The typical HUS is caused by infection. Patients with STEC-induced HUS suffer from – first watery, then bloody – diarrhea, nausea, vomiting, abdominal pain, hematuria and anemic pallor (Salvadori and Bertoni, 2013). Characteristic for HUS are schistocytes in the peripheral blood smear. These are fragmented parts of an erythrocyte (Egan et al., 2004). The diagnosis of HUS can be confirmed by a triad of hemolytic anemia, thrombocytopenia and acute renal failure with elevated retention parameters, hematuria and proteinuria (Besser et al., 1993). During the epidemic in 2011, the picture of HUS was a special one as compared to cases described before. Many patients initially presented to the emergency department with diarrhea, vomiting, abdominal cramps and nausea. A few days later the same patients presented with renal involvement and rapidly developing neurological symptoms with amnestic aphasia, pareses and even epileptic seizures (Harendza, 2011). HUS cases during

this epidemic were - in a substantial proportion - complicated with severe neurological impairment, sepsis and renal and respiratory failure. The diagnosis for STEC serotype O104:H4-induced gastroenteritis was carried out by stool culture and fecal Shiga toxin detection (Braune et al., 2013).

2.2 Epidemiology of hemolytic uremic syndrome

The epidemic form of HUS is the Shiga toxin-producing EHEC-associated HUS (STEC-HUS). It is predominantly observed in young children and rarely in adults. In 10 – 20 % of cases with EHEC infection the patients develop the complication of HUS (Ducker et al., 2011). STEC-HUS usually presents with diarrhea in children younger than 2-3 years. Acute kidney injury is caused in 55-70% of STEC-HUS patients, but generally with a favourable prognosis. Recovery in renal function is observed in 70-85% of patients with typical HUS (Tarr et al., 2005). In children, HUS is the major cause for acute kidney injury (Siegler and Oakes, 2005). The first description of HUS dates back to 1955, where Gasser et al. characterized the disease as bilateral necrosis of the renal cortex in acute acquired hemolytic anemia (Gasser et al., 1955). The most common serotype of E. coli causing HUS is O157:H7. In Western Europe and North America, it causes 70% of STEC-HUS cases. From May to August 2011 an epidemic of EHEC-infections caused by the novel strain O104:H4 of Shiga toxin producing E.coli took place in Germany, predominantly in Northern Germany. The outbreak was characterized by many cases of STEC-induced diarrhea with rapidly increasing severity of clinical symptoms and the development of hemolytic uremic syndrome with a high frequency. Until then, HUS cases were mainly described in young children. The epidemic in 2011 remarkably affected young adults and apart from this, healthy individuals without pre-existing medical conditions (Kielstein et al., 2012). The outbreak affected more women with a female: male ratio of 3: 1 (Braune et al., 2013). In six hospitals in Hamburg, Germany, 106 patients with STEC-HUS were admitted to the Intensive Care Units. 66 % out of these 106 patients developed neurological symptoms and 48.1% developed sepsis. Of these, 25.4 % developed septic shock. The overall mortality rate in the six observed hospitals in Hamburg was 4.7%. During the outbreak, 3.816 patients in Germany suffered from STEC-induced gastroenteritis, 845 patients developed HUS and 54 patients died (Braune et al.,

2013). 88% of HUS patients were adults (Buchholz et al., 2011) with a median age of 40 years (Braune et al., 2013).

2.3 Causes of hemolytic uremic syndrome

Other than infections with viruses like Epstein-Barr virus (EBV), Parvovirus B19 and Cytomegalovirus (CMV), bacterial infections with Shigella dysenteriae, Streptococcus pneumonia and enterohemorrhagic E. coli can lead to the development of a typical HUS (Riedl et al., 2014). E. coli bacteria are part of the normal gut flora of humans and warm-blooded animals. However, we also find human pathogenic strains like EAEC (enteroaggregative E. coli), EHEC (enterohemorrhagic E. coli), EIEC (enteroinvasive E. coli), EPEC (enteropathogenic E. coli) and ETEC (enterotoxigenic E. coli). These pathotypes can be transmitted via smear infection, contaminated water or food (Gonzalez Garcia, 2002). EHEC cause disease by producing a toxin which is, after its similarity to the Shiga toxin (Stx) produced by Shigella dysenteriae (S. dysenteriae), called Shiga-like toxin (Sajjan and Forstner, 1990). Since it is cytotoxic to Vero cells of African green monkey kidney cells, this toxin is also called verotoxin (Ruggenenti et al., 2001). E. coli bacteria capable of producing this toxin are called STEC which stands for Shiga-like toxin-producing E. coli (Clawson et al., 2009). Only the minority of the more than 100 serotypes of E. coli bacteria producing Shiga-like toxins (SLTs) is harmful to humans. The common serotype O157:H7 is able to produce two toxins, the SLT type 1, neutralizable by anti-Shiga-toxin antibodies, and the SLT type 2, not neutralizable by anti-Shiga-toxin antibodies. The neutralization with antisera of S. dysenteriae shows the similarity of SLTs to Shiga toxins and classifies the two types of SLTs in Stx-1 and Stx-2 (Ruggenenti et al., 2001) of which several subtypes exist. The Stx-2c mostly causes severe courses of the disease (Ducker et al., 2011). STEC infections originate from contaminated food or water infected by mostly ruminants to which the bacterium is not pathogenic (Buchholz et al., 2011).

The novel strain causing the epidemic in 2011 was originally thought to be a common EHEC. Later it was identified as serotype O104:H4 Shiga toxin-producing E. coli which had acquired the features of adhesion to enterocytes and production

of extended-spectrum beta lactamases (Rohde et al., 2011). Initially, fresh vegetables were suspected to be the most likely source of the EHEC bacteria because there had been a suspicious accumulation of having eaten fresh vegetables within the days before infection and the outbreak of the disease. In June 2011, the German Federal Institute for Risk Assessment (BfR), the Federal Office of Consumer Protection and Food Safety (BVL) and the Robert Koch Institute (RKI) issued a press release that the EHEC bacteria presumably originated from sprouts. The primary alert concerning fresh tomatoes, salad and cucumbers were withdrawn. The agriculture minister of Lower Saxony identified later-on a farm in Bienenbüttel as a potential source and a laboratory in Lower Saxony isolated the outbreak strain of EHEC in discarded sprouts (Harendza, 2011, Borgatta et al., 2012).

2.4 Therapy of hemolytic uremic syndrome

Before 2011, the recommendation for treating HUS has been a symptomatic therapy in order to treat anemia, coagulation disorder and kidney injury. To treat HUS, plasma has to be infused or exchanged in order to counteract the platelets consumption. In some severe cases, the removal of the spleen or both kidneys might be necessary (Ruggenenti et al., 2001). During the epidemic in 2011, HUS treatment was a different one. Despite the fact, that Harendza *et al.* from the University Medical Center Hamburg-Eppendorf had put EHEC-patients directly from the beginning of clinical HUS symptoms under plasmapheresis, many patients did not recover and in many patients neurological symptoms still aggravated (Harendza, 2011). Patients often required renal replacement therapy, intubation, non-invasive ventilation and mechanical ventilation. In addition to supportive therapy, therapeutic plasma exchange was applied (Braune et al., 2013). Later-on, the monoclonal anti-C5 antibody eculizumab was used as off-label (Harendza, 2011). Overall, during the epidemic, 91.5 % of HUS patients were treated with plasma exchange and 47.2 % with eculizumab. At discharge, 21.7 % of patients still showed neurological symptoms. Six months after admission, no patient needed renal replacement therapy anymore (Braune et al., 2013).

2.5 Pathophysiology of hemolytic uremic syndrome

The primary event in the pathogenesis of HUS is the endothelial cell damage. This crucial lesion consists of thrombotic microangiopathy and red blood cell fragmentation (Siegler and Oakes, 2005). During an infection with foodborne STEC, the bacteria reach the gastrointestinal tract and adhere to gastrointestinal mucosa cells. By means of this process, the bacteria injure the intestinal epithelial cells in order to prevent being dislodged from the mucosa. The subsequent impairment in the brush border's function leads to hemorrhagic ulcerative lesions and subsequently to non-bloody and bloody diarrhea. Further, STEC produce Shiga-like toxins which pass through epithelial cells of the gastrointestinal tract. How the toxins pass into the systemic circulation is not yet fully understood and the toxin is not detectable in blood analysis. However, SLTs enter the blood stream and - by binding to white blood cells and platelets - reach vital organs where they bind to the Gb3 receptor. This receptor is found on glomerular epithelial and endothelial cells, tubular and mesangial cells in the kidneys. The Gb3 receptor also appears in the gastrointestinal tract and the central nervous system. Moreover, *in vitro* models have shown that polymorphonuclear leucocytes (PMNs) bind SLTs and release them to target cells in the kidneys. SLTs bind to the Gb3 receptor on their target organs and when internalized, prevent the cells from protein synthesis and thus promote the cells' apoptosis (Ruggenenti et al., 2001). Further, it has been shown that PMNs migrating to the intestine as innate response to a STEC-infection transmigrate across intestinal epithelial cells (IECs). This process decreases the barrier function and thus increases the paracellular permeability promoting the translocation of Shiga toxins across IECs. Since STEC induce PMN migration (Hurley et al., 2001) and PMNs are suspected to contribute to thrombus formation, these findings indicate an important role of PMNs in the pathogenesis of STEC-induced HUS.

2.6 The role of neutrophils and plasma DNA

It is known that neutrophils are part of the innate immune response to inflammation (Page and Good, 1958). In response to infection, neutrophils are able to undergo a cytolytic cell death pathway to release granular, cytoplasmic and nuclear components (Fuchs et al., 2012b). The nuclear content consists of DNA fibers with

histones and neutrophil enzymes which all together form a trap in the extracellular space (Brinkmann and Zychlinsky, 2012). This neutrophil extracellular trap (NET) then traps and immobilizes pathogens. The process of forming these NETs is called NETosis. During NETosis, compact nuclear DNA filaments are unfolded and the nuclear envelope dismantles (Fuchs et al., 2007) (Papayannopoulos et al., 2010), releasing NETs into the extracellular space (Yipp et al., 2012). Further, NETs can be found in venous thrombi of animals and patients with venous thromboembolism (Brill et al., 2012) (Savchenko et al., 2014).

Neutrophil elastase (NE) and peptidylarginine deiminase type 4 (PAD4) regulate NETosis by modifying histones and enabling DNA decondensation. In mice, PAD4- or NE-knockout neutrophils are not able to form NETs, a fact that protects those knockout mice from experimental thrombosis (Papayannopoulos et al., 2010) (Li et al., 2010). Markers for NETosis are myeloperoxidase, citrullinated histone 3 and calgranulin (Fuchs et al., 2007). It was shown that markers for NETosis are elevated in plasma from patients with acute TMAs (Fuchs et al., 2012b).
NETs have been discovered to be a stimulus for thrombus formation (Fuchs et al., 2010, Massberg et al., 2010). *In vitro*, NETs stimulate platelet adhesion as well as fibrin formation (Martinod and Wagner, 2014) (Massberg et al., 2010). The histones in NETs activate platelets and aggravate platelet aggregation (Fuchs et al., 2010, Fuchs et al., 2011, Semeraro et al., 2011) whereas DNA in NETs along with neutrophil serine proteases stimulate fibrin formation through the factor XII-pathway (Massberg et al., 2010) (von Bruhl et al., 2012). NETs also present an optimal framework for formation of blood clots (Fuchs et al., 2010). It was shown that acute TMAs like TTP and HUS are associated with increased levels of plasma DNA, hypothetically due to a decreased DNase1-mediated DNA degradation. Furthermore, increased DNA concentrations were identified in plasma of TMA patients, including HUS patients, during the acute disease state. Extracellular DNA promotes blood clotting *in vitro* and in animal models (Fuchs et al., 2012b) and may contribute to the formation of a thrombus in TMA patients. Altogether, by binding and activating platelets and clotting factors, NETs promote tissue damage and coagulation and thus contribute to thrombosis. This implies that efficient dissolution of NETs is required to prevent thrombus formation in HUS.

STEC induce PMN migration (Hurley et al., 2001) and PMNs like neutrophils are able to bind SLTs (Ruggenenti et al., 2001). PMNs further promote the translocation of SLTs across IECs (Hurley et al., 2001). These findings point toward the importance of PMNs in the pathogenesis of STEC-induced HUS. The fact that prothrombotic NETs are suspected to contribute to thrombus formation indicates a decisive role of neutrophils in the aggravation of an ongoing STEC infection and thus in the development of HUS and its treatment or prevention.

2.7 The role of plasma DNase1

Since NETs are composed of double-stranded DNA filaments (Brinkmann et al., 2004), they are digestable by DNase1s (Nassberger et al., 1989). In circulation there are primarily two extracellular DNase1s, namely DNase1 and DNase1γ (Napirei et al., 2005) (Yeh et al., 2003). It is known that serum DNase1 is required to efficiently degrade NETs (Hakkim et al., 2010) and we have previously shown that NETs are degraded by plasma DNase1 rather than by plasma DNase1γ (Jimenez-Alcazar et al., 2015). In healthy condition, there is sufficient DNase1 in the circulation. Plasma DNase1 reduces or cancels the prothrombotic effects of NETs by disassembling the NET-scaffold. If the activity of circulating DNase1 is impaired, the prothrombotic NETs cannot efficiently be degraded. NETs would then be stable in plasma, would accumulate prothrombotic extracellular DNA and could promote or aggravate excessive microvascular thrombosis as found in patients with acute TMAs.

Conclusively, the effective elimination of NETs may be essential for prohibiting thrombotic diseases. However, not much is known about how plasma DNase1 and free extracellular DNA, such as NETs, interact in thrombotic diseases. The endonuclease DNase1 cleaves chromatin from apoptotic and necrotic cells (Napirei et al., 2004). For autoimmune disorders, persisting cell detritus is understood to be a stimulus for inducing anti-nuclear immunity (Yasutomo et al., 2001) (Dittmar et al., 2009) (Malickova et al., 2011). In patients with systemic lupus erythematosus (SLE), NETs could be identified as an origin of auto-antigens against which the organism develops auto-antibodies (Nassberger et al., 1989) (Schnabel et al., 1995). Since sera from SLE patients are deficient in DNase1 leading to impaired NET-degradation (Hakkim et al., 2010) and DNase1-deficient mice develop

spontaneously SLE (Napirei et al., 2000), DNase1 seems to play an important role in autoimmunity.

In patients with thrombotic microangiopathies such as HUS, the role of DNase1 has only recently been addressed. In TMAs, there are occlusive thrombi in the microvasculature (Rosove, 2014). As previously shown, free extracellular DNA is elevated during the acute disease state in TMA patients (Fuchs et al., 2012b); a fact that triggers blood clotting and may lead to the aggravation of thrombus formation. For thrombotic diseases, we could show that plasma DNase1s prevent thrombosis. *In vitro*, DNase1 prevents activation of platelets and clotting factors by degrading the NET-scaffold (Jimenez-Alcazar et al., 2015). *In vivo*, experimental thrombus formation in mice is inhibited by infusing recombinant DNase1 (Fuchs et al., 2012b). In the previously performed experiments we included TMA patients, but the HUS cohorts were small. Altogether, the role of plasma DNA and DNase1 in HUS patients is not yet understood.

2.8 Aims

To decipher the role of plasma DNA and DNase1 in the pathogenesis of HUS, we compare patients with non STEC-induced diarrheal symptoms, STEC-induced diarrhea, STEC-induced HUS and healthy controls regarding their DNA levels and DNase1 activity in plasma. A comparison of plasma samples collected in the acute disease state of HUS and in remission was conducted in order to find an answer to our research objective if plasma DNA and/or plasma DNase1 recover. Furthermore, we want to classify the potential of plasma DNA and/or plasma DNase1 to serve as follow-up parameters, allowing a better surveillance of the disease during therapy. Time courses of plasma DNA and DNase1 levels of all patients who had developed a HUS should be analysed and the question should be answered if there is a correlation between those two parameters or with other diagnostic parameters in order to investigate the origins of plasma DNA and plasma DNase1. We want to see if and in what manner plasma DNA and plasma DNase1 can predict the development of HUS. We aim to find a new diagnostic parameter indicating the impending danger of a complication with HUS of an ongoing STEC-infection prior to the acute disease state and a new therapeutic target for thrombotic diseases such as HUS.

3 MATERIAL AND METHODS

3.1 Material

Reagents

I used SYBRSafe [10000X] (Invitrogen, product number: S33102) for DNA stainings, DNA from salmon testes (Sigma-Aldrich, product number: D1626), stored at 4°C, MES [2-(N-morpholino)ethanesulfonic acid] (Sigma-Aldrich, product number: 0332-100G), stored at 4°C, MgCl2 hexahydrate (Roth, product number: HN03.3), CaCl2 dihydrate (Roth, product number: HN04.3), Agarose GP-36 (Gerbu, product number: 01139-64), NaOH pellets (J.T. Baker, product number: BAKR3728), horizontal tray with lid (dimensions 22.5 cm x 22.5 cm x 2.5 cm), 96 well white plate (non-sterile), tube (Falcon, 50 ml), Parafilm, transparencies for fotocopies (Folex), beaker (400 ml), beaker (1L), graduated cylinder (1L), erlenmeyer flask (500 ml), jar (1L), pipette tips (10-200µl), pipette tips (100-1000µl). If not stated otherwise, the reagents were stored at room temperature.

Equipment

I used sealing tape (Sarstedt, Alu-Sealing Tape, pierceable, pcs. 100, REF 95.1995), microtest Plate 96 Well (Sarstedt, pcs. 25, REF 82.1583), epMotion 5070 pipetting tool (Eppendorf), soft roller (Daper, Soft Faced Wallpaper Seam Roller, Stock No.: 42594, Part No.: DSRSG), single channel pipette (2-20µl), single channel pipette (100-1000µl), scalpel, needle, microwave, culture incubator (37ºC), water bath incubator (50ºC), fluorescence scanner (Bio-Rad), pH meter, magnetic stirrer.

Stocks

Stocks of 1L MgCl2, CaCl2 and MES pH 6.5 were prepared, each of them having a concentration of 1M in H2O as diluent. 10 ml ssDNA with concentration 10 g/L diluted in H2O were stored at 4°C.

Diagnostic parameters

From the Institute of Clinical Chemistry and Laboratory Medicine at the UKE, I got access to all clinical data of all patients who were included in the sample collection during the epidemic. According to these medical records, I could examine which

patient belongs to which cohort and I was able to classify the disease state of the samples. Patients agree to the use of their clinical data for study purposes in advance of their admission.

3.2 Ethics vote

This study and the use of included clinical data was approved by the local ethics committee in the letter of ethics application acceptance with the number PV4447 from April 25th in 2013 from the Ethik-Kommission der Ärztekammer Hamburg.

3.3 Blood samples

Blood collection during the STEC epidemic in 2011

During the STEC O104:H4 epidemic in 2011, which took place from April to October 2011 in northern Germany (Wieler et al., 2011), heparinized plasma samples were collected from patients who applied to the emergency ward of the University Medical Center Hamburg-Eppendorf with symptoms of gastro-intestinal disease such as diarrhea. Single inclusion criterion was the ordered microbiological screening for EHEC despite the result. If the screening was negative and the patient was discharged, the STEC biobank then only has got this one sample collected at the screening day. Of patients who had to stay in hospital because their screening was positive, samples were collected randomly at different timepoints during the patients' stay in hospital. Since the collections in 2011, all collected plasma tubes are stored at -80 degrees in a freezer on the campus of the University Hospital Hamburg-Eppendorf.

Blood collection of healthy donors

With the kind support of the Institute of Transfusion Medicine of the University Medical Center Hamburg-Eppendorf, blood samples were collected in June 2014 from 88 healthy adults who are regularly donating in the Institute. Blood was only taken from donors who did not donate for the first time and blood samples were anonymized. Blood was only taken during the initial predonating-sampling process. Here, 30 ml blood is collected in a smaller blood bag before the blood runs into the bigger blood bag for blood donation. Out of the smaller bag which is primarily filled,

blood is collected for pre-transfusion testings such as the screening for infectious diseases. If the amount of required blood tubes was collected, a heparinized plasma tube was filled with the residual blood. This is blood which would have been discarded otherwise because it is not part of the blood reserve. Blood donors did not have to sign an informed consent for the use of their blood in this particular scientific study because this is already part of the agreement for the donation of blood in the Institute of Transfusion Medicine of the University Medical Center Hamburg-Eppendorf.

3.4 Preparation of STEC biobank for high throughput-screening

Inventory

The goal was to create a well-arranged inventory of samples collected at the UKE during the STEC epidemic in 2011 with all sample and patient dates as well as all available diagnostic parameters additionally to samples of healthy blood donors serving as controls. The patient samples were classified into three diagnosis groups: patients with non STEC-induced diarrheal symptoms, STEC-induced diarrheal symptoms or STEC-induced HUS.

Since, during the epidemic in 2011, samples were randomly collected at several collection time points during the patients' stay, it was important to find out which samples represented which state of the patient's disease progression. In order to distinguish between samples collected when the patient was in the acute state of disease and those samples collected when the patient was in remission, it was necessary to define those states and organize the STEC biobank systematically.

The STEC biobank consists of 15 cryoboxes with space for up to 100 plasma tubes each. In each box, the positions go from 1 to 100. The labelling for a tube is, for example, B5P23, which means that this particular sample is stored in box 5 on position 23. In some boxes some positions are empty. Thus, not every box contains exactly 100 samples.

Our first goal was to get an aliquot of each sample in a 96-wellplate/ microtiterplate (MTP) with V-bottom. I wanted to guarantee that, after aliquotation, there is still enough (at least 50%) volume left in the original tube for further investigations.

I had to specify the unknown initial volume of each tube so that I could decide if there is enough sample volume for aliquots.
I prepared standard tubes with volumes from 50 µl to 4700 µl in steps of 100 µl and labelled them. I compared the original sample tubes to those standards and noted their volumes.

Aliquotation

I aimed to store the aliquots in the same layout of 10 x 10 samples as the original sample tubes. Since the aliquot MTPs provide dimensions of 8 x 12 samples, two MTPs would carry the samples of one cryobox. I filled the empty positions with orange-coloured test tubes as place holders to avoid frameshift during the aliquotation procedure.
Knowing the approximate volume of each sample, I calculated which maximum amount could be taken as aliquot without depleting a sample tube. The maximum fitting volume in a well for the aliquots was 250 µl. From samples with more than 500 µl initial volume, I took 250 µl aliquot. Of samples with less than 500 µl, I took 50% of the initial volume (**Table 1**).
After aliquotation, the aliquot plates were sealed with transparent sealing tape, fixed with a soft seam roller, labelled and stored in a -80°C freezer until use. As result of the aliquotation, there is a duplicate of the STEC biobank stored in 96-wellplates.

Table 1. The filling volume of the original tubes in the STEC biobank was detected. All tubes showed enough volume for taking an aliquot without depleting a tube. On the basis of the initial filling volume of the original tubes, the aliquot volume was calculated.

Sample volume	Number of samples	% of total	Aliquot
empty	102	7	None
< 100 µl	17	1	50 %
l00 µl < x < 300 µl	83	6	50 %
300 µl < x < 500 µl	110	7	250 µl
500 µl < x < 1000 µl	308	21	250 µl
> 1000 µl	860	58	250 µl
Sum	1480	100	

3.5 Experiments

Plasma preparation

The aliquot MTPs, stored at -80°, were placed – still sealed - on a bacterial shaker at 37° for 20 minutes. Since I have observed fibrin formations in the aliquots due to multiple thawing or a long-term storage, the samples should be centrifuged before use so that the fibrin polymers would stick to the bottom. Thus, I centrifuged the Aliquots at 3600 RPM for another 20 minutes.

After centrifugation, the sealing tape was removed and the samples were ready to use for the following DNA and DNase1 measurements.

Commercial DNA

DNA from salmon testes from Sigma Aldrich was diluted in PBS containing 0.1 % BSA.

Commercial DNase1

Human recombinant DNase1 from Roche (dornase alpha, Pulmozyme) was diluted in SRED buffer containing 0.1% BSA.

DNA measurement

The quantification of circulating DNA in the plasma is based on a previously described method (Fuchs et al., 2012b) with modifications.

In brief, 2 µl of plasma were diluted in 98 µl of PBS containing 0.1% bovine serum albumin. To the diluted patient's plasma, 100 µl PBS containing 2 µM Sytox Green nucleic acid stain (Invitrogen) were added. A MTP reader (Tecan) with a 485 nm excitation and 535 nm emission filter set was used to measure the DNA fluorescence. Since it was considered to measure plasma autofluorescence as background, the same samples were measured once again only diluted in PBS and without Sytox Green. If in samples with no or very low amounts of DNA the procedure resulted in negative values, those were omitted for graphical plotting. Known plasma DNA concentrations served as a standard curve to calculate the concentrations of the measured plasma samples.

DNase1 measurement

The DNase1 activity was measured by the SRED assay (Nadano et al., 1993) which measures the nuclease activity of DNase1 1 by measuring the formation of dark circles in an agarose gel containing fluorescent-marked DNA. The SRED assay was used with some modifications by Miguel Jiménez-Alcázar as previously described (Jimenez-Alcazar et al., 2015).

In brief, 0.13 mg/ml double-stranded DNA from salmon testes (Sigma-Aldrich) were dissolved in a buffer solution containing 100 mM MES, 20 mM $MgCl_2$, 2 mM $CaCl_2$ and 2x SYBR SAFE (Life Technologies). After heating the mixture at 50°C for 10 minutes, the same volume of 2% agarose GP-36 (Nacalai tesque) was added. The fluid gel was poured into trays (22.5 x 22.5 x 2.5) and wells of radius 1 mm were prepared. After solidification, 2 µl of each sample were loaded in the wells and the gels were, placed in a moist chamber, incubated for 10 hours at 37°C. The fluorescence was measured with a fluorescence scanner (Molecular Imager FX, Bio-Rad). The pictures were analysed with ImageJ (NIH), by checking the intensity and the size of the DNA-degradation circle.

Sample Restorage

After measuring, transparent sealing tape (Sarstedt) was fixed with a soft faced wallpaper seam roller (Draper) onto the MTPs. For a long-term storage, the MTPs were put in a -80° freezer.

3.6 Statistical analysis

Results were analyzed by using the program GraphPad Prism. Non-parametric one-way analysis of variance tests (Kruskal-Wallis test) or non-parametric Mann-Whitney test were done for each chart. Data are presented as values for each patient with medians. Means, standard deviation plus standard error were calculated by using GraphPad Prism. Difference with p value <0.05 was considered as statistically significant.

4 RESULTS

4.1 Organization of STEC biobank

Inventory and Aliquotation

The Institute of Clinical Chemistry / Central Laboratories of the University Medical Center Hamburg-Eppendorf provided access to all available data by means of which I could identify the patients' diagnosis and allocate samples to its belonging donor. The result of the manual volume detection and aliquotation was a duplicate of the entire STEC biobank in 96-wellplates with an inventory containing the following information for each sample: sample code, number of cryobox in which the original sample tube is stored, position in this cryobox, number of MTP in which the belonging aliquot is stored, position number in MTP, aliquot volume, residual volume in original sample tube, patient's ID, case number, diagnosis group, age, date of birth, gender, sample collecting date, sample collecting time and if available the values for 149 laboratory parameters. The result of my inventory work is a well-arranged biobank in the storage of the Central Laboratories of the University Medical Center Hamburg-Eppendorf with aliquots in MTPs for further studies.

Classification of cohorts

The single including criterion for the sample collections during the ongoing epidemic was the ordered microbiological EHEC screening. The STEC biobank contains plasma samples of all patients who were - during April to October 2011 - suspected for having EHEC due to their clinical symptoms and therefore were tested in the microbiological laboratory for EHEC. Thus, the biobank also includes sample material of patients who eventually did not have the diagnosis EHEC because their diarrheal or other gastrointestinal symptoms were not EHEC-induced. But since those patients presented to the emergency department with clinical symptoms during the ongoing epidemic, they were initially suspected of having EHEC and were as a precaution put through the EHEC screening. Those patients form the diagnosis group "EHEC (-)" for all experiments. The diagnosis group "EHEC (+)" is defined by clinical symptoms which means diarrhea and a positive EHEC screening but without any symptoms of HUS. Patients with an EHEC infection developing HUS, form the diagnosis group "HUS". The control group of healthy donors without any symptoms

form the cohort healthy controls (HCs). Based on this classification, the following division of the cohorts as shown in **Table 2** with epidemiological data results.

The entire STEC biobank consists of 1395 samples collected from 394 patients during the epidemic in 2011 (175 male, 219 female) and from 88 healthy donors (54 male, 34 female) in 2014. The patients' age ranges from 5 to 102 years. Those patients were hospitalized in the University Medical Center Hamburg-Eppendorf between April 6th, 2011 and November 11th, 2011. The sample collections took place between May 31st, 2011 and October 17th, 2011 by the team of the clinical chemistry and laboratory medicine at the UKE.

Table 2. The cohorts for the following experiments were classified by means of their clinical symptoms and their diagnosis. EHEC (-), EHEC (+) and HUS patients suffered from clinical symptoms such as diarrhea. EHEC (-) did not have the EHEC-bacteria, whereas EHEC (+) and HUS patients did have the infection, identified by the microbiological EHEC screening. EHEC (+) patients did not develop a HUS as compared to patients in the HUS cohort. The diagnostic tool to identify which patient belongs to the HUS cohort was the patients` medical report. As explained in material and methods, the following classification with epidemiological data results for the biobank.

	Diarrhea	EHEC	HUS	Individuals	Male	Female	Age		Samples
							Range [Years]	Average [Years]	
HCs	-	-	-	88	54	34	18 - 68	43	88
EHEC (-)	+	-	-	238	125	113	15 - 102	59	314
EHEC (+)	+	+	-	80	30	50	13 - 84	49	213
HUS	+	+	+	76	20	56	5 - 78	42	780
Entire STEC biobank				482	229	253	5 - 102	48	1395

Arrangement of plasma samples

There are 314 samples from 238 patients for the cohort EHEC(-), 213 samples from 80 patients for the EHEC(+) diagnosis group, 780 samples from 76 patients for the HUS group and 88 samples from 88 healthy controls (=HCs).

Of 307 individuals there is only one sample in the biobank and of 175 individuals there are multiple samples. Of 33 patients (mainly of the HUS diagnosis group) there are more than ten samples available (**Table 3**).

Table 3. In order to identify the number of patients applicable for a time course analysis, the available samples in the STEC biobank of each patient in each group were counted. 87% of the HUS patients showed more than one sample allowing the analysis of time courses.

	Individuals	Samples	= 1 sample		1< x >= 10 samples		> 10 samples	
			[no. of pat.]	[%]	[no. of pat.]	[%]	[no. of pat.]	[%]
HCs	88	88	88	100	0	0	0	0
EHEC (-)	238	314	183	77	55	23	0	0
EHEC (+)	80	213	26	33	52	65	2	2
HUS	76	780	10	13	35	46	31	41
Entire STEC biobank	482	1395	307	65	142	29	33	7

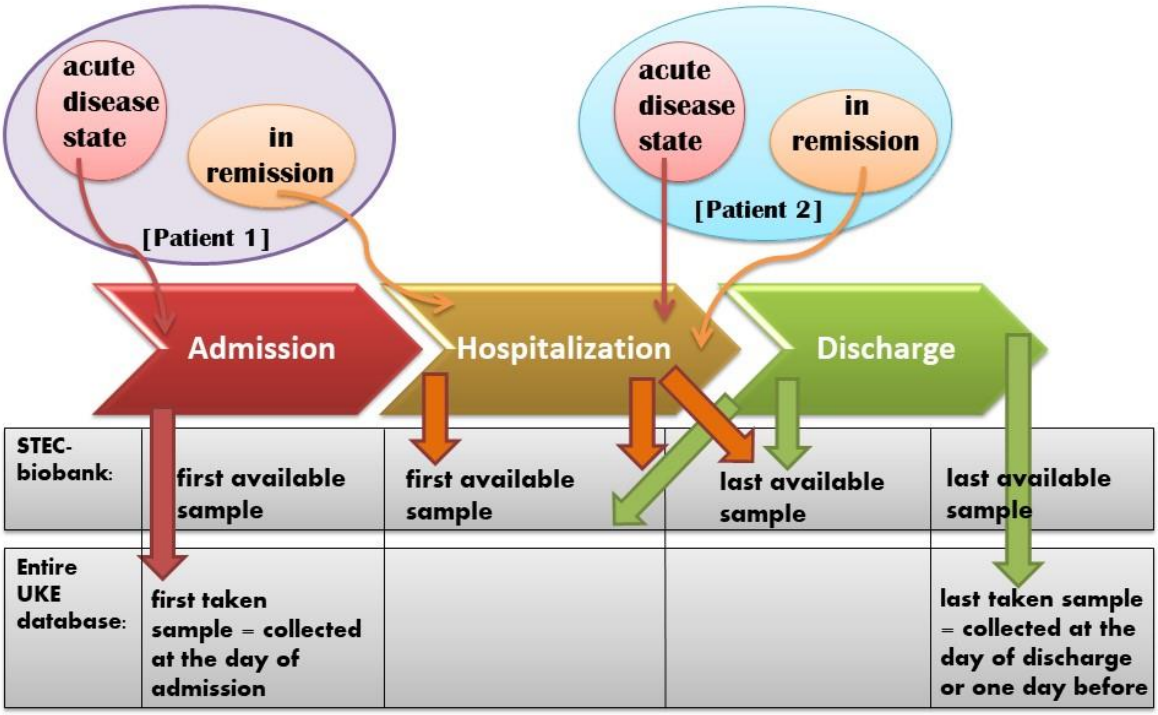
Classification of samples

During the epidemic in 2011, samples were randomly collected at several time points during the patients' stay in hospital. Sometimes sample collection did not take place directly at admission but several days later, perhaps at day 7 of the patient's stay in hospital (**Table 4**). At that particular time point, the patient could have already been under treatment and his constitution could have already been improved. If considered those kinds of samples as acute state of HUS, this would give a wrong population. Thus, I identified those samples collected directly at admission. It occurs that there are patients of whom there is no sample collected at admission but several days later. Therefore, I classified the samples into four groups: collected at *admission*, *first available* ones in the STEC biobank, *last available* ones in the STEC biobank and collected at *discharge*.

Collected at admission is defined as the sample collection for the STEC biobank took place the very same day at admission; whereas at discharge is defined as the sample collection for the STEC biobank took place the day or one day before discharge because not every patient had been seen the day of admission and I assume that the disease state one day before the date of admission had been the same. A patient who did not receive a sample collection for the STEC biobank at admission had been seen several days after admission and this sample is then the first sample which is available of that particular patient in the STEC biobank. The same applies when a patient did not receive a sample collection at or one day before discharge. Then, the STEC biobank would show a last sample which is available for that particular patient. Since this sample could have been collected two weeks before discharge (in a state where the patient could have been in the acute disease state), considering it as the last sample of the patient`s stay in hospital and equating it as the state of remission, would give a wrong population. Amongst the first available samples there are also the one which were collected the date of admission and amongst the last available samples, there are also the samples collected at or the day before discharge.

For the comparison of extracellular DNA and DNase1 activity amongst the cohorts, I performed the experiments, created graphs and calculated statistics with all available samples from the STEC biobank. When selecting the first available samples, the data showed comparable results according to the significance of differences amongst the cohorts and yet, the samples at admission showed a higher significance. Thus, I focused on the better sample selection meaning the samples at admission and discharge in order to investigate the acute disease state and remission.

Table 4. The sample collection of the first collected sample for the STEC biobank could be immediately at admission or at any time during the hospitalization, irrespective of the disease state. To ensure that the patient was not already under treatment when the first sample for the STEC biobank was collected, a second group of samples was defined as “first taken sample” where it was checked that the sample collection date was the very same date as the date of admission, in addition to the “first available sample” in the STEC biobank which only states that it is the first sample in the STEC biobank which is available for that patient but it does not give the information if the this sample was collected during the first blood sampling of that patient or during a blood sampling process later. The same problem results for the last sample of a patient. In the STEC biobank, the last available sample of a patient could have been collected several days before discharge, even many days before, perhaps when the patient was still in an acute disease state. Thus, I defined the last taken samples as samples collected at or the day before discharge.



Definition of HUS in acute disease state and in remission

Since the sample collections for the STEC biobank weren’t defined at particular timepoints, I still could not be sure that, as assumed, a patient was in the acute disease state at admission and in remission at discharge. There are patients who first present with EHEC and do not fulfill all the criteria of a HUS. If so, it would give a wrong cohort of samples, if only taking the first taken samples at admission and presuming that these samples would have been collected in the acute state of HUS. Thus, I searched the UKE database for all the diagnostic parameters which define the state of HUS (as shown in **Table 5**) for all patients of the STEC biobank.

In order to distinguish among the HUS patients between those samples being in acute disease state and in remission, the acute disease state of HUS was defined

as reduced Hemoglobin and elevated LDH for hemolytic anemia, reduced platelet-count for thrombocytopenia and an increase in serum creatinine level over 50 % over baseline for renal failure (**Table 5**). Since the individual baseline values were not known, I defined the acute renal failure as 50% increase over reference range for female and male (Besser et al., 1993). The state of remission was defined as state when all the parameters diagnosing HUS were in its reference range.

To investigate if extracellular DNA and DNase1 activity levels recover in HUS patients, I examined the samples at admission of HUS patients which fulfilled the above-mentioned criteria for the acute disease state at admission. Of those patients, I searched the biobank if there was also a sample available at discharge. In order to avoid that the sample at discharge could reflect an acute disease state, I checked if the samples at discharge fulfilled the above-mentioned criteria for remission.

Table 5. The reference ranges for the laboratory parameters for the triad of HUS symptoms were identified. The acute state of disease was defined as state where all the four parameters were out of its reference range. The state of remission was defined as state where all parameters were in the reference range again.

Symptom	Laboratory analyzing	Reference range	Acute disease state	Remission
Hemolytic anemia	Hemoglobin	Adults: ♀: 12 – 16 g/dl, ♂: 13.5 – 17.5 g/dl	reduced	in reference range
		Children: 2-6 years: 11.5–13-5 g/dl 6-12 years: 11.5-15.5 g/dl 12-18 years: ♀: 12-16 g/dl, ♂: 13-16 g/dl		
	LDH	Adults: 120 – 240 U/l	elevated	in reference range
		Children: 3-5 years: <339 U/l 6-11 years: ♀ <319 U/l, ♂ <420 U/l 12-16 years: ♀<240 U/l, ♂< 376 U/l		
Thrombo-cytopenia	Platelet count	Adults: 150 – 400 x 10^9/Liter	reduced	in reference range
		Children: 150 – 500 x 10^9/Liter		
Renal failure	Serum Creatinine	Adults: ♀ ≤ 1.2 mg/dl, ♂ ≤ 1.4 mg/dl	reduced	in reference range
		Children: 0.2 – 1.0 mg/dl		

4.2 Comparison of diagnosis groups

For all experiments and the following comparisons, the whole STEC biobank with its 1395 samples was thawed and samples were centrifuged. For the DNA measurements, in PBS diluted patient's plasma was stained with Sytox Green and

the fluorescence was checked by an ELISA reader. For the DNase1 measurements, DNA degradation by patient's plasma was measured by means of fluorescent Sytox Green staining dye and measured with a fluorescence scanner. The results were compared to plasma from healthy donors measured in the same way for plasma DNA levels respectively for plasma DNase1 activity.

In order to investigate whether the amount of plasma DNA differs in the three patient groups compared to healthy donors, it was necessary to identify those patients' samples taken in an active disease state so that I would not analyse a sample where the patient had already been treated, meaning the patient had received plasmapheresis yet.

The results showed that the plasma DNA levels in HUS patients are significantly elevated in comparison to all other three groups, and yet the significance was even increased when analysing those samples taken right at admission instead of all first available samples.

The other method, meaning the first available samples were also examined, but here the results were less clear, probably because in many cases, the first available sample had been collected several days after admission. In this state, therapy had already been initiated and his health condition could have been improved or the therapy status could not be determined exactly or a significant improvement has already occurred through intrinsic healing processes. Accordingly, the focus was on the samples taken at admission as they were the best-characterized and more precisive data collective to analyse. Hence, I searched the STEC biobank for the first taken sample at admission of each patient and arranged samples in the three diagnosis groups of EHEC (-), EHEC (+) and HUS.

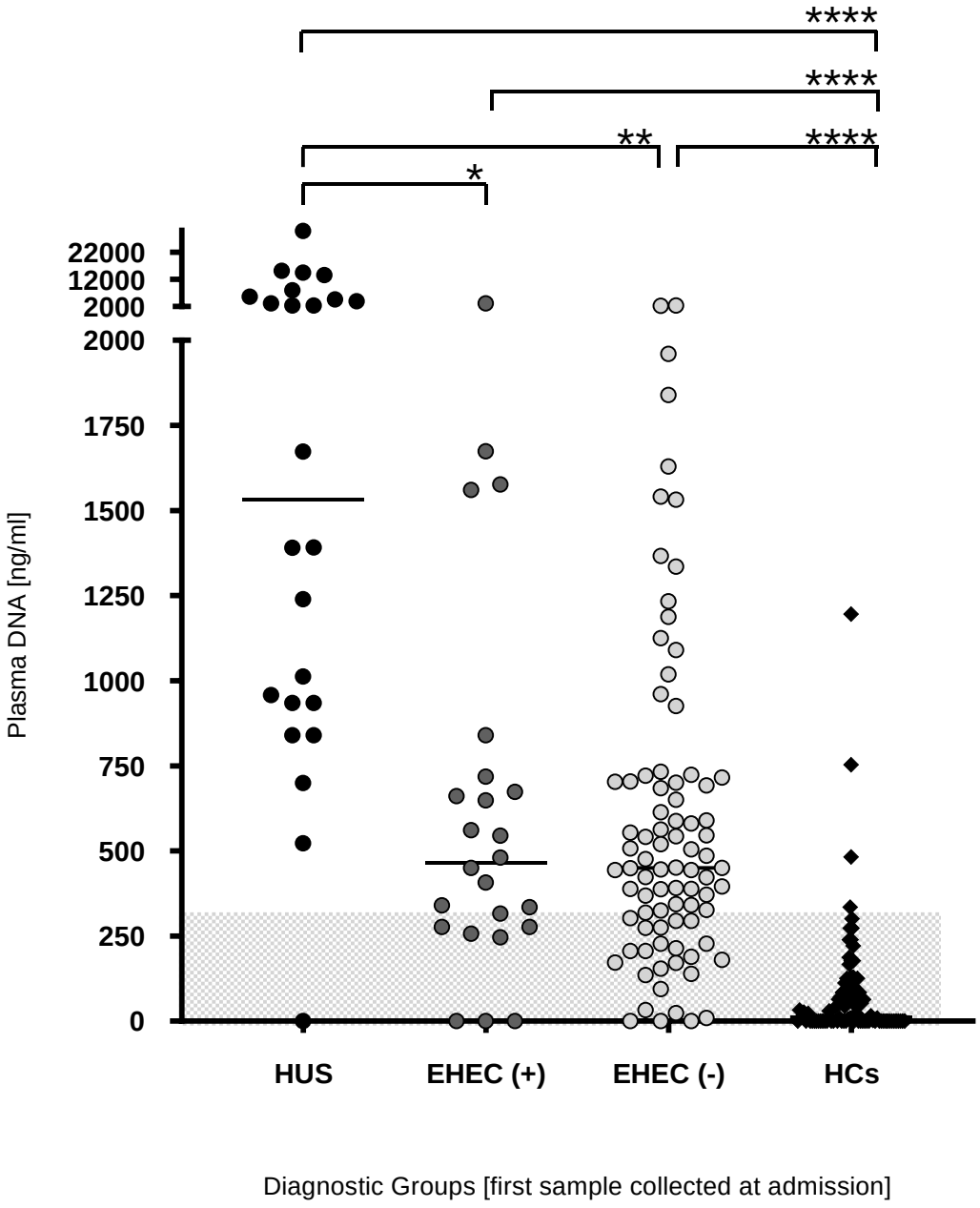


Figure 1. Plasma DNA levels at admission: The amount of circulating DNA measured by Sytox Green in the first taken sample at admission of each patient of each diagnosis group was compared to the amount of circulating DNA measured in the same way in healthy donors' sera. The comparison shows significantly lower DNA levels in healthy controls as compared to patients with diarrheal symptoms, with or without EHEC infection. The horizontal axis lists the different diagnosis groups. Each dot stands for one patient. The vertical axis represents the range of measured DNA values. Medians are marked with a line. α = 0.05, ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; circle = diarrheal symptoms; rhomb = no clinical symptoms; gray shaded rectangle background = 0 - 95 percentile range of healthy controls (one-sided). One-way ANOVA: number of treatments (columns) 4, number of values (total) 220, Kruskal-Wallis test: p value < 0.0001, p value approximate, p value summary ****, do the medians vary significantly ($p < 0.05$)? Yes. Number of groups 4, Kruskal-Wallis statistic 124.5.

Table 6. Statistics of plasma DNA levels at admission: Statistics of Figure 1 show significantly lower DNA levels at admission in healthy controls as compared to patients with diarrheal symptoms, with or without EHEC infection. α = 0.05, ns = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Statistics	HUS	EHEC (+)	EHEC (-)	HCs
Number of values	24	24	84	88
Minimum	0	0	0	0
25% Percentile	934.6	276.7	280.1	0
Median	1533	465.4	449.9	10.72
75% Percentile	5375	707.2	703.7	94.74
Maximum	29816	3190	2442	1196
Mean	4817	668.1	593.1	80.73
Std. Deviation	6987	708.2	505.5	168.6
Std. Error	1426	144.6	55.16	17.97
Lower 95% CI of mean	1867	369	483.4	45.01
Upper 95% CI of mean	7767	967.2	702.8	116.5
Sum	115609	16035	49817	7104

Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
HUS vs EHEC (+)	55.54	P < 0.05	*
HUS vs EHEC (-)	53.06	P < 0.01	**
HUS vs HCs	135.5	P < 0.001	***
EHEC (+) vs EHEC (-)	-2.479	P > 0.05	ns
EHEC (+) vs HCs	79.96	P < 0.001	***
EHEC (-) vs HCs	82.44	P < 0.001	***

As expected, the data in **Figure 1** and **Table 6** show significantly (p value < 0.001) increased plasma DNA levels in HUS patients with a maximum of 29816 ng/ml and way higher outliers as compared to healthy controls with a maximum of only 1196 ng/ml. HUS patients show a median of 1533 ng/ml and a mean of 4817 ng/ml and (SD 6987 ng/ml ± 1426 ng/ml) whereas healthy controls show a median of 10,72 ng/ml and a mean of 80.73 ng/ml (SD 168.6 ng/ml ± 17.97 ng/ml). The mean is 60-fold and the median 143-fold higher in HUS patients than in healthy controls (**Table 6**). Plasma DNA levels in patients with diarrheal symptoms with and without

diagnosed EHEC infection are increased compared to healthy controls and decreased compared to HUS patients. EHEC (+) show a median of 465.4 ng/ml and a mean of 668.1 ng/ml and (SD 708.2 ng/ml ± 144.6 ng/ml) which is less than a seventh of the mean, respectively a third of the median of HUS patients (p value < 0.05) and 8.28-fold higher than the mean, respectively 43.41-fold higher than the median of healthy controls (p value < 0.001). EHEC (-) show a median of 449.9 ng/ml and a mean of 593.1 ng/ml and (SD 505.5 ng/ml ± 55.16). This is a 7.35-fold higher mean and a 41.97-fold higher median as compared to healthy controls and less than an eighth of the mean and less than a third of the median of HUS patients. EHEC (+) and EHEC (-) do not differ significantly as their means (668.1 ng/ml respectively 593.1 ng/ml) and medians (465.4 ng/ml respectively 449.9 ng/ml) are not far apart from each other.

Taken together, these data show higher DNA levels for all patient groups with diarrheal symptoms, namely EHEC (-), EHEC (+) and HUS in comparison to people without diarrhea (HCs). HUS patients have even higher plasma DNA levels than patients with non-HUS and non-EHEC diarrhea. And yet, also EHEC and diarrhea patients stand out from healthy people due to significantly elevated DNA levels. Thus, an increase in extracellular DNA is not specific for HUS and leads to the question where the measured DNA originates from. The figure gives the idea of increased DNA levels in gastrointestinal diseases. In HUS patients, we find a wide distribution range of plasma DNA levels. Some values reach the medians of EHEC(+) and EHEC(-) and some go up to the highest values of HUS patients. In conclusion, these findings suggest that plasma DNA levels are increased with disease severity.

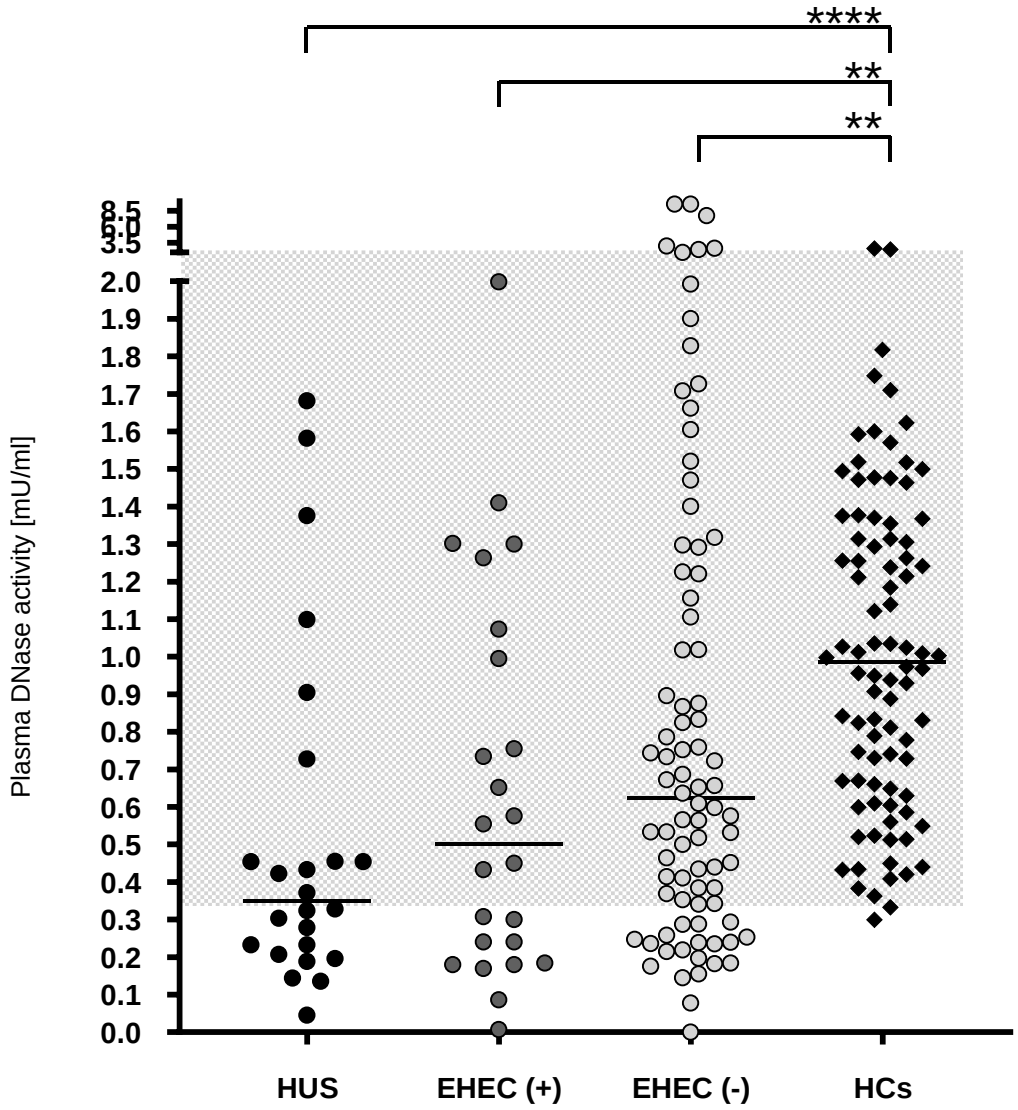


Figure 2. Plasma DNase1 levels at admission: The activity of circulating DNase1 measured by the previously described SRED assay in the first taken sample at admission of each patient of each diagnosis group was compared to the activity of circulating DNase1 measured in the same way in healthy donors' sera. The healthy controls show significantly elevated DNase1 levels as compared to patients with diarrheal symptoms, with or without EHEC infection. The horizontal axis lists the different diagnosis groups. Each dot stands for one patient. The vertical axis represents the range of measured DNase1 values. Medians are marked with a line. α = 0.05, ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; circle = diarrheal symptoms; rhomb = no clinical symptoms; gray shaded rectangle background = 2.5 – 97.5 percentile range of healthy controls (two-sided). One-way ANOVA: number of treatments (columns) 4, number of values (total) 220, Kruskal-Wallis test p value < 0.0001, approximate p value, do the medians vary significantly ($p < 0.05$)? Yes. Number of groups 4, Kruskal-Wallis statistic 30.05

Table 7. Statistics of plasma DNase1 levels at admission: The statistics of Figure 2 show significantly elevated DNase1 levels in healthy controls as compared to patients with diarrheal symptoms, with or without EHEC infection. α = 0.05, ns = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Statistics	HUS	EHEC (+)	EHEC (-)	HCs
Number of values	24	24	84	88
Minimum	0.04534	0.007009	0	0.2997
25% Percentile	0.2139	0.1985	0.3412	0.6519
Median	0.3504	0.5025	0.6231	0.9857
75% Percentile	0.6597	1.054	1.225	1.345
Maximum	1.682	1.999	9.591	2.63
Mean	0.5244	0.6417	1.086	1.023
Std. Deviation	0.4644	0.5205	1.658	0.4574
Std. Error	0.09479	0.1063	0.1809	0.04876
Lower 95% CI of mean	0.3283	0.4219	0.7257	0.9259
Upper 95% CI of mean	0.7205	0.8615	1.445	1.12
Sum	12.59	15.4	91.19	90.01

Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
HUS vs EHEC (+)	-16.33	P > 0.05	ns
HUS vs EHEC (-)	-35.91	P > 0.05	ns
HUS vs HCs	-68.25	P < 0.001	***
EHEC (+) vs EHEC (-)	-19.58	P > 0.05	ns
EHEC (+) vs HCs	-51.91	P < 0.01	**
EHEC (-) vs HCs	-32.34	P < 0.01	**

Simultaneously to the DNA measurements, the thawed plasma samples were analysed by the SRED assay (see Material and Methods) to measure the activity of DNase1 in plasma. In **Figure 2,** the same samples as in **Figure 1** were analysed. The results show a significant (p < 0.001) difference when comparing HUS to HCs. The median of 0.3504 mU/ml and the mean of 0.5244 mU/ml of HUS patients are lower by a factor of 1.95 respectively 2.81. The median of HUS is at the level of the 2.5 percentile of HCs. The medians of EHEC(+) and EHEC(-) are found in the range of the 2.5 – 97.5 percentile range of HCs but both cohorts show significantly (p <

0.01) impaired DNase1 levels as compared to HCs with a median of 0.5025 mU/ml and a mean of 0.6417 mU/ml (SD 0.5205 mU/ml ± 0.1063 mU/ml) for EHEC(+) and a median of 0.6231 mU/ml and a mean of 1.086 mU/ml (SD 1.658 ± 0.1809 mU/ml) for EHEC(-). There is no significance found when comparing HUS, EHEC(+) and EHEC(-). The three cohorts show a wide range of values, laying in the range of HCs, and yet, the three diagnosis groups also show lower DNase1 levels which are outside the range of HCs (**Table 7**).

Taken together, these data indicate that plasma DNase1 levels are decreased in patients with diarrheal symptoms, whether with or without EHEC infection. Although the medians do not differ significantly amongst the diagnosis groups, they give the idea of more and more decreasing DNase1 levels in patients with gastrointestinal symptoms due to the severity of symptoms. This could be due to a lack of DNase1 activity which is initially altered in patients and leads to their outcome or due to the fact that the DNase1 activity is more and more impaired due to the overload of DNA and cannot recover or the recovery takes more time as DNases have to be resynthesized.

4.3 Analysis of HUS patients

To investigate whether the initially elevated plasma DNA levels of HUS patients would improve over time, the last samples at discharge of each HUS patient were identified and compared to the first taken samples at admission (**Figure 3, Table 8**).

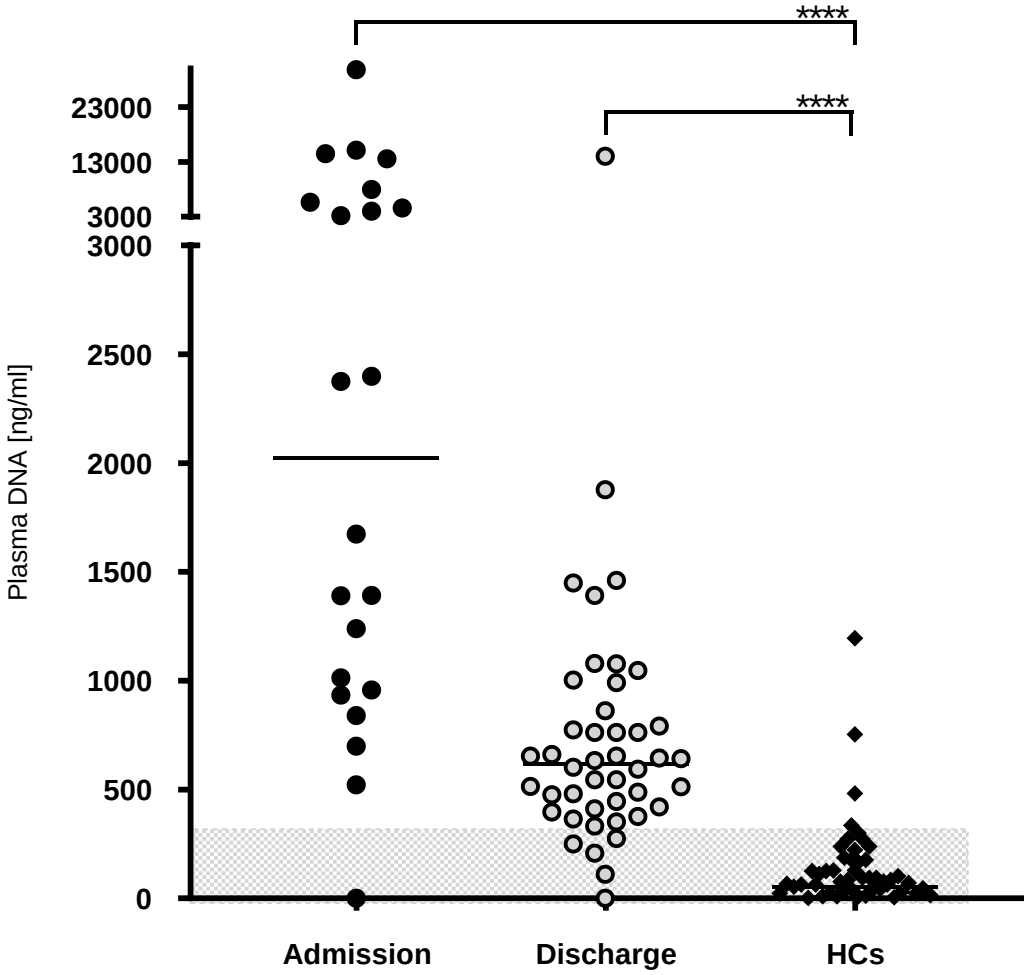


Figure 3. Plasma DNA levels of HUS patients at admission and discharge: The amount of circulating DNA measured by Sytox Green nucleic acid stain in the first taken sample at admission and in the last taken sample before discharge of each HUS patient was compared to the amount of circulating DNA measured in the same way in healthy donors' sera. Those healthy controls show significantly impaired DNA levels as compared to HUS patients, irrespective of the sample collection time point. The horizontal axis lists the time points for the sample collection. Each dot stands for one HUS patient. The vertical axis represents the range of measured DNA values. $\alpha = 0.05$, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, **** = $p < 0.0001$. Gray shaded background: 0- to 95 percentile range of healthy controls. One-way ANOVA: number of treatments (columns) 3, number of values (total) 154, Kruskal-Wallis test: p value < 0.0001, approximate p value, p value summary ****, do the medians vary significantly ($p < 0.05$)? Yes. Number of groups 3, Kruskal-Wallis statistic 100.6

Table 8. Statistics of plasma DNA levels of HUS patients at admission and discharge: The statistics show significantly impaired DNA levels in healthy controls as compared to HUS patients, irrespective of the sample collection time point. α = 0.05, ns = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001, **** = p < 0.0001.

Statistics	Admission	Discharge	HCs
Number of values	22	44	88
Minimum	0	0	-234.5
25% Percentile	952.2	414.3	-45.01
Median	2025	617,8	10.72
75% Percentile	6225	844.6	94.74
Maximum	29816	14054	1196
Mean	5174	971.3	53.74
Std. Deviation	7202	2053	186.4
Std. Error	1535	309.5	19.87
Lower 95% CI of mean	1981	347	14.26
Upper 95% CI of mean	8367	1596	93.23
Sum	113835	42736	4729

Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
At Admission vs At Discharge	23.81	P > 0.05	ns
At Admission vs HCs	87.17	P < 0.0001	****
At Discharge vs HCs	63.36	P < 0.0001	****

Extracellular DNA shows significantly (p < 0.001) higher levels in HUS patients at admission with a median of 2025 ng/ml and a mean of 5174 ng/ml (SD 7202 ± 1535 ng/ml) as compared to HCs with a median of 10.72 ng/ml and a mean of 53.74 ng/ml (SD 186.4 ± 19.87 ng/ml). DNA levels at discharge with a median of 617,8 ng/ml and a mean of 971.3 ng/ml (SD 2053 ng/ml ± 309.5 ng/ml) are significantly (p < 0.001) increased as compared to HCs, but not significantly (p > 0.05) impaired as compared to at admission. The medians at admission and discharge are higher than the 95th percentile of HCs.

The data show that HUS patients have many times higher extracellular DNA levels in the acute state of the disease as compared to healthy controls. And yet, HUS patients show even elevated DNA levels at discharge. This leads to the picture of a lack of significance between admission and discharge. In fact, in **Figure 3** we see a trend of highly elevated DNA levels at admission and in comparison, there is a clear difference to the lower DNA levels at discharge. When checking the values and even when looking at the figure, admission and discharge appear to be different populations.

I investigated the first and last available samples in order to expand the populations and could find the same trend of elevated DNA levels in the first available samples and lower DNA levels in the last available samples (data not shown). In addition, they reached the first significance level ($p < 0.05$). Thus, I speculated that the lack of significance in **Figure 3** between admission and discharge could be due to a lack of power what should be confirmed in further studies. I planned another experiment with an even more narrowly defined population of samples (**Figure 5**) in order to look closer at each patient.

The data suggest that initially increased DNA levels decrease under therapy. In order to investigate whether the initially increased and eventually decreased DNA levels would originate from an initially impaired DNase1 activity which normalizes gradually, the first and last taken samples of all HUS patients were compared concerning their DNase1 levels (**Figure 4, Table 9**).

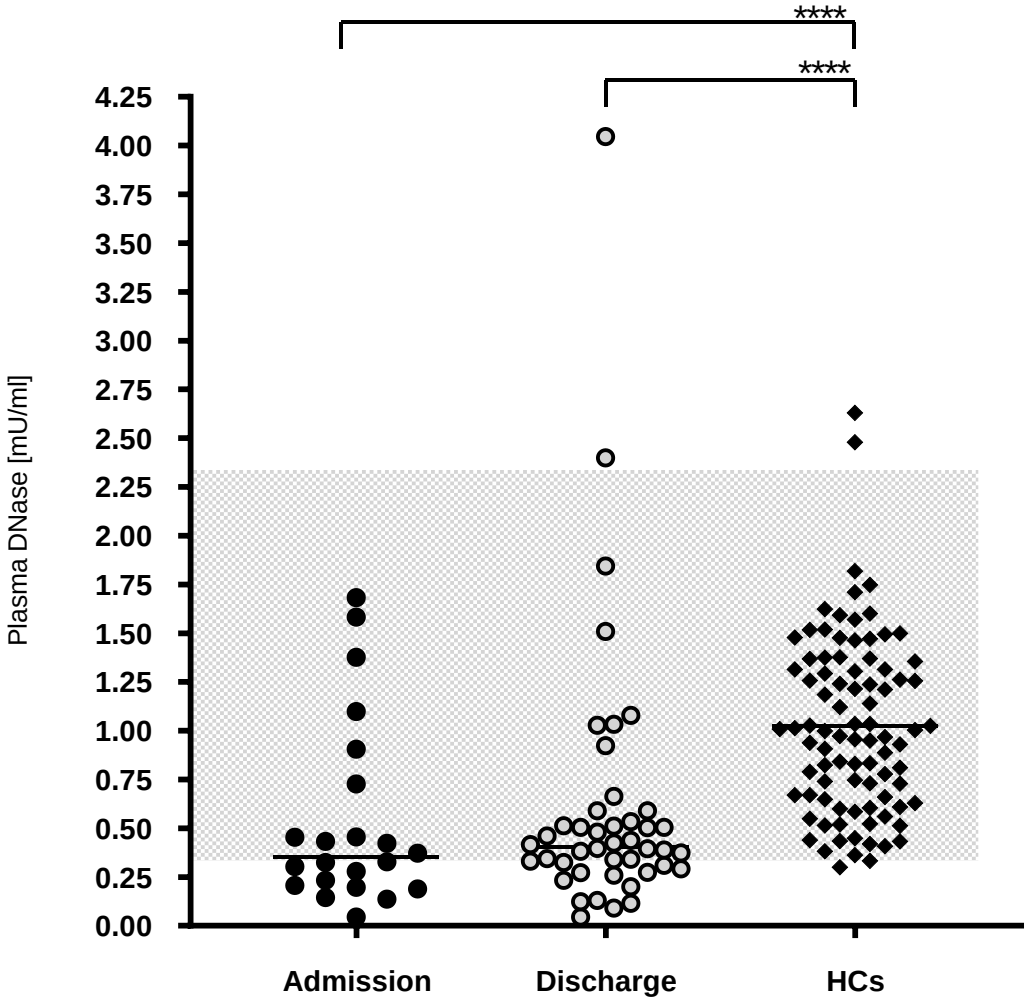


Figure 4. Plasma DNase1 levels of HUS patients at admission and discharge: The amount of circulating DNase1 measured by the previously described SRED assay in the first taken sample at admission and in the last taken sample at discharge of each HUS patient was compared to the amount of circulating DNase1 measured in the same way in healthy donors' sera. Those healthy controls show significantly elevated DNase1 levels as compared to HUS patients, irrespective of the sample collection timepoint. The horizontal axis lists the timepoints for the sample collection. Each dot stands for one HUS patient. The vertical axis represents the range of measured DNase1 values. $\alpha = 0.05$, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Gray shaded rectangle background = 2.5 – 97.5 percentile range of healthy controls (two-sided). One-way ANOVA: number of treatments (columns) 3, number of values (total) 154, Kruskal-Wallis test: p value < 0.0001, approximate p value, p value summary ****, do the medians vary significantly ($p < 0.05$)? Yes. Number of groups 3, Kruskal-Wallis statistic 43.38

Table 9. Statistics of plasma DNase1 levels of HUS patients at admission and discharge: The statistics show significantly elevated DNase1 levels in healthy controls as compared to HUS patients, irrespective of the sample collection timepoint. α = 0.05, ns = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001, **** = p < 0.0001.

Statistics	Admission	Discharge	HCs
Number of values	22	44	88
Minimum	0.04534	0.04511	0.2997
25% Percentile	0.2047	0.2966	0.6519
Median	0.3504	0.4068	0.9857
75% Percentile	0.7721	0.5754	1.345
Maximum	1.682	4.046	2.63
Mean	0.5408	0.6126	1.023
Std. Deviation	0.4813	0.6987	0.4574
Std. Error	0.1026	0.1053	0.04876
Lower 95% CI of mean	0.3274	0.4002	0.9259
Upper 95% CI of mean	0.7542	0.825	1.12
Sum	11.9	26.95	90.01

Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
At Admission vs At Discharge	-2,591	P > 0.05	ns
At Admission vs HCs	-49,53	P < 0.0001	****
At Discharge vs HCs	-46,94	P < 0.0001	****

I could not observe a significant change in plasma DNase1 levels in the course of hospitalization. The charts do not show significant one-way analysis of variances. The median at admission is 0.3504 mU/ml and the mean 0.5408 mU/ml (SD 0.4813 mU/ml ± 0.1026 mU/ml), likewise a median of 0.4068 mU/ml and a mean of 0.6126 mU/ml (SD 0.6987 ± 0.1053 mU/ml) at discharge.

These data do not show a significant ($p < 0.01$) decrease in plasma DNA levels from admission to discharge and the plasma DNase1 activity does neither show an increase from admission to discharge, nor negatively correlate with the DNA levels. Nevertheless, the DNA graph shows a clear trend of decreasing DNA levels from admission to discharge.

In conclusion, these data suggest that DNA levels correlate with the acute disease state and the patients' health condition improves with decreasing DNA levels but the results do not show that this is explicit due to increasing DNase1 levels. The DNase1 activity in HUS patients seems to stay the same regardless to the disease state and yet, DNase1 activity is significantly impaired in disease as compared to under healthy conditions. With discharge, patients could not be examined further on to see whether the DNase1 activity would recover.

Since I could still not be certain that the, for the analysis selected, first taken samples at admission would represent, as assumed, the acute disease state of HUS patients presenting with symptoms, I wanted to select those samples collected during exacerbation of the illness. Thus, samples were correlated with all available laboratory parameters which were analysed at the same date and the same time as the STEC biobank sample collection. The acute disease state of HUS was defined as previously described with a modification concerning serum creatinine level. The STEC biobank was searched for all samples collected when the patient's laboratory parameters required for diagnosing a HUS were as follows:
Hemoglobin less than 12 g/dl (if female) and less than 13.5 g/dl (if male); Lactatdehydrogenase above 250 U/Liter; platelet count less than 150 x 10^9/Liter and serum creatinine level above 1.2 mg/dl (if female) and above 1.4 mg/dl (if male). Chronologically, the first sample of each HUS patient fulfilling those criteria, in other words representing the acute exacerbation of the disease was compared to the first and last taken sample to see whether the DNA and DNase1 level would be altered in relation.

Since it is possible that at the time of sample collection of the last available sample and even at discharge, the patient could still have had some HUS symptoms, I wanted to identify those samples collected at a time point where the patient had been clinically in remission. The state of remission was defined as time point when all the laboratory parameters for diagnosing a HUS are concomitantly renormalized. I searched the STEC biobank for those samples which had simultaneously shown normal hemoglobin, lactatdehydrogenase, platelets and serum creatinine levels and thus, represent the state of remission, as defined in **Table 5**. All those samples

collected in remission were compared to all samples collected in the acute disease state at admission.

For the HUS cohort, patients of whom the first available sample was collected immediately at admission were identified. Of these 22 HUS patients, the first sample collected in an acute disease state was identified. Among these, 15 patients could be identified of whom the STEC biobank contains also the last collected sample right at discharge and in state of remission (as defined above).

Figure 5 shows a significant (p value < 0.0001) decline in the DNA levels from admission with a median of 2399 ng/ml and a mean of 6237 ng/ml (SD 8007 ng/ml ± 2067 ng/ml), until discharge with a significantly lower median of 544.9 ng/ml and a mean of 545.7 ng/ml (SD 236.0 ng/ml ± 60.93 ng/ml, Fehler! Verweisquelle konnte nicht gefunden werden.). In conclusion, these data suggest that the DNA could be interesting to serve as a time course parameter for the therapy control. The grey lines illustrate the predominantly decreasing process.

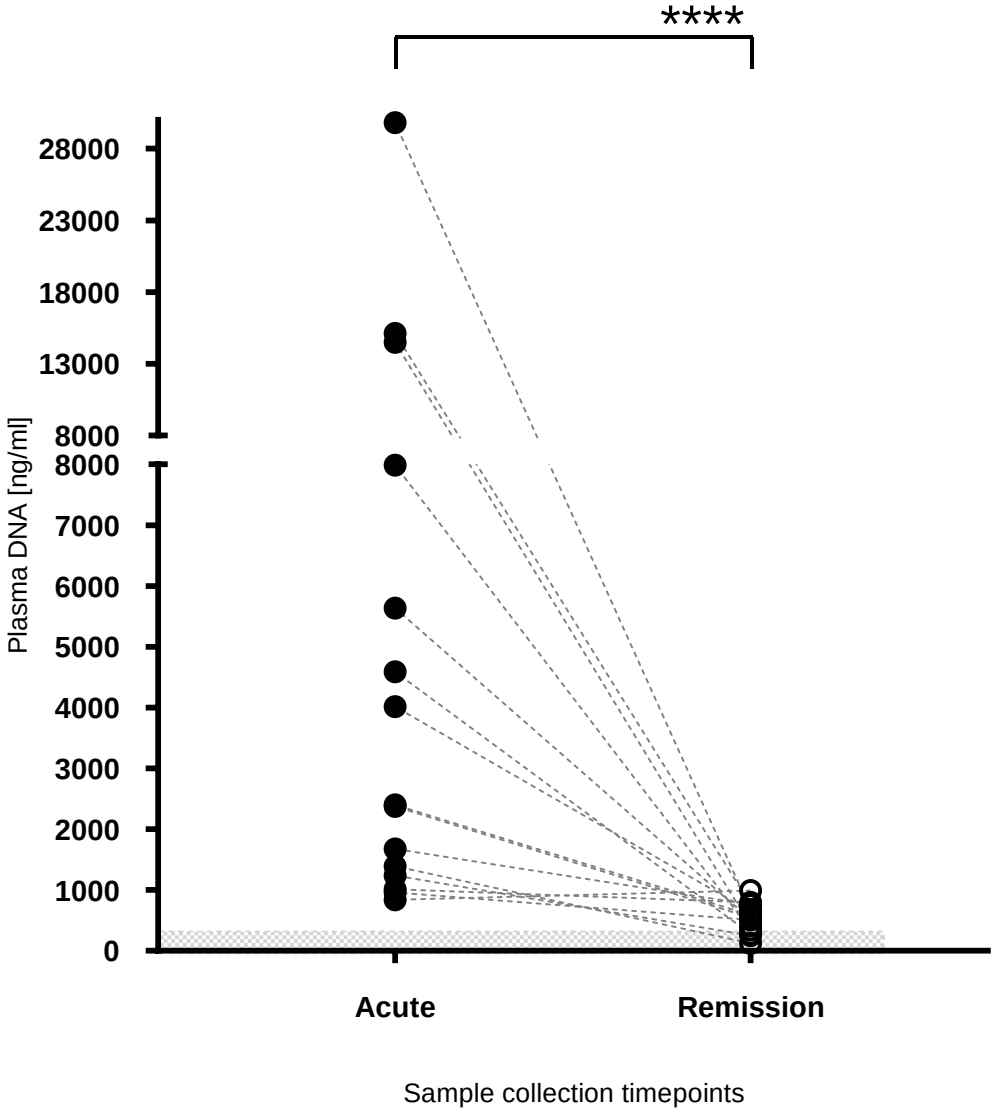


Figure 5. Plasma DNA level comparison of HUS patients at admission and at discharge: The amount of plasma DNA measured by Sytox Green nucleic acid stain in all samples of HUS patients fulfilling the previously defined criteria for the sample at admission was compared to the amount of plasma DNA measured in the same way in samples of these particular patients at discharge. Plasma DNA levels at discharge are significantly impaired as compared to at admission. The horizontal axis lists the sample collection timepoints. The vertical axis represents the range of measured DNA values. Gray shaded rectangle background = 0 - 95 percentile range of healthy controls. Gray lines: connect the samples belonging to one patient. α = 0.05, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, **** = $p < 0.0001$. Mann-Whitney test: p value <0.0001, exact p value, value summary ****, significantly different ($p < 0.05$)? yes, two-tailed p value, sum of ranks in column Admission, Discharge: 343, 122, Mann-Whitney U: 2. Difference between medians: Median of column Admission: 2399, n=15, median of Discharge: 544, n=15, Difference: Actual -1854, Difference: Hodges-Lehmann -2032.

Table 10. Statistics of plasma DNA level comparison of HUS patients at admission and at discharge: The statistics indicate that plasma DNA levels at discharge are significantly impaired as compared to at admission.

Statistics	Admission	Discharge
Number of values	15	15
Minimum	839,7	125
25% Percentile	1240	344,3
Median	2399	544,9
75% Percentile	7986	761,8
Maximum	29816	990,8
Mean	6237	545,7
Std. Deviation	8007	236
Std. Error	2067	60,93
Lower 95% CI of mean	1803	415
Upper 95% CI of mean	10671	676,4
Sum	93555	8186

DNase1 level comparison (**Figure 6**) of the very same samples of those 15 patients as in **Figure 5** do not show a significant decreasing trend from the acute disease state with a median of 0.4555 mU/ml and a mean of 0.7024 mU/ml (SD 0.5130 mU/ml ± 0.1325 mU/ml) until discharge with a median of 0.3954 mU/ml and a mean of 0.6133 mU/ml (SD 0.6335 mU/ml ± 0.1636 mU/ml, **Table 10**). Six patients show an increase in DNase1 activity from acute disease state to remission whereas nine patients show even a decrease in DNase1 activity; perhaps because DNase1s are more and more consumed during the disease. These findings suggest that DNase1 activity in HUS patients cannot be increased all of a sudden as reaction to inflammation and thrombus formation. It is conceivable that the recovery of DNases after acute HUS takes more time than patients in remission stay in hospital and thus, the state of fully recovery several days or even weeks after discharge would not be included in the STEC biobank.

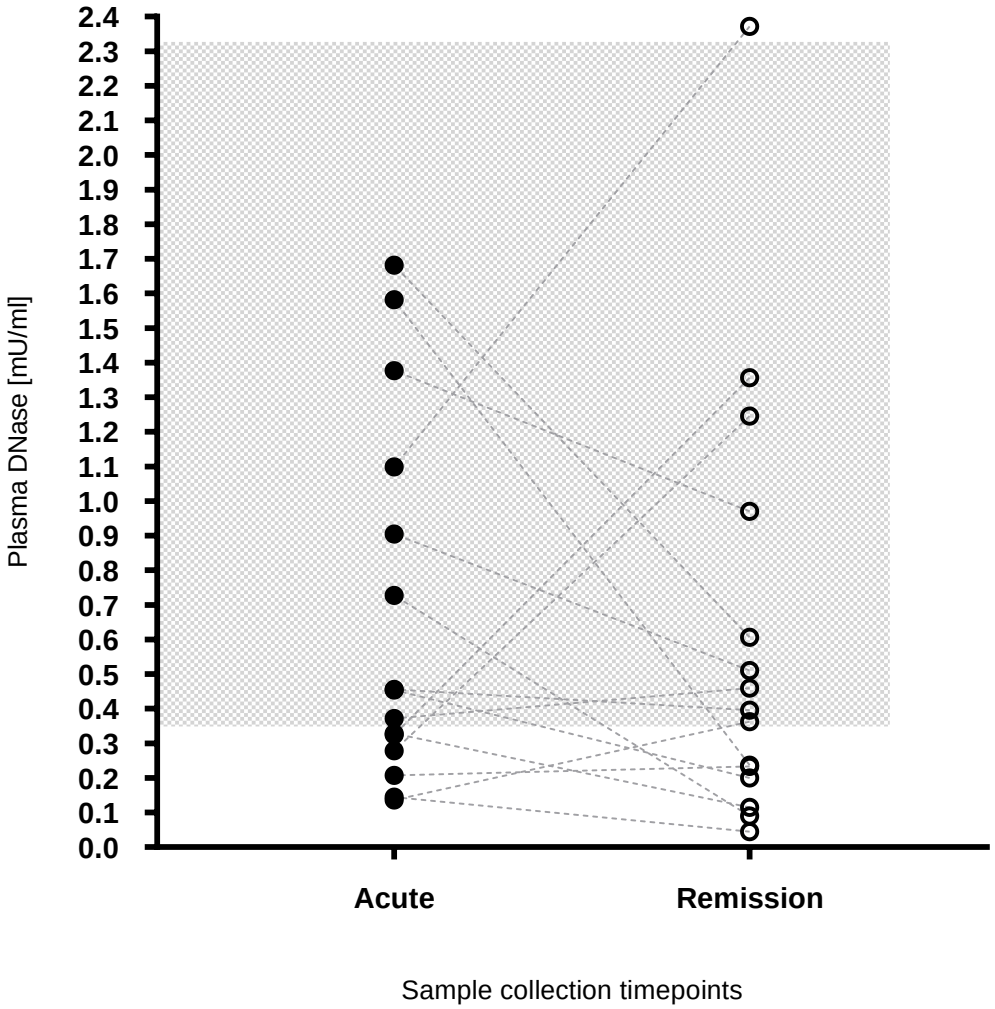


Figure 6. Plasma DNase1 level comparison of HUS patients in the acute disease state compared to at admission and at discharge: The amount of plasma DNase1 measured by the previously described SRED assay in all samples of HUS patients fulfilling the previously defined criteria at admission was compared to the amount of plasma DNase1 measured in the same way in samples of these particular patients at discharge. The DNase1 levels don't show a significant difference at admission as compared to at discharge. The horizontal axis lists the sample collection timepoints. The vertical axis represents the range of measured DNase1 values. Gray shaded rectangle background = 2.5 – 97.5 percentile range of healthy controls (two-sided). Grey lines: connect the samples belonging to one patient. $\alpha = 0.05$, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, **** = $p < 0.0001$. Mann-Whitney test: p value 0.5668, exact p value, value summary ns, significantly different ($p < 0.05$)? no, two-tailed p value, sum of ranks in column Admission, Discharge: 247, 218, Mann-Whitney U: 98. Difference between medians: Median of column Admission: 0.4542, n=15, median of Discharge: 0.3954, n=15, Difference: Actual -0.05878, Difference: Hodges-Lehmann -0.09091.

Table 10. Statistics of plasma DNase1 level comparison of HUS patients at admission compared to at discharge: The statistics don't show a significant difference in DNase1 levels at admission as compared to at discharge.

Statistics	Admission	Discharge
Number of values	15	15
Minimum	0,136	0,04511
25% Percentile	0,2789	0,1999
Median	0,4542	0,3954
75% Percentile	1,099	0,9704
Maximum	1,682	2,372
Mean	0,6716	0,6133
Std. Deviation	0,5308	0,6335
Std. Error	0,137	0,1636
Lower 95% CI of mean	0,3777	0,2625
Upper 95% CI of mean	0,9655	0,9641
Sum	10,07	9,199

4.4 Correlation of parameters

Diagnostic parameters

The UKE database was searched for all diagnostic parameters compiled at the same collection date and time of the samples in the STEC biobank in order to check whether the laboratory parameters diagnosing a HUS would be available for all samples so that I could differentiate between samples. There is still little known about the origin of DNA and DNase1 in plasma. However, it has been shown that cell free DNA in plasma in different health conditions can originate from NETs during NETosis (Grabuschnig et al., 2020).

I wanted to investigate whether there are correlations of DNA and DNase1 in plasma to some laboratory parameters. If, for example the DNase1 would correlate positively with a kidney enzyme, it could indicate that the DNase1 activity is impaired because of its origin from the disease-affected kidneys. Furthermore, I wanted to see if DNA and DNase1 correlate strongly with a well-established diagnostic parameter or if they could stand as a diagnostic parameter for itself.

For the entire biobank in total, there are 149 diagnostic parameters available with not 100% availability for each patient and each sample. Of those 149 parameters, there are 17 available for more than half of the biobank (for more than 653 samples). Those parameters are creatinine, erythrocytes, erythrocytes distribution width (RDW), hemoglobin, hematocrit, Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Leucocytes, Platelets, Urea, Lactate Dehydrogenase (LDH), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Potassium, Sodium, Creatine Kinase (CK). The required laboratory parameters were the ones for diagnosing HUS, analysing the patient's disease state by identifying the means of the parameters. If summarized to different diagnostic fields, most of the parameters are available for the hematologic, kidney and liver diagnostic, but not for the diagnostic of coagulation, blood gas analysis or electrolyte metabolism (**Table 11**).

Table 11. Laboratory parameters per diagnostic group were counted to find out if the ones relevant for diagnosing a HUS are available for the majority of HUS patients in order to identify samples collected in an acute state of disease and in remission by means of the laboratory parameters.

	EHEC (-)		EHEC (+)		HUS		Total	
	314 samples		213 samples		780 samples		1307 samples	
	[no. of samples]	[%]	[no. of. samples]	[%]	[no. of samples]	[%]	[no. of samples]	[%]
Hematology	309	98	207	97	711	91	1226	94
Electrolyte	99	32	76	36	230	29	405	31
Blood gas analysis	15	5	9	4	232	30	257	20
Kidney	294	94	202	95	707	91	1204	92
Enzymes	260	83	197	92	449	58	906	69
Coagulation	28	9	8	4	241	31	277	21

There are lead enzymes for several organs when damaged such as AST and ALT for liver cell damage. Enzymes located in cells disaggregating become released into the plasma. When measuring higher amounts of a certain lead enzyme, it could indicate cell damage of the corresponding organ. We can observe a strong positive correlation between DNA levels and the diagnostic parameters leucocytes, AP, LDH, AST whereas there is a negative correlation between the DNA levels and Albumin, Hap, Sodium, Calcium. These data support the results that the DNA is elevated in the acute disease state and normalized in the state of remission. Leucocytes positively correlate with DNA with a Spearman correlation r of 0.780698 and a p-value of 5.38602E-24 because the inflammation cells could be the source or cause for the elevated DNA levels. Either they release DNA by means of NETosis or they cause higher DNA amounts in the plasma through performing scaffolding for extracellular DNA fibers. The DNA significantly correlates with the AST with a Spearman correlation r of 0.61 and a p-value of $2.24E^{-07}$. The AST is an enzyme found in several cells, but high concentrations occur in liver and muscle cells. The AST is mostly located in mitochondria (Kondoh et al., 1989) whereas the ALT is mostly located in the cytosol (Anemaet et al., 2010). ALT is a liver-specific enzyme (Wedemeyer et al., 2010) but its correlation with the DNA (Spearman r of 0.40 and a p-value of $1.22E^{-03}$) is not as high as the correlation of AST with DNA. This leads

to the assumption that the disease includes vast cell damage. Since some DNases can originate from the liver (Shiokawa and Tanuma 2001), liver cell damage could lead to a lack of DNase1 resynthesis.
Moreover, Neutrophils also contain mitochondria, which could be the source of the measured AST.

The measured DNase1 levels of all 1307 samples negatively correlate with uric acid. For uric acid there is only an n of 7 which could lead to the presumed correlation but yet, interesting is that uric acid is the end product of nucleic acid metabolism, in other words the end product of DNA degradation. High amounts of DNA present in the plasma lead to high amounts of uric acid. Although the parameter has only rarely been determined, one could assume that the exhaustion of the DNAse1 due to high amounts of DNA having to be broken down is reflected here. But whether it is really a matter of a depletion of the DNase or whether this collective generally has lower values cannot be reflected by this correlation.
The parameters serum creatinine, urea, LDH and bilirubin show a negative correlation to the DNAse1. In total, these parameters represent the severity of HUS. An increase in these parameters could lead to - as cause or consequence – decreased DNase activity. As hemolysis proceeds, thrombi cannot be degraded due to reduced DNase1 activity and stable NETs could aggregate and could accumulate in the kidneys. This could suggest that kidney function is negatively impacted by low DNase1 activity. If the kidneys would be one source of the DNase1, it would aggravate the impairment of DNase1 activity as there would be no rapid regeneration of the enzyme.
Since there is no strong positive correlation for the DNase1, it would be predestined to serve as a new diagnostic parameter and would not be redundant to a yet well-established diagnostic parameter. But also, the correlation to the disease itself seems not to be simultaneously to clinical symptoms and it remains a diffuse correlation to the clinical state of the disease itself.
DNase1 seems to be delayed giving the idea of rather serving for retrospective statements.

Table 12. Correlation of DNA to all available diagnostic parameters: The measured DNA values of all 1307 patients' samples were correlated to all 149 available diagnostic parameters from the UKE database. Spearman correlation r, number of available samples for the particular parameter and p value were calculated. α = 0.05. List of abbreviations in Annex.

	spearman r	p value	n of XY pairs
Leucocytes	0,780698	5,38602E-24	111
AP	0,7412587	0,005801151	12
LDH	0,6511831	1,42229E-15	118
AST	0,6062786	2,24018E-07	61
Lipase	0,4398555	0,06776167	18
Urea	0,4126751	1,00023E-05	107
ALT	0,4013906	0,001223395	62
Mg	0,3498982	0,1546256	18
MtHb	0,3359757	0,009924707	58
FibD	0,3288986	0,01844546	51
CRP	0,2787623	0,008934262	87
COHb	0,2196412	0,09462731	59
Krea	0,2049182	0,02601663	118
CK	0,1997791	0,1642287	50
Protein	0,1759492	0,1904673	57
GGT	0,1294786	0,5559843	23
Bilirubin	0,1218522	0,3579017	59
pH	0,1133329	0,3969601	58
Erythrocytes	0,09160356	0,3389759	111
Uric acid	0,09009375	0,8476721	7
TZ	0,05940767	0,7121598	41
Potassium	0,05478641	0,7207587	45
DNase	0,02588581	0,7808242	118
Hematocrit	0,001316335	0,9890602	111
Hemoglobin	-0,01085998	0,9099314	111
PO2	-0,06010921	0,6539955	58
MCHC	-0,06020292	0,5302222	111
SO2	-0,06789098	0,6125956	58
RDW	-0,1288022	0,1778921	111
K	-0,1303179	0,3670384	50
HCO3	-0,1974573	0,1338609	59
INR	-0,2203722	0,0935069	59
Glucose	-0,2830285	0,1906659	23
Phos	-0,3043147	0,07550382	35
Na	-0,3243237	0,02299624	49
MCH	-0,3833483	3,28274E-05	111
Thrombocytes	-0,416276	1,00227E-05	105
MCV	-0,4611203	3,52085E-07	111
Albumin	-0,5004809	0,01083403	25
Hap	-0,5214286	0,04621923	15
Sodium	-0,5684392	4,63027E-05	45
Calcium	-0,5869594	0,000816773	29

Table 13. Correlation of DNase to all available diagnostic parameters: The measured DNase values of all 1307 patients' samples were correlated to all 149 available diagnostic parameters from the UKE database. Spearman correlation r, number of available samples for the particular parameter and p value were calculated. α = 0.05. List of abbreviations in Annex.

	spearman r	p value	n of XY pairs
AP	0,4055944	0,1908359	12
GGT	0,3251792	0,1300181	23
TZ	0,2613241	0,09886891	41
Potassium	0,2070808	0,1722854	45
MCV	0,1746099	0,06681555	111
CK	0,1669308	0,2465884	50
Lipase	0,1393908	0,5811992	18
MCH	0,1350772	0,1575078	111
Protein	0,1103531	0,41382	57
RDW	0,09245608	0,3344809	111
FibD	0,08290343	0,5630154	51
MtHb	0,07228372	0,5897358	58
K	0,06597705	0,6489474	50
AST	0,06400254	0,6241074	61
Leucocytes	0,04843494	0,6136898	111
Hematocrit	0,04638326	0,6288072	111
Thrombocytes	0,03878482	0,6944549	105
Hemoglobin	0,03329077	0,7286921	111
DNA	0,02588581	0,7808242	118
ALT	0,02320221	0,8579374	62
Ca	0,007395541	0,969628	29
Erythrocytes	0,004773898	0,9603399	111
CRP	0,004684161	0,9656543	87
pH	0,002399557	0,9857372	58
Glucose	-0,00098961	0,9964244	23
Albumine	-0,005000962	0,9810722	25
Sodium	-0,008696197	0,9547879	45
Na	-0,02240074	0,8785747	49
MCHC	-0,05626576	0,5575053	111
Creatinine	-0,06004083	0,5183762	118
HCO3	-0,0904574	0,4956507	59
Hap	-0,1142857	0,6850658	15
COHb	-0,1174131	0,3758119	59
INR	-0,127014	0,3377459	59
Phos	-0,1303404	0,4554908	35
LDH	-0,1410321	0,1276793	118
AT	-0,1904762	0,6514015	8
Urea	-0,2145007	0,026511	107
PO2	-0,2760286	0,03596298	58
SO2	-0,2867909	0,02906057	58
Bilirubin	-0,3020389	0,02007445	59
Uric acid	-0,7027312	0,07823745	7

5 DISCUSSION

Organization of STEC biobank

Sample collections took place in 2011 and I received the whole biobank in 2014 to analyze the role of plasma DNA and DNase in HUS. What should be taken into account is the preanalytical process of my study. Sample collections took place under real life conditions in an epidemic and samples were stored at -80 ° at all times. Even if there is no guarantee that the DNases remain fully stable over this time, my results go in line with previous studies that show impaired DNase1 activity in TMAs (Jiménez-Alcázar et al., 2015) and even in HUS (Leffler et al., 2016). HUS patients showed predominantly low DNase1 activity compared to HCs and a mean under the mean of HCs. Only some rare cases showed increased activity compared to HCs (Leffler et al., 2016) as shown in **Figure 2 and 4**. My observations reflect the findings of Leffler et al. (2016), even though samples used in our study were stored for three years prior analysis. I even received similar results when performing my experiments four times at different timepoints within two years due to a trial of a high-throughput automized screening with a self-programmed robot and slight modifications in the procedure.

Another limitation of the study is the limited time range of sample collections during hospitalization without the possibility of reevaluating the patients after discharge.

All in all, elevated DNA levels correlate with initial untreated symptoms seen in patients. At discharge, DNA levels are not at the level of healthy controls but they are already lower than at admission leading to the assumption that the amount of circulating cell-free DNA (cfDNA) correlates with the severity of symptoms and could serve as a novel diagnostic parameter. It remains to be elucidated if DNase1 is the cause or effect of impaired NET-degradation as DNase1 activity is significantly impaired in HUS. Understanding the mechanisms underlying NETosis, the role of circulating DNA and circulating DNase1, can provide insights into the pathogenesis of various diseases and potentially lead to the development of novel therapeutic strategies.

Since plasma collections of patients took place during an ongoing epidemic, there was no standardized protocol for the collection date and time. The additional plasma sample tube for the STEC biobank for post hoc analyses was collected randomly

whenever blood samples needed to be taken for laboratory diagnostics. I arranged the samples in the STEC biobank, established an inventory with all available clinical data, defined subgroups and excluded samples which could have been collected under already started therapy or which were not precisely enough to allocate because of missing data.

The first available samples in the STEC biobank were not a sufficiently precise collective because, as mentioned, the patients could have already undergone therapy. Therapy might affect DNA and DNase1 levels, this indicates the possibility that among the first available samples there are some which do not accurately represent the DNA and DNase1 levels in an ongoing HUS disease and thus were excluded from further analysis. The sample collective taken at admission reflects the untreated disease state because those samples were taken simultaneously with the very first blood collection of each patient meaning with the laboratory conducted at admission and, therefore, when the patient was still untreated. Thus, I focused on the cohort where samples were collected at admission and discharge instead of the first and last available samples.

Comparison of diagnosis groups

Patients with HUS symptoms could have higher DNA levels (**Figure 1**) in plasma because of variable cell death pathways due to the infection and increased NETosis which leads to pro-thrombotic NETs and/or because of reduced DNase1 activity causing the persistence of NETs.

Recently, it was shown that Shiga toxin-producing enterohemorrhagic bacterial infections promote NETs formation by activating neutrophils through a pathway of Shiga toxin 2 amplifying the expression of the cell adhesion protein P-selectin on platelets. This leads to increased aggregation of platelets and neutrophils and consequently to NETosis by these neutrophils. Landoni et al. also showed that Shiga toxin 2a induces NETosis in healthy neutrophils and that neutrophils derived from STEC-HUS patients show spontaneous NETosis (Landoni et al., 2022). Additionally, DNA and MPO co-localized in immunofluorescent images in the acute state and in remission in STEC-HUS patients (Feitz et al., 2021).

In terms of these findings, the elevated DNA levels in HUS patients of my research are likely to originate from NETs. Since EHEC (+) and EHEC (-) do not differ significantly, their elevated plasma DNA levels could be due to diarrheal symptoms since this is a common feature of both cohorts. Here, it would be interesting to conduct a study with patients presenting to the emergency to investigate in which diseases DNA levels are altered.

The activity of free extracellular DNase1 in HUS patient plasma is impaired as compared to DNase1 levels in control plasma of healthy donors. The DNase1 levels of HUS patients stay impaired under therapy and seem not to recover during the period of hospitalization (**Figure 4** and **Figure 6**).

Due to an impaired activity of plasma DNase1 in HUS patients in the acute disease state as compared to healthy controls, free circulating DNA in plasma is suspected to not be efficiently degraded. Higher amounts of free circulating DNA in plasma could also originate from damaged tissue caused by thrombotic microangiopathies. Pro-thrombotic NETs could accumulate because of the reduced NET degradation and thus aggravate the course of the disease. Indeed, previous experiments showed that NETs are stable after exposure to plasma from TMA patients whereas plasma from healthy donors degrades NETs efficiently (Jimenez-Alcazar et al., 2015).

The three observed HUS cohorts showed a significant difference in DNase1 levels compared to healthy controls (**Figure 2**) and no significant differences amongst the cohorts. However, there is a trend to lower DNase1 activity in HUS patients compared to EHEC(+) and EHEC(-), and a lower DNase1 activity in EHEC(+) than in EHEC(-). For the DNase1 activity we can observe - contrarily to the plasma DNA – an impairment. DNase1 activity is more and more impaired with disease activity from diarrhea to EHEC and HUS. The statistical significance was calculated using Dunn's Multiple Comparison Test which is considered to be rather conservative. A follow-up study could provide considerable added value here.

The impairment of DNase1 activity in HUS patients could have existed before the infection, due to reduced DNase1 baseline levels or due to genetic mutations. In other diseases such as SLE, it is known that DNases are mutated (Keyel et al.,

2017) leading to the possibility of increased susceptibility in these individuals. On the other hand, the impairment of DNase1 activity could occur during disease due to a somehow blocking process or as an effect of the disease itself. It is plausible that DNase1 is depleted during massive extracellular DNA accumulation. Still lower DNase1 activity at hospital discharge could be due to the resynthesis process. As with discharge, all patients were gone and I could not measure the DNase1 activity in samples collected after discharge, I could solely investigate the state at discharge. I hypothesize that patients were not fully recovered upon discharge from hospital, causing persistently low DNase1 activity. It would be interesting to investigate the same individuals nowadays to compare if their individual DNase1 activity is lower or comparable to HCs.

Analysis of HUS patients

The data show that the amount of free extracellular DNA circulating in the plasma of patients suffering from HUS is substantially elevated at admission and decreases during therapy from the acute state to remission. Only in a few recovering HUS patients, DNA levels reach the range of healthy control plasma (**Figure 3**) but DNA levels are substantially and significantly lower in the state of remission than in the acute state (**Figure 5**). Extracellular DNA released by neutrophils during NETosis may be increased in various diseases. Some examples include inflammatory diseases such as rheumatoid arthritis (Wright et al., 2021), SLE (Reshetnyak et al., 2023), chronic obstructive pulmonary disease (Trivedi et al., 2021), and sepsis (Alsabani et al., 2022). In addition, increased extracellular DNA has also been observed in cancers (Cristinziano et al., 2021) such as lung cancer (Inoue et al., 2018) and breast cancer (Pan et al., 2023). However, it is important to note that the increase in extracellular DNA is not specific to a particular disease and can occur in other conditions as well. DNA levels could be influenced by health behaviour and are higher in men than in women. Smoking and frailty were recently associated with higher cfDNA levels and vegetable consumption with lower cfDNA levels (Kananen et al., 2022). An accurate diagnosis therefore requires a comprehensive assessment of clinical symptoms and further diagnostic tests.

Circulating DNA derived from neutrophils undergoing NETosis promotes thrombus formation (Fuchs et al., 2010). During acute HUS, high amounts of DNA are present

in the plasma (**Figure 3, Figure 5**), likely originating from neutrophil-derived NETs. My results go in line with other findings in STEC-HUS patients which show significantly higher serum DNA levels as compared to healthy donors (Leffler et al., 2016).

As plasma DNase1 is the counterpart for free extracellular DNA circulating in the plasma, it degrades the high amounts of plasma DNA and thus it could be the cause of the remission of the disease. Since HUS patients had immediately been put under plasmapheresis at the onset of the disease (Harendza, 2011), the reduction of the highly elevated plasma DNA levels could be also due to the supportive therapy. This could lead to the assumption that DNase1 is not responsible for the reduction of plasma DNA and its impairment would not have an effect of the disease's aggravation. But it would also predestine plasma DNA to be a good marker for the therapy control.
Circulating cfDNA is already proposed to be used as a biomarker in different diseases such as autoimmune rhematic disease and sepsis (Duvvuri et al., 2019, Jing et al., 2022).

Since the time from the acute disease state to remission seems to be not long enough to observe a DNase1 recovery, DNase1 levels stay impaired in the acute disease state and in remission (**Figure 4, Figure 6**). Another explanation could be that the DNase1 activity is not increased or decreased in a patient but rather remains at an individual baseline, irrespective of the disease status. Those individuals, whose DNase1 activity would be impaired in contrast to others may be more likely to develop HUS when become acquiring EHEC.
In STEC-HUS patients it has already been shown that anti-dsDNA antibodies do not impede NET -degradation as this is a known mechanism in SLE patients (Hakkim et al., 2010, Leffler et al., 2012).
Thus, DNase1 may not be as swiftly replenished than the here given period under review as I could not examine the same patients after their discharge, in a state of DNase1 repletion. Further investigations of DNase1 activity time courses in donors could give valuable information.

In Long Covid patients, Fogarty and colleagues investigated alongside other parameters extracellular DNA and DNase1 activity. More than eight weeks after discharge or resolution of symptoms, endotheliopathy persisted whereas extracellular DNA and DNase1 activity in convalescent patients were comparable to healthy controls (Fogarty et al., 2021). Other studies suggest that NETosis and NET-clearance deficiencies are part of acute Covid-19 (Arcanjo et al., 2020, Veras et al., 2020). Deficient NET-clearance in Covid-19 could be DNase1-mediated. If so, the enzyme could then more and more be depleted and would show impaired DNase1 activity in the acute state. But comparable DNase1 activity in Long Covid patients after more than eight weeks indicate that the activity of DNase1 is able to recover. Hypothetically, it takes more time for the DNase1 to be resynthesized than the given time in hospitalization. The here observed period of STEC-HUS patients was only during hospitalization and it could be that after eight weeks or more after discharge, the DNase1 was replenished in HUS patients.

Several diseases like vasculitis, psoriasis, SLE, pancreatitis and thrombosis are associated with a lack of efficient degradation of NETs (Engavale et al. 2021). In HUS patients, the elevated plasma DNA levels (**Figure 1, Figure 5**) are suspected to indicate inefficient NET degradation and thus, accumulation of NETs. Reduced host DNase1 activity (**Figure 2, Figure 4**) is suspected to be responsible for the inefficient NET clearance and thus, plays a key role in the origin of other diseases like SLE (Engavale et al. 2021). If the plasma DNase1 activity does not suffice to degrade plasma DNA and NETs, this could contribute to the formation of HUS. The fact that the DNase1 activity before-after graph (**Figure 6**) presents such a diffuse pattern and that some samples of HUS patients in remission show DNase1 levels above the median of healthy controls (**Figure 4**), could be due to a supportive therapy with glucocorticoids which are suspected to improve DNase1 activity (Zhang et al., 2014). The few DNase1 levels of HUS patients at admission above the median of healthy controls (**Figure 4**) could show that DNase1 activity was not immediately impaired, but rather during the course of disease.

It was already shown that sera of STEC-HUS patients fail to degrade NETs efficiently and that there is a correlation between NET degradation and nuclease activity (Leffler et al., 2016).

As already shown, NET degradation in vitro is reduced by plasma of TMA patients (Jimenez-Alcazar et al., 2015). Unlike in some patients with the autoimmune

disease systemic lupus erythematosus due to protective anti-NET-antibodies and/or DNase1-inhibitors (Hakkim et al., 2010), reduced DNase1 levels were shown to be the reason for the impairment of NET degradation in TMA patients. Our lab previously showed that impaired NET-degradation of TMA patient plasma is due to the lack of DNase1 activity and not due to DNase1 inhibitors (Jimenez-Alcazar et al., 2015). These results suggest that patients, whose individual plasma DNase1 baseline activity is impaired, are more likely to develop HUS as a complication of an ongoing EHEC-infection than patients with plasma DNase1 activity comparable with those of HCs. If so, this would suggest that DNase1 activity in EHEC-patients could be a prediction marker for the development of HUS. **Figure 2** shows that there are overlaps in the DNase1 activity of HCs and HUS patients. This could suggest that amongst healthy individuals there are some with an impaired DNase1 baseline activity. Yet, HUS patients have a significantly lowerDNase1 activity compared to HCs (**Figure 2**). On the other hand, if DNase1 is depleted in the acute state and not fully resynthesized in remission but several weeks later, it could probably be an interesting parameter to estimate disease. Further trials are required to answer this question conclusively.

Investigations of healthy cohorts should be done in order to establish a reference range for plasma DNase1 as a diagnostic parameter. The same applies to the reference range for plasma DNA as a disease state and follow-up marker of an ongoing HUS disease.

Recombinant human DNase1 is already an FDA-approved drug (Pulmozyme®, dornase alfa. Genentech, South San Francisco, CA, USA) and is an aerosolized used in the treatment of cystic fibrosis (CF) to improve pulmonary function (Ranasinha et al., 1993, Witt and Anderson, 1996). High amounts of extracellular DNA in the bronchial secret of CF patients are degraded by DNase1 what enables the patient to expectorate the less viscous mucus (Shak et al., 1990, Ranasinha et al., 1993).

In the technical information of Roche's Pulmozyme® 2 it is declared that healthy humans received intravenous treatment with dornase alfa in order to examine whether antibodies would be produced. Here, Roche states that the intravenous treatment had been well tolerated by all six test persons (Roche, Pulmozyme, package leaflet, 2017). Work is already underway to target NETs and other

pathologies of extracellular DNA. Neutrolis is a clinical stage therapeutics company working on therapeutic options.
In Covid-19 patients, aerosolized Pulmozyme® showed a degradation of NETs and contributed to the patients' recovery (Fisher et al., 2021).
Recombinant human DNase1 could be valuable for the therapy for HUS as well. Since it degrades plasma DNA, it could be used in the acute state of HUS to eliminate thrombi in the microvasculature. Administered in good time, initial inhibition of thrombus formation by DNase1 is also conceivable. It has been shown that combined DNase1 and DNase 1 like 3 (DNase1L3) activities lead to efficient DNA degradation and respectively NET degradation ex vivo (Englert et al., 2023). In murine models, it has recently been shown that DNase1 and even more DNase1L3 knock-out mice develop NETs-associated inflammation. Additionally, the application of DNase1 (Dornase alfa, Roche) reduced the formation of NETs-associated peritoneal adhesions *in vivo* (Elrod et al., 2023). Further studies for the intravenous application of DNases could offer great benefits for therapeutic strategies not only in TMAs.

Correlation of parameters

It is speculated that the DNA originates from NETs which are released by neutrophils during TMAs (Fuchs et al., 2012b) such as HUS. The cellular origin of circulating cfDNA in the extracellular space is poorly understood. As Shiga toxins are bound to their Gb3 receptor which is expressed on several cell types (Meyers and Kaplan, 2000), the elevated DNA levels in the acute disease state of HUS may exclusively originate from the cell damage due to the infection. Since the AST originates from mitochondria (e.g. of liver cells) and shows a significant correlation with elevated DNA levels, it indicates vast cell damage.
The fact that the DNA positively correlates with those diagnostic parameters which are increased in HUS and also correlates with the clinical presence of symptoms and negatively correlates with those parameters which are decreased in HUS allows to conclude that the elevated DNA levels represent the state of the ongoing disease. In other diseases such as myelofibrosis, it has been observed that high amounts of plasma DNA correlate with markers of systemic inflammation (De Luca et al., 2023). The results of correlating DNase1 levels with laboratory parameters suggest a negative effect from impaired DNase1 levels on kidney function. Kidney function

could be negatively influenced by stable NET aggregation because of impaired NET degradation by DNase1. In mice, it was shown that renal ischemia reperfusion injury leads to the formation of NETs in the kidneys and to elevated plasma DNA (Jansen et al., 2017) which may consequently result in DNase1 depletion.

In order to answer the question if DNA and DNase1 could serve as diagnostic parameters, correlations of parameters and disease state are relevant. High extracellular DNA levels correlate with HUS severity. But since the significance is also high for patients with EHEC but without HUS and even for patients only with diarrheal symptoms but without EHEC, extracellular DNA is not specific for the disease and elevated DNA levels are already associated with several other diseases (Kubota, 2023, Bruschi et al., 2021). DNA could serve as a follow-up parameter with a good correlation to the severity of the disease. Here, it is questionable if extracellular DNA would offer new information or if well-established laboratory parameters give the same information and are sufficient for the diagnostic such as the number of leucocytes and LDH.
For further studies, it would be interesting to investigate if the highest DNA levels are found on the same date when the disease activity is at its climax. Furthermore, investigations of patients presenting to the emergency with other diseases would be necessary to see if DNA levels are also elevated in the acute state of other diseases. For example, late-onset sepsis and necrotizing enterocolitis are associated with high DNA levels (Nguyen et al., 2017). In some cancer patients, it was shown that high cfDNA levels come from neutrophils (Mattox et al., 2023). Elevated cfDNA levels are found in Covid-19 patients (Zuo et al., 2020, Englert et al., 2021).
In addition, it would be interesting in which other diseases DNA levels are increased. For further studies, patients presenting to the emergency should be investigated to see in which diseases elevated DNA levels are found.

It is questionable, if DNase1 is able to serve as a new diagnostic parameter yet. Its significance to the disease activity seems to correlate in the acute state with impaired DNase1 activity compared to healthy controls **(Figure 1).** For further studies, it would be necessary to investigate the very same patients in state of health, disease and recovery. Since the STEC-biobank is now a well-arranged extended biobank, it would be possible to establish a screening for those patients

including when they present to the University Medical Center Hamburg-Eppendorf in the future due to other symptoms and to measure their individual DNase1 activity then.

In addition, it would be of interest to examine if the measured extracellular DNA originates from NETs. Therefore, staining for the NET markers myeloperoxidase-DNA (MPO-DNA) and citrullinated histone H3 (Cit-H3) (Masuda et al., 2016) should be performed for the STEC-biobank samples.

Since there is no strong correlation between DNase1 activity and a laboratory parameter, it is feasible that DNase1 activity is capable of giving us new information about the state of a patient. In the light of my findings as well as according to the current status of research, I attribute DNase1 a retrospective value.

It would be interesting to establish a regularly longitudinal collecting process from healthy donors before the blood donation process. Measuring DNase1 activity in the same donors over a long time could give information about whether there is a collective of people who have lower baseline levels for DNase1. If so, this would predestine for risk prediction. If not, DNase1 is reduced over the course of the disease which would predestine for progression marker for (subclinical) progression.

6 ABSTRACT

Hemolytic uremic syndrome (HUS) is a life-threatening disease characterized by acute thrombotic microangiopathy (TMA). The most common form of the disease is caused by infection with Shiga toxin-producing enterohemorrhagic Escherichia coli bacteria (STEC). TMAs are characterized by disseminated thrombi in the microvasculature and are associated with the formation of neutrophil extracellular traps (NETs). These NETs are composed of DNA fibers and are sensitive to digestion by plasma DNase1 *in vitro*.

I hypothesize that HUS patients have large amounts of free extracellular DNA and lack efficient DNA resolution due to reduced DNase1 activity. To this end, I examined plasma DNA levels and plasma DNase1 activity in HUS patients in the acute stage of the disease and in remission compared to patients with non-STEC diarrhea symptoms, non-HUS-STEC infection and in healthy subjects.

In this study, I show that HUS patients have elevated DNA plasma levels and decreased DNase1 activity at admission and in the acute disease state of HUS compared to healthy controls. Plasma DNA levels of HUS patients recover during therapy until discharge, whereas plasma DNase1 activity of HUS patients remains impaired compared to healthy controls. The plasma DNA content in the acute state is also increased in patients with non-HUS-STEC infection and non-STEC diarrhea symptoms compared to healthy controls, although the values in HUS patients are sometimes significantly higher. DNase1 activity is also reduced in patients with non-HUS-STEC infection and non-STEC diarrheal symptoms compared to healthy subjects, yet the picture emerges that HUS patients show the lowest DNase1 activities. High levels of plasma DNA appear to represent the acute disease state with excessive thrombi in the microvasculature. Impaired plasma DNase1 basal activity and thus inadequate plasma DNA or NET degradation can predestine a STEC patient for the development of HUS.

More likely, DNase1 activity is depleted in the presence of large amounts of plasma DNA and requires more time to recover and be resynthesized in convalescent patients. To treat excessive thrombus formation in thrombotic diseases such as HUS, DNase1-mediated degradation of NETs may be crucial. TMAs have been shown to have impaired DNase1 activity, which may lead to impaired degradation of NETs. This lack of DNA degradation may contribute to HUS. Because NETs are

known to trigger clotting through activation of platelets and clotting factors, inhibiting NET formation or eliminating it may prevent or treat the disease. It is conceivable that DNase1 could function as a drug against HUS, while DNase1 activity could serve as a retrospective parameter and plasma DNA levels could be used to monitor therapy.

ZUSAMMENFASSUNG

Das lebensbedrohliche hämolytisch-urämische Syndrom (HUS) wird in seiner häufigsten Form durch eine Infektion mit Shiga-Toxin produzierenden enterohämorrhagischen Escherichia coli-Bakterien (STEC) verursacht. HUS ist gekennzeichnet durch eine akute thrombotische Mikroangiopathie (TMA). TMAs zeichnen sich durch disseminierte Thromben im Mikrogefäßsystem aus und sind mit der Bildung von extrazellulären Neutrophilenfallen (NETs) verbunden. Diese NETs bestehen aus DNA-Fasern und reagieren *in vitro* empfindlich auf die Verdauung durch Plasma-DNase1.

Ich stelle die Hypothese auf, dass bei HUS-Patienten große Mengen an freier extrazellulärer DNA vorhanden sind und es aufgrund verringerter DNase1-Aktivität an einer effizienten DNA-Auflösung mangelt. Hierfür untersuchte ich die Plasma-DNA-Spiegel und die Aktivität von Plasma-DNase1 bei HUS-Patienten im akuten Krankheitsstadium und in Remission im Vergleich zu Patienten mit Nicht-HUS-STEC-Infektion, Nicht-STEC-Durchfallsymptomen und bei Gesunden.

In dieser Studie zeige ich, dass HUS-Patienten bei Aufnahme und im akuten Krankheitszustand im Vergleich zu gesunden Kontrollpersonen erhöhte DNA-Plasmaspiegel und eine verminderte DNase1-Aktivität aufweisen. Die Plasma-DNA-Spiegel von HUS-Patienten erholen sich während der Therapie bis zur Entlassung, wohingegen die Plasma-DNase1-Aktivität im Vergleich zu gesunden Kontrollpersonen beeinträchtigt bleibt. Der Gehalt an Plasma DNA im akuten Zustand ist auch in Patienten mit Nicht-HUS-STEC-Infektion und Nicht-STEC-Durchfallsymptomen im Vergleich zu gesunden Kontrollen erhöht, wobei die Werte von HUS-Patienten teilweise deutlich höher liegen. DNase1-Aktivität ist auch in Patienten mit Nicht-HUS-STEC-Infektion und Nicht-STEC-Durchfallsymptomen im Vergleich zu Gesunden vermindert, dennoch zeigt sich das Bild, dass HUS-Patienten die niedrigsten DNase1-Aktivitäten aufweisen.

Hohe Mengen an Plasma-DNA scheinen den akuten Krankheitszustand mit übermäßigen Thromben im Mikrogefäßsystem darzustellen. Eine beeinträchtigte Plasma-DNase1-Basisaktivität und damit ein unzureichender Plasma-DNA- bzw. NET-Abbau können einen STEC-Patienten für die Entwicklung von HUS prädestinieren. Wahrscheinlicher ist, dass die DNase1-Aktivität in Gegenwart großer Mengen an Plasma-DNA erschöpft ist und bei rekonvaleszenten Patienten mehr Zeit benötigt, um sich zu erholen und neu synthetisiert zu werden. Um übermäßige Thrombusbildung bei thrombotischen Erkrankungen wie HUS zu behandeln, könnte der DNase1-vermittelte Abbau von NETs von entscheidender Bedeutung sein. Es wurde gezeigt, dass TMAs eine beeinträchtigte DNase1-Aktivität aufweisen, was zu einem beeinträchtigten Abbau von NETs führen kann. Dieser Mangel an DNA-Abbau kann zu HUS beitragen. Da NETs bekanntermaßen die Gerinnung durch die Aktivierung von Blutplättchen und Gerinnungsfaktoren auslösen, kann die Hemmung der NET-Bildung oder deren Eliminierung die Krankheit verhindern oder behandeln. Es ist denkbar, dass DNase1 als Medikament gegen HUS fungieren könnte, während die DNase1-Aktivität als retrospektiver Parameter dienen und Plasma-DNA-Spiegel zur Therapiekontrolle verwendet werden könnten.

7 ABBREVEATIONS

ALT	Alanin-Aminotransferase
AP	Alkaline Phosphatase
AST	Aspartat-Aminotransferase
BSA	Bovine serum albumin
cfDNA	cell-free DNA
Covid-19	Corona-virus-disease-19
D3	Calcitriol (= 1.25-Dihydroxy-Cholecalciferol, Vitamine D3)
DNA	Deoxyribonucleic acid
DNase1	Deoxyribonuclease
E. coli	Escherichia coli
EHEC	Enterohemorrhagic Escherichia coli
Gb3	Globotriaosylceramide
GGT	Gamma-Glutamyl-Transferase
GLDH	Glutamat Dehydrogenase
Hb	Hemoglobin
HUS	Hemolytic uremic syndrome
HsTNT	High sensitive Troponin T
H3cit	Citrullinated histone H3
INR	International Normalized Ratio
LDH	Lactate dehydrogenase
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MPO	Myeloperoxidase
NETs	Neutrophil extracellular traps
Neutrophil(s)	Neutrophil granulocyte(s); Polymorphonuclear neutrophil(s)
PBS	Phosphate buffered saline
PMNs	Polymorphonuclear leukocytes
PTH	Parathyroid hormone
PTT	Partial Thromboplastin Time
RDW	Red blood cell distribution width
SD	Standard deviation

sF	Supplementary figure
SLE	Systemic lupus erythematosus
SLT	Shiga-like toxin
STEC	Shiga toxin-producing E. coli
Stx	Shiga toxin

8 REFERENCES

Alsabani M, Abrams ST, Cheng Z, Morton B, Lane S, Alosaimi S, Yu W, Wang G, Toh CH (2022) Reduction of NETosis by targeting CXCR1/2 reduces thrombosis, lung injury, and mortality in experimental human and murine sepsis. Br J Anaesth. 128(2):283-293.

Anemaet IG, González JD, Salgado MC, Giralt M, Fernández F, Baanante IV, Metón I (2010) Transactivation of cytosolic alanine aminotransferase gene promoter by p300 and c-Myb. J Mol Endocrinol. 45(3):119-32.

Arcanjo A, Logullo J, Menezes CCB, de Souza Carvalho Giangiarulo TC, Dos Reis MC, de Castro GMM, da Silva Fontes Y, Todeschini AR, Freire-de-Lima L, Decoté-Ricardo D, Ferreira-Pereira A, Freire-de-Lima CG, Barroso SPC, Takiya C, Conceição-Silva F, Savino W, Morrot A (2020) The emerging role of neutrophil extracellular traps in severe acute respiratory syndrome coronavirus 2 (COVID-19). Sci Rep. 10(1):19630.

Besser RE, Lett SM, Weber JT, Doyle MP, Barrett TJ, Wells JG, Griffin PM (1993) An outbreak of diarrhea and hemolytic uremic syndrome from Escherichia coli O157:H7 in fresh-pressed apple cider. JAMA. 269(17):2217-20.

Borgatta B, Kmet-Lunaček N, Rello J (2012) E. coli O104:H4 outbreak and haemolytic-uraemic syndrome. Med Intensiva. 36(8):576-83.

Braune SA, Wichmann D, von Heinz MC, Nierhaus A, Becker H, Meyer TN, Meyer GP, Müller-Schulz M, Fricke J, de Weerth A, Hoepker WW, Fiehler J, Magnus T, Gerloff C, Panzer U, Stahl RA, Wegscheider K, Kluge S (2013) Clinical features of critically ill patients with Shiga toxin-induced hemolytic uremic syndrome. Crit Care Med. 41(7):1702-10.

Brill A, Fuchs TA, Savchenko AS, Thomas GM, Martinod K, De Meyer SF, Bhandari AA, Wagner DD (2012) Neutrophil extracellular traps promote deep vein thrombosis in mice. J Thromb Haemost. 10(1):136-44.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A (2004) Neutrophil extracellular traps kill bacteria. Science. 303(5663):1532-5.

Brinkmann V, Zychlinsky A (2012) Neutrophil extracellular traps: is immunity the second function of chromatin? J Cell Biol. 198(5):773-83.

Bruschi M, Moroni G, Sinico RA, Franceschini F, Fredi M, Vaglio A, Cavagna L, Petretto A, Pratesi F, Migliorini P, Manfredi A, Ramirez GA, Esposito P, Negrini S, Trezzi B, Emmi G, Santoro D, Scolari F, Volpi S, Mosca M, Tincani A, Candiano G, Prunotto M, Verrina E, Angeletti A, Ravelli A, Ghiggeri GM (2021) Neutrophil Extracellular Traps in the Autoimmunity Context. Front Med (Lausanne). 8:614829.

Cristinziano L, Modestino L, Antonelli A, Marone G, Simon HU, Varricchi G, Galdiero MR (2022) Neutrophil extracellular traps in cancer. Semin Cancer Biol. 79:91-104.

Buchholz U, Bernard H, Werber D, Böhmer MM, Remschmidt C, Wilking H, Deleré Y, an der Heiden M, Adlhoch C, Dreesman J, Ehlers J, Ethelberg S, Faber M, Frank C, Fricke G, Greiner M, Höhle M, Ivarsson S, Jark U, Kirchner M, Koch J, Krause G, Luber P, Rosner B, Stark K, Kühne M (2011) German outbreak of Escherichia coli O104:H4 associated with sprouts. N Engl J Med. 365(19):1763-70.

Clawson ML, Keen JE, Smith TP, Durso LM, McDaneld TG, Mandrell RE, Davis MA, Bono JL (2009) Phylogenetic classification of Escherichia coli O157:H7 strains of human and bovine origin using a novel set of nucleotide polymorphisms. Genome Biol. 10(5):R56.

De Luca G, Lev PR, Camacho MF, Goette NP, Sackmann F, Castro Ríos MA, Moiraghi B, Cortes Guerrieri V, Bendek G, Carricondo E, Enrico A, Vallejo V, Varela A, Khoury M, Gutierrez M, Larripa IB, Marta RF, Glembotsky AC, Heller PG (2023) High cell-free DNA is associated with disease progression, inflammasome activation and elevated levels of inflammasome-related cytokine IL-18 in patients with myelofibrosis. Front Immunol. 14:1161832.

Dittmar M, Bischofs C, Matheis N, Poppe R, Kahaly GJ (2009) A novel mutation in the DNASE1 gene is related with protein instability and decreased enzyme activity in thyroid autoimmunity. J Autoimmun. 32(1):7-13.

Dücker C, Dautel P, Wagner K, Przewozna J, Oehlerking S, Repenthin J, Brüning R, Meyer TN, Faiss S (2011) Klinische Symptomatik, Therapie und Verlauf Stationär Behandelter EHEC/EHEC-HUS Patienten [Clinical symptoms, treatment and outcome of EHEC and EHEC-HUS patients treated as in-patients]. Dtsch Med Wochenschr. 136(36):1770-6.

Duvvuri B, Lood C (2019) Cell-Free DNA as a Biomarker in Autoimmune Rheumatic Diseases. Front Immunol. 10:502.

Egan JA, Hay SN, Brecher ME (2004) Frequency and significance of schistocytes in TTP/HUS patients at the discontinuation of plasma exchange therapy. J Clin Apher. 19(4):165-7.

Elrod J, Heuer A, Knopf J, Schoen J, Schönfeld L, Trochimiuk M, Stiel C, Appl B, Raluy LP, Saygi C, Zlatar L, Hosari S, Royzman D, Winkler TH, Lochnit G, Leppkes M, Grützmann R, Schett G, Tomuschat C, Reinshagen K, Herrmann M, Fuchs TA, Boettcher M (2023) Neutrophil extracellular traps and DNases orchestrate formation of peritoneal adhesions. iScience. 26(12):108289.

Engavale M, McCord J, Mapp B, Nzimulinda N, Bengtson E, Sutton RB, Keyel PA (2021) Dnase1 Family in Autoimmunity. Encyclopedia. 1(3):527-541.

Englert H, Rangaswamy C, Deppermann C, Sperhake JP, Krisp C, Schreier D, Gordon E, Konrath S, Haddad M, Pula G, Mailer RK, Schlüter H, Kluge S, Langer F, Püschel K, Panousis K, Stavrou EX, Maas C, Renné T, Frye M (2021) Defective NET clearance contributes to sustained FXII activation in COVID-19-associated pulmonary thrombo-inflammation. EBioMedicine. 67:103382.

Englert H, Göbel J, Khong D, Omidi M, Wolska N, Konrath S, Frye M, Mailer RK, Beerens M, Gerwers JC, Preston RJS, Odeberg J, Butler LM, Maas C, Stavrou EX,

Fuchs TA, Renné T (2023) Targeting NETs using dual-active DNase1 variants. Front Immunol. 14:1181761.

Feitz WJC, Suntharalingham S, Khan M, Ortiz-Sandoval CG, Palaniyar N, van den Heuvel LP, van de Kar NCAJ, Licht C (2021) Shiga Toxin 2a Induces NETosis via NOX-Dependent Pathway. Biomedicines. 9(12):1807.

Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A (2007) Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 176(2):231-41.

Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD Jr, Wrobleski SK, Wakefield TW, Hartwig JH, Wagner DD (2010) Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A. 107(36):15880-5.

Fuchs TA, Bhandari AA, Wagner DD (2011) Histones induce rapid and profound thrombocytopenia in mice. Blood. 118(13):3708-14.

Fuchs TA, Brill A, Wagner DD (2012) Neutrophil extracellular trap (NET) impact on deep vein thrombosis. Arterioscler Thromb Vasc Biol. 32(8):1777-83.

Fuchs TA, Kremer Hovinga JA, Schatzberg D, Wagner DD, Lämmle B (2012) Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies. Blood. 120(6):1157-64.

Fogarty H, Townsend L, Morrin H, Ahmad A, Comerford C, Karampini E, Englert H, Byrne M, Bergin C, O'Sullivan JM, Martin-Loeches I, Nadarajan P, Bannan C, Mallon PW, Curley GF, Preston RJS, Rehill AM, McGonagle D, Ni Cheallaigh C, Baker RI, Renné T, Ward SE, O'Donnell JS; Irish COVID-19 Vasculopathy Study (iCVS) investigators (2021) Persistent endotheliopathy in the pathogenesis of long COVID syndrome. J Thromb Haemost. 19(10):2546-2553.

Gasser C, Gautier E, Steck A, Siebenmann RE, Oechslin R (1955) Hämolytisch-urämische Syndrome: bilaterale Nierenrindennekrosen bei akuten erworbenen hämolytischen Anämien [Hemolytic-uremic syndrome: bilateral necrosis of the renal

cortex in acute acquired hemolytic anemia]. Schweiz Med Wochenschr. 85(38-39):905-9.

George JN, Nester CM (2014) Syndromes of thrombotic microangiopathy. N Engl J Med. 371(19):1847-8.

González Garcia EA (2002) Animal health and foodborne pathogens: enterohaemorrhagic O157:H7 strains and other pathogenic Escherichia coli virotypes (EPEC, ETEC, EIEC, EHEC). Pol J Vet Sci. 5(2):103-15.

Grabuschnig S, Bronkhorst AJ, Holdenrieder S, Rosales Rodriguez I, Schliep KP, Schwendenwein D, Ungerer V, Sensen CW (2020) Putative Origins of Cell-Free DNA in Humans: A Review of Active and Passive Nucleic Acid Release Mechanisms. Int J Mol Sci. 21(21):8062.

Hakkim A, Fürnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, Herrmann M, Voll RE, Zychlinsky A (2010) Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A. 107(21):9813-8.

Harendza S (2011) "HUS diary" of a German nephrologist during the current EHEC outbreak in Europe. Kidney Int. 80(7):687-9. doi: 10.1038/ki.2011.238. PMID: 21734639.

Hurley BP, Thorpe CM, Acheson DW (2001) Shiga toxin translocation across intestinal epithelial cells is enhanced by neutrophil transmigration. Infect Immun. 69(10):6148-55. doi: 10.1128/IAI.69.10.6148-6155.2001. PMID: 11553554; PMCID: PMC98745.

Inoue M, Nakashima R, Enomoto M, Koike Y, Zhao X, Yip K, Huang SH, Waldron JN, Ikura M, Liu FF, Bratman SV (2018) Plasma redox imbalance caused by albumin oxidation promotes lung-predominant NETosis and pulmonary cancer metastasis. Nat Commun. 9(1):5116.

Jansen MP, Emal D, Teske GJ, Dessing MC, Florquin S, Roelofs JJ (2017) Release of extracellular DNA influences renal ischemia reperfusion injury by platelet activation and formation of neutrophil extracellular traps. Kidney Int. 91(2):352-364. doi: 10.1016/j.kint.2016.08.006. Epub 2016 Sep 28. PMID: 27692564.

Jiménez-Alcázar M, Napirei M, Panda R, Köhler EC, Kremer Hovinga JA, Mannherz HG, Peine S, Renné T, Lämmle B, Fuchs TA (2015) Impaired DNase1-mediated degradation of neutrophil extracellular traps is associated with acute thrombotic microangiopathies. J Thromb Haemost. 13(5):732-42.

Jiménez-Alcázar M, Rangaswamy C, Panda R, Bitterling J, Simsek YJ, Long AT, Bilyy R, Krenn V, Renné C, Renné T, Kluge S, Panzer U, Mizuta R, Mannherz HG, Kitamura D, Herrmann M, Napirei M, Fuchs TA (2017) Host DNases prevent vascular occlusion by neutrophil extracellular traps. Science. 358(6367):1202-1206.

Jing Q, Leung CHC, Wu AR (2022) Cell-Free DNA as Biomarker for Sepsis by Integration of Microbial and Host Information. Clin Chem. 2022 Sep 1;68(9):1184-1195.

Kananen L, Hurme M, Bürkle A, Moreno-Villanueva M, Bernhardt J, Debacq-Chainiaux F, Grubeck-Loebenstein B, Malavolta M, Basso A, Piacenza F, Collino S, Gonos ES, Sikora E, Gradinaru D, Jansen EHJM, Dollé MET, Salmon M, Stuetz W, Weber D, Grune T, Breusing N, Simm A, Capri M, Franceschi C, Slagboom E, Talbot D, Libert C, Raitanen J, Koskinen S, Härkänen T, Stenholm S, Ala-Korpela M, Lehtimäki T, Raitakari OT, Ukkola O, Kähönen M, Jylhä M, Jylhävä J (2023) Circulating cell-free DNA in health and disease - the relationship to health behaviours, ageing phenotypes and metabolomics. Geroscience. 45(1):85-103.

Keyel PA (2017) Dnases in health and disease. Dev Biol. 429(1):1-11.

Kielstein JT, Beutel G, Fleig S, Steinhoff J, Meyer TN, Hafer C, Kuhlmann U, Bramstedt J, Panzer U, Vischedyk M, Busch V, Ries W, Mitzner S, Mees S, Stracke S, Nürnberger J, Gerke P, Wiesner M, Sucke B, Abu-Tair M, Kribben A, Klause N, Schindler R, Merkel F, Schnatter S, Dorresteijn EM, Samuelsson O, Brunkhorst R;

Collaborators of the DGfN STEC-HUS registry (2012) Best supportive care and therapeutic plasma exchange with or without eculizumab in Shiga-toxin-producing E. coli O104:H4 induced haemolytic-uraemic syndrome: an analysis of the German STEC-HUS registry. Nephrol Dial Transplant. 27(10):3807-15.

Kondoh M, Aoki Y, Ogawa Z, Hata Y, Suzuki H, Itoh H (1989) Effect of chlorpromazine on transmigration of mitochondrial aspartate aminotransferase in rat liver. Kitasato Arch Exp Med. 62(4):181-6.

Kubota T (2023) An Emerging Role for Anti-DNA Antibodies in Systemic Lupus Erythematosus. Int J Mol Sci. 24(22):16499.

Landoni VI, Pittaluga JR, Carestia A, Castillo LA, Nebel MC, Martire-Greco D, Birnberg-Weiss F, Schattner M, Schierloh P, Fernández GC (2022) Neutrophil Extracellular Traps Induced by Shiga Toxin and Lipopolysaccharide-Treated Platelets Exacerbate Endothelial Cell Damage. Front Cell Infect Microbiol. 12:897019.

Leffler J, Martin M, Gullstrand B, Tydén H, Lood C, Truedsson L, Bengtsson AA, Blom AM (2023) Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. J Immunol. 188(7):3522-31.

Leffler J, Prohászka Z, Mikes B, Sinkovits G, Ciacma K, Farkas P, Réti M, Kelen K, Reusz GS, Szabó AJ, Martin M, Blom AM (2017) Decreased Neutrophil Extracellular Trap Degradation in Shiga Toxin-Associated Haemolytic Uraemic Syndrome. J Innate Immun. 9(1):12-21.

Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y (2010) PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J Exp Med. 207(9):1853-62.

Malíčková K, Duricová D, Bortlík M, Hrušková Z, Svobodová B, Machková N, Komárek V, Fučíková T, Janatková I, Zima T, Lukáš M (2011) Impaired

deoxyribonuclease I activity in patients with inflammatory bowel diseases. Autoimmune Dis. 2011:945861.

Martinod K, Wagner DD (2014) Thrombosis: tangled up in NETs. Blood. 123(18):2768-76.

Masuda S, Nakazawa D, Shida H, Miyoshi A, Kusunoki Y, Tomaru U, Ishizu A (2016) NETosis markers: Quest for specific, objective, and quantitative markers. Clin Chim Acta. 459:89-93.

Massberg S, Grahl L, von Bruehl ML, Manukyan D, Pfeiler S, Goosmann C, Brinkmann V, Lorenz M, Bidzhekov K, Khandagale AB, Konrad I, Kennerknecht E, Reges K, Holdenrieder S, Braun S, Reinhardt C, Spannagl M, Preissner KT, Engelmann B (2010) Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. Nat Med. 16(8):887-96.

Mattox AK, Douville C, Wang Y, Popoli M, Ptak J, Silliman N, Dobbyn L, Schaefer J, Lu S, Pearlman AH, Cohen JD, Tie J, Gibbs P, Lahouel K, Bettegowda C, Hruban RH, Tomasetti C, Jiang P, Chan KCA, Lo YMD, Papadopoulos N, Kinzler KW, Vogelstein B (2023) The Origin of Highly Elevated Cell-Free DNA in Healthy Individuals and Patients with Pancreatic, Colorectal, Lung, or Ovarian Cancer. Cancer Discov. 13(10):2166-2179.

Meyers KE, Kaplan BS (2000) Many cell types are Shiga toxin targets. Kidney Int. 57(6):2650-1.

Moake JL (2002) Thrombotic microangiopathies. N Engl J Med. 347(8):589-600.

Nadano D, Yasuda T, Kishi K (1993) Measurement of deoxyribonuclease I activity in human tissues and body fluids by a single radial enzyme-diffusion method. Clin Chem. 39(3):448-52.

Napirei M, Karsunky H, Zevnik B, Stephan H, Mannherz HG, Möröy T (2000) Features of systemic lupus erythematosus in Dnase1-deficient mice. Nat Genet. 25(2):177-81.

Napirei M, Wulf S, Eulitz D, Mannherz HG, Kloeckl T (2005) Comparative characterization of rat deoxyribonuclease 1 (Dnase1) and murine deoxyribonuclease 1-like 3 (Dnase1l3). Biochem J. 389(Pt 2):355-64.

Napirei M, Wulf S, Mannherz HG (2004) Chromatin breakdown during necrosis by serum Dnase1 and the plasminogen system. Arthritis Rheum. 50(6):1873-83.

Nässberger L, Jonsson H, Sjöholm AG, Sturfelt G, Heubner A (1989) Circulating anti-elastase in systemic lupus erythematosus. Lancet. 1(8636):509.

Nguyen DN, Stensballe A, Lai JC, Jiang P, Brunse A, Li Y, Sun J, Mallard C, Skeath T, Embleton ND, Berrington JE, Sangild PT (2017) Elevated levels of circulating cell-free DNA and neutrophil proteins are associated with neonatal sepsis and necrotizing enterocolitis in immature mice, pigs and infants. Innate Immun. 23(6):524-536.

PAGE AR, GOOD RA (1958) A clinical and experimental study of the function of neutrophils in the inflammatory response. Am J Pathol. 34(4):645-69.

Pan J, Zhang L, Wang X, Li L, Yang C, Wang Z, Su K, Hu X, Zhang Y, Ren G, Jiang J, Li P, Huang J (2023) Chronic stress induces pulmonary epithelial cells to produce acetylcholine that remodels lung pre-metastatic niche of breast cancer by enhancing NETosis. J Exp Clin Cancer Res. 42(1):255.

Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A (2010) Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol. 191(3):677-91.

Pulmozyme® Roche, package leaflet, April 2017, [online source] URL: https://german-public-reg-elibrary.roche.com/api/documents/PYM/SPC/2500E-nebsol/PDF [status: March 19, 2024, 8 pm]

Ranasinha C, Assoufi B, Shak S, Christiansen D, Fuchs H, Empey D, Geddes D, Hodson M (1993) Efficacy and safety of short-term administration of aerosolised recombinant human DNase I in adults with stable stage cystic fibrosis. Lancet. 342(8865):199-202.

Reshetnyak T, Nurbaeva K, Ptashnik I, Kudriaeva A, Belogurov A Jr, Lila A, Nasonov E (2023) Markers of NETosis in Patients with Systemic Lupus Erythematosus and Antiphospholipid Syndrome. Int J Mol Sci. 24(11):9210.

Riedl M, Orth-Höller D, Würzner R (2014) An update on the thrombotic microangiopathies hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Semin Thromb Hemost. 40(4):413-5.

Rohde H, Qin J, Cui Y, Li D, Loman NJ, Hentschke M, Chen W, Pu F, Peng Y, Li J, Xi F, Li S, Li Y, Zhang Z, Yang X, Zhao M, Wang P, Guan Y, Cen Z, Zhao X, Christner M, Kobbe R, Loos S, Oh J, Yang L, Danchin A, Gao GF, Song Y, Li Y, Yang H, Wang J, Xu J, Pallen MJ, Wang J, Aepfelbacher M, Yang R (2011) E. coli O104:H4 Genome Analysis Crowd-Sourcing Consortium. Open-source genomic analysis of Shiga-toxin-producing E. coli O104:H4. N Engl J Med. 365(8):718-24.

Rosove MH (2014) Thrombotic microangiopathies. Semin Arthritis Rheum. 43(6):797-805.

Ruggenenti P, Noris M, Remuzzi G (2001) Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. Kidney Int. 60(3):831-46.

Sajjan SU, Forstner JF (1990) Characteristics of binding of Escherichia coli serotype O157:H7 strain CL-49 to purified intestinal mucin. Infect Immun. 58(4):860-7.

Salvadori M, Bertoni E (2013) Update on hemolytic uremic syndrome: Diagnostic and therapeutic recommendations. World J Nephrol. 2(3):56-76.
Savchenko AS, Martinod K, Seidman MA, Wong SL, Borissoff JI, Piazza G, Libby P, Goldhaber SZ, Mitchell RN, Wagner DD (2014) Neutrophil extracellular traps form predominantly during the organizing stage of human venous thromboembolism development. J Thromb Haemost. 12(6):860-70.

Schnabel A, Csernok E, Isenberg DA, Mrowka C, Gross WL (1995) Antineutrophil cytoplasmic antibodies in systemic lupus erythematosus. Prevalence, specificities, and clinical significance. Arthritis Rheum. 38(5):633-7.

Semeraro F, Ammollo CT, Morrissey JH, Dale GL, Friese P, Esmon NL, Esmon CT (2011) Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. Blood. 118(7):1952-61.

Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL (1990) Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. Proc Natl Acad Sci U S A. 87(23):9188-92.

Shiokawa D, Tanuma S (2001) Characterization of human DNase I family endonucleases and activation of DNase gamma during apoptosis. Biochemistry. 2001 Jan 9;40(1):143-52.

Siegler R, Oakes R (2005) Hemolytic uremic syndrome; pathogenesis, treatment, and outcome. Curr Opin Pediatr. 17(2):200-4.

Tarr PI, Gordon CA, Chandler WL (2005) Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome. Lancet. 365(9464):1073-86.

Trachtman H, Austin C, Lewinski M, Stahl RA (2012) Renal and neurological involvement in typical Shiga toxin-associated HUS. Nat Rev Nephrol. 8(11):658-69.

Trivedi A, Khan MA, Bade G, Talwar A (2021) Orchestration of Neutrophil Extracellular Traps (Nets), a Unique Innate Immune Function during Chronic Obstructive Pulmonary Disease (COPD) Development. Biomedicines. 9(1):53.

von Brühl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Köllnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, Eckart A, Haidari S, Rudelius M, Schulz C, Echtler K, Brinkmann V, Schwaiger M, Preissner KT, Wagner DD, Mackman N, Engelmann B, Massberg S (2012) Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med. 209(4):819-35.

Veras FP, Pontelli MC, Silva CM, Toller-Kawahisa JE, de Lima M, Nascimento DC, Schneider AH, Caetité D, Tavares LA, Paiva IM, Rosales R, Colón D, Martins R, Castro IA, Almeida GM, Lopes MIF, Benatti MN, Bonjorno LP, Giannini MC, Luppino-Assad R, Almeida SL, Vilar F, Santana R, Bollela VR, Auxiliadora-Martins M, Borges M, Miranda CH, Pazin-Filho A, da Silva LLP, Cunha LD, Zamboni DS, Dal-Pizzol F, Leiria LO, Siyuan L, Batah S, Fabro A, Mauad T, Dolhnikoff M, Duarte-Neto A, Saldiva P, Cunha TM, Alves-Filho JC, Arruda E, Louzada-Junior P, Oliveira RD, Cunha FQ (2020) SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. J Exp Med. 217(12):e20201129.

Wedemeyer H, Hofmann WP, Lueth S, Malinski P, Thimme R, Tacke F, Wiegand J (2010) ALT als Screeningparameter für Lebererkrankungen: eine kritische Evaluation der Evidenz [ALT screening for chronic liver diseases: scrutinizing the evidence]. Z Gastroenterol. 48(1):46-55.

Wieler LH, Semmler T, Eichhorn I, Antao EM, Kinnemann B, Geue L, Karch H, Guenther S, Bethe A (2011) No evidence of the Shiga toxin-producing E. coli O104:H4 outbreak strain or enteroaggregative E. coli (EAEC) found in cattle faeces in northern Germany, the hotspot of the 2011 HUS outbreak area. Gut Pathog. 3(1):17.

Witt DM, Anderson L (1996) Dornase alfa: a new option in the management of cystic fibrosis. Pharmacotherapy. 16(1):40-8. PMID: 8700791.

Wright HL, Lyon M, Chapman EA, Moots RJ, Edwards SW (2021) Rheumatoid Arthritis Synovial Fluid Neutrophils Drive Inflammation Through Production of Chemokines, Reactive Oxygen Species, and Neutrophil Extracellular Traps. Front Immunol. 11:584116.

Yasutomo K, Horiuchi T, Kagami S, Tsukamoto H, Hashimura C, Urushihara M, Kuroda Y (2001) Mutation of DNASE1 in people with systemic lupus erythematosus. Nat Genet. 28(4):313-4.

Yeh TM, Chang HC, Liang CC, Wu JJ, Liu MF (2003) Deoxyribonuclease-inhibitory antibodies in systemic lupus erythematosus. J Biomed Sci. 10(5):544-51.

Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, Pittman K, Asaduzzaman M, Wu K, Meijndert HC, Malawista SE, de Boisfleury Chevance A, Zhang K, Conly J, Kubes P (2012) Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med. 18(9):1386-93.

Zhang S, Shu X, Tian X, Chen F, Lu X, Wang G (2014) Enhanced formation and impaired degradation of neutrophil extracellular traps in dermatomyositis and polymyositis: a potential contributor to interstitial lung disease complications. Clin Exp Immunol, 177(1):134-41.

Zuo Y, Yalavarthi S, Shi H, Gockman K, Zuo M, Madison JA, Blair C, Weber A, Barnes BJ, Egeblad M, Woods RJ, Kanthi Y, Knight JS (2020) Neutrophil extracellular traps in COVID-19. JCI Insight. 5(11):e138999.

9 ACKNOWLEDGEMENTS

A number of people have given valuable information and motivation during the course of my work.

I am grateful to my family, especially to my mother and to my father, for always believing in me.

I am particularly grateful to my soulmate and to my son for their love, their understanding for my work effort and their support.

I thank my fellow lab mate and good friend Dr. Luke Syndey Hopf for our professional discussions and our mutual support.

I thank Prof. Dr. med. Hans-Ulrich Kauczor from Heidelberg University Hospital for encouraging me in performing research and for being a valuable mentor for my medical career since my childhood.

I thank Prof. Dr. med. Caner Süsal from Heidelberg and Koç University Istanbul for introducing me into the world of medical laboratory science and research and for stating that I am highly gifted.

I am thankful to Dr. med. Dipl. Psych. Michael Schwab for our valuable medical exchange and the willingness to look outside the box.

I thank Dr. Jochen Veigel, who aroused my interest in clinical chemistry and laboratory medicine during his classes.

Many thanks to Dr. rer. nat. Tobias Fuchs for teaching me scientific thinking and for supervising my research.

My special thank goes to Dr. med. Dr. rer. nat. Ulrich C. Klostermeier for his valuable comments, his spirit and his tremendous support.

I would like to express my sincere gratitude to Prof. Dr. Dr. Thomas Renné.
I thank him very much for his valuable guidance in science and research and for teaching his outstanding knowledge and expertise. His trust is empowering and support in a high qualification.

10 CURRICULUM VITAE

entfällt aus datenschutzrechtlichen Gründen

11 EIDESSTATTLICHE ERKLÄRUNG

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Hamburg, den 19. April 2024

Unterschrift: .....  .....