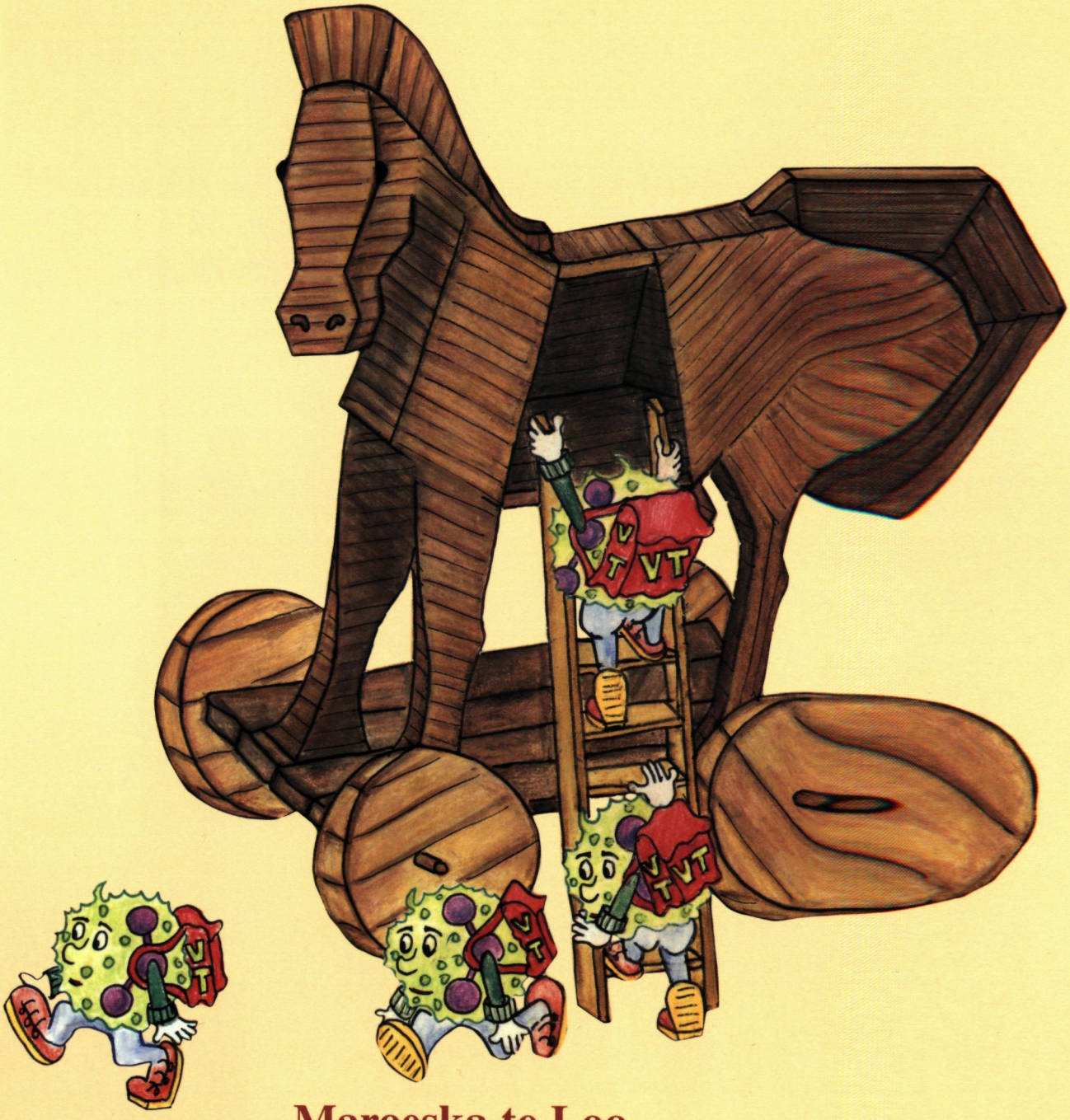
# THE PATHOGENESIS OF THE HEMOLYTIC UREMIC SYNDROME:

## The role of the granulocyte




Maroeska te Loo

# THE PATHOGENESIS OF THE HEMOLYTIC UREMIC SYNDROME :
## The role of the granulocyte

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**van de Medische Wetenschappen**

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Dedicated to my mom and dad

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**ABBREVIATIONS**

| | |
|---|---|
| CAPD | continuous ambulatory peritoneal dialysis |
| *E. coli* | *Escherichia coli* |
| ELISA | enzyme linked immunosorbent assay |
| ET | endothelin |
| FITC | fluorescein isothiocyanate |
| Gb3 | globotriaosylceramide |
| Gb4 | globotetraosylceramide |
| GMVEC | glomerular microvascular endothelial cells |
| HUS | hemolytic uremic syndrome |
| LPS | lipopolysaccharide |
| NO | nitric oxide |
| eNOS | endothelial nitric oxide synthase |
| iNOS | inducible nitric oxide synthase |
| PAI-I | plasminogen activator inhibitor I |
| PCR | polymerase chain reaction |
| PMN | polymorphonuclear leukocytes |
| SC-35 | splicing factor 35 |
| SD | standard deviation |
| SEM | standard error of the mean |
| TLC | thin layer chromatography |
| TNFα | tumor necrosis factor alpha |
| TTP | thrombotic thrombocytopenic purpera |
| TUNEL | terminal deoxynucleotidyl nick-end labeling |
| VEGF | vascular endothelial growth factor |
| VT | verocytotoxin |
| VTEC | verocytotoxin producing *Eschericha coli* |

# INTRODUCTION AND OUTLINE OF THE THESIS

## Clinical features

The hemolytic uremic syndrome (HUS) was for the first time described in 1955 by Gasser et al (1) and is defined by a triad of clinical features that include hemolytic anemia, thrombocytopenia, and acute renal failure HUS is mostly seen in children, especially below the age of five years although the syndrome is increasingly recognized in adults (2-4) Children that present with HUS are pale and restless Petechiae and bruises can be found due the presence of thrombocytopenia Fragmented erythrocytes or so-called 'Burr cells' can be found in the peripheral blood smear of HUS patients Oliguria or even anuria is often present at the time of admission to the hospital (5) Hyperglycemia and the development of insulin-dependent diabetes mellitus as a consequence of pancreas dysfunction belong to the rare, but severe complication of HUS (6-8) HUS is the leading cause of acute renal failure in childhood, at least in developing countries (9) HUS can be broadly divided in two subtypes, the typical or epidemical form that in most cases is preceded by a prodromal phase of acute, often bloody diarrhea and has therefore been termed D+ HUS It is believed that D+ HUS is caused by an infection with a verocytotoxin producing *Escherichia coli* (10) Severity of D+ HUS is associated with young age, anuria during the prodromal phase and a white blood cell count greater than 20x $10^9$ cells/L (11,12) The second form of HUS we know is the atypical or sporadic form and resembles in most cases a disease more common in adults namely thrombotic thrombocytopenic purpera (TTP) The atypical form of HUS is not preceded by a prodromal phase and has been termed D- HUS Several causes for the atypical form of HUS have been described like factor H deficiency and recently the involvement of a vWF-cleaving protease deficiency in some cases with atypical HUS has been suggested This thesis focuses on the typical form of HUS

## Epidemiology

Several studies in the past had noted that the majority of HUS cases were preceded by diarrhea and an infectious cause was suspected In 1983, Karmali and colleagues demonstrated for the first time a linkage between the presence of infection with VTEC serotype O157 H7 and the occurrence of sporadic D+ HUS (10) In the beginning of the same year, a study was published describing a relationship between the presence of VTEC O157 H7 infection and hemorrhagic

colitis (13). The designation "O" represents a specific lipopolysaccharide or O-antigen and the designation "H" represents a specific flagellar antigen. In the years that followed it became clear that in 90% of the cases of D+ HUS evidence of VTEC infection could be found. It has been suggested that only 6-8% of the population infected with VTEC will develop HUS (14). However, it is difficult to estimate the true incidence of VTEC infection and in this way the occurrence of D+ HUS, since many persons with only mild clinical symptoms will not need or search medical care. The incidence rate of D+ HUS varies among different countries. For example, the incidence in Argentina is about 20 cases per 100 000 children a year whereas in the Netherlands 2-3 cases pr 100 000 children below the age of five years are seen (15-17). Serotype O157: H7 can be found in Western Europe in 70% of the cases. In addition, some other non-O157 serotypes can also be important in causing disease, in particular serotypes O26, O103, and O111 (18-21). The majority of outbreaks with VTEC are linked with the consumption of improperly cooked contaminated ground beef (22,23) although infections as a consequence of contaminated water or other food products, like apple cider, potatoes and yogurt have been described (24). Another route of transmission of the infection is from person-to-person (25,26). Although D+ HUS is mostly seen in early childhood, it can occur in all age groups. (27). Outbreaks have occurred in nursing homes and day care centers (28,29). Besides, it seems that there is a seasonal variation considering the occurrence of D+ HUS and VTEC infection, with most cases seen during the summer and autumn. The cause for this is not clear, but it has been suggested that the ecology (relationship with environment and life circumstances) of the animals that are a source for VTEC infection might play a role. Another explanation might be that during the summer months more products are incompletely cooked. In this way the bacteria can survive and be ingested.

**Passage of the intestinal barrier**

As mentioned before, infection with VTEC mostly occurs through consumption of contaminated food. After ingestion, the bacteria can survive the acidity of the stomach and can colonize in the distal small bowel and the large bowel. The adhesion of VTEC to intestinal epithelial cells demonstrates a pattern of attaching and effacing (A/E) lesions that have been previously described in enteropathogenic *E. coli* strains (30-32). For the interaction and adhesion to the enterocytes a variety of genes is necessary, some of them encoded on a pathogenecity island known as the locus of enterocyte effacement (LEE) region (33,34). One of the genes found in this locus is the *eae* gene, encoding intimin, an outer membrane protein that plays an important role in the adherence process (35). Another gene that can be found in the LEE region is the *tir* gene, encoding a receptor for intimin. The exact function of *tir* is not

known but it has been suggested that *tir* functions as a receptor for intimin on the surface of cells (33). The LEE region is not present in normal *E. coli* flora, but can be found in strains that are capable of producing A/E lesions like VTEC. The A/E lesions are characterized by intimate adherence between the bacterium and the epithelial cell membrane associated with effacement of the epithelial microvilli (34). Cytoskeletal changes are seen just below the adherent bacterium, including the accumulation of polymerized actin resulting in the formation of adhesion pedestals (Figure 1). It is still not clear, how VT moves across the intestinal mucosa after adherence to the intestinal epithelial wall. The fact that VTEC is not invasive and human intestinal epithelial cells do not possess the classical receptor for VT, the Gb3, makes it even more difficult (36,37). However, several possible routes by which the toxin may gain access to the circulation have been suggested (38,39). *In vitro* studies have shown that SLT-1 is able to translocate via an energy requiring process from the apical to the basolateral surface of intestinal epithelial cell lines without loss of monolayer integrity. Toxin that moved across the barrier retained its biological activity.

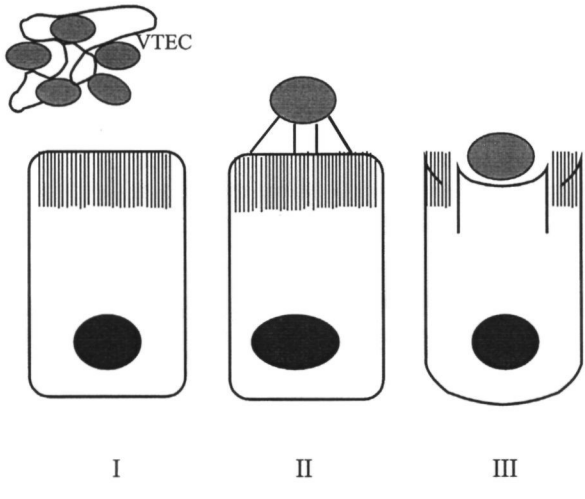


Figure 1: Schematic representation of the adherence of the *E. coli* bacteria and formation of pedestals. The bacteria can connect with the host cell (I+II) and forms attaching and effacing lesions (III). There are cytoskeletal changes just below the bacterium resulting in the formation of pedestrals (III).

**Nomenclature**

In 1977, Konowalchuk and colleagues reported that certain diarrheagenic *E coli* strains make a cytotoxin that was able to kill cultured Vero-cells, a epithelial cell line isolated from African green monkey kidneys, hence these cytotoxins were called verocytotoxins (40) The striking similarity in structure and biological activity of verocytotoxins (VT) with Shiga toxin, a cytotoxin produced by *Shigella dysenteria* type I and the fact that VT could be neutralized by anti-Shiga toxin was the reason that VT have also been called Shiga-like toxin or Shiga toxin (41,42) In this thesis, the original nomenclature, verocytotoxin or VT will be used

**Characteristics of VT**

The structural genes that encode for VT are incorporated in bacteriophage DNA of VTEC in contrast to Shiga toxin that is encoded by the chromosomes of the Shigella bacteria It's important to realize that the pathogenic property can be transferred between E *coli* strains, with potentially serious clinical and epidemiological consequences (43,44) Several forms of toxins are currently purified and cloned, VT-1, VT-2, VT-2c, VT-2d, VT-2e and the just recently discovered VT-2f (45-47) Three of them, VT-1, VT-2, and VT2c, are mostly associated with pathogenic diseases in humans although infections with VT2d and VT2e have been described (45,48) VT-2e is associated with edema disease in pigs, that is characterized by vascular necrosis, neurological signs and edema in the central nervous system and gastrointestinal tract (49) VT-1 differs only one amino acid at position 45 with Shiga toxin that is produced by *Shigella Dysenteria type 1* On the contrary, the structural genes of VT-1 and VT-2 share only 58% overall nucleotide and 56% amino acid sequence homologies Human pathogenic VTEC can produce more than one type of toxin Infections with strains that produce VT-2 increase the likelihood of developing D+ HUS (50) Although the variants of VT might differ in amino acid sequence, they are structurally the same They consist of two parts, one part that is responsible for the binding of the toxin (B-subunits) and an enzymatic active part (A-subunit) The binding part of the toxin consists of five small B-subunits (7 kDa) that are noncovalently bound to the A-subunit (32 -kDa)(51,52)

**Cellular internalization and cytotoxicity**

In *vitro* and in *vivo* studies have shown that VT binds to specific glycosphingolipids in order to exert its cytotoxic effect The general structure of glycosphingolipids consists of a polar head with one or more sugars and a non-polar ceramide group VT can bind through his B-subunits to specific neutral sphingoglycolipids

The classical receptor known for VT, globotriaosylceramide (Gb3), has a terminal Galα1-4Gal residue, and can be found in human renal tissue, human lymphocytes, human erythrocytes (Pk-antigen) and human endothelial cells (53-55) In this regard, it is important to realize that cultured confluent endothelial cells expose this receptor only in very small amounts on their surface Stimulation of these cells with inflammatory cytokines such as tumor necrosis factor alpha induces the expression of the VT receptor Gb3 (55,56) Not only the sugar residues determine the binding affinity of VT but also the ceramide part plays an important role. Fatty acid chain length and degree of saturation have been shown to markedly affect the ability of the toxin to bind to glycosphingolipids (57,58) After binding of the holotoxin to the classical receptor Gb3, the toxin can be internalized by clatrin-endocytotic mechanism (59)

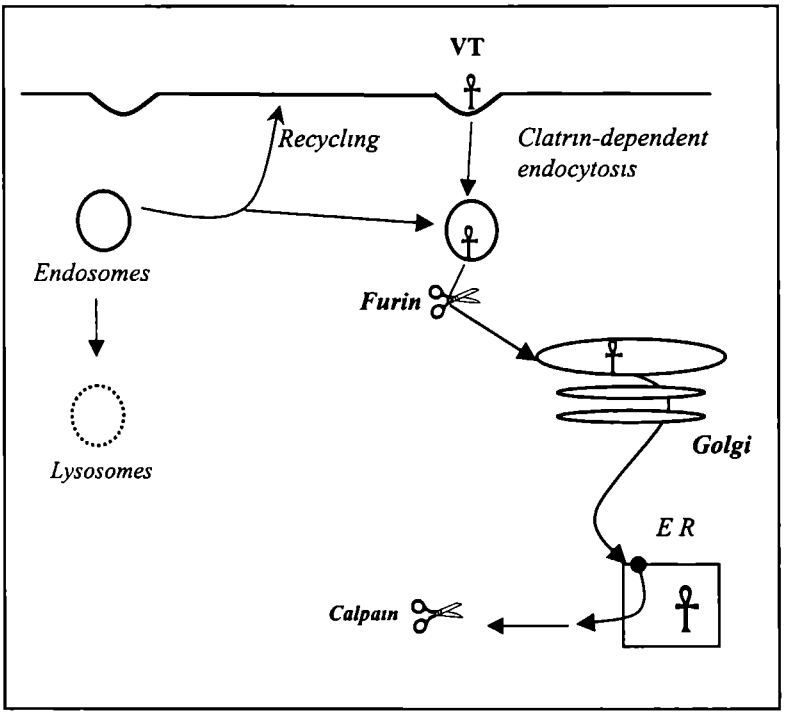


Figure 2 *Schematic representation of the internalization of verocytotoxin Somewhere during the routing of the toxin, the A-subunit of the toxin is cleaved into two fragments (A1+A2) by either furin or calpain leading to activation*

The toxin, once endocytosed is either delivered to the endosomal compartment and from there transported to the trans-*Golgi* network or recycled back to the cell surface, and delivered to the lysosomes for degradation or transcytosed to the opposite surface (Figure 2).

Retrograde transport of the toxin via the Golgi apparatus to the rough endoplasmic reticulum occurs and even proceeds to the nuclear envelopes (60,61). It is interesting to notice that the B-subunits of the toxin alone also can be transported retrogradely indicating that the A-subunit is not required for this process. The retrograde transport through the endoplasmic reticulum seems to be very important for the cytotoxic effect of the toxin because when the toxin is inhibited from entry of the Golgi apparatus, cells are protected against the toxin actions (59,62). Sandvig and colleagues were able to demonstrate that this transport was calcium, temperature and pH dependent (63-65). Somewhere during the routing of the toxin, the A-subunit of the toxin is cleaved into two fragments, one of 27- kDa (A1) and one of 4-kDa (A2). The B-subunits of the toxin remains associated with the smaller fragment. The A1 subunit forms an active intracellular enzyme N-glycosidase and can cleave the N-glycoside bound of adenine at position 4324 from the 5' end of 28S ribosomal RNA. This prevents ribosomal elongation factor dependent binding of aminoacyl-transfer RNA molecules to the 60 S ribosomal unit (59,62,66). If protein synthesis is completely inhibited, cell death occurs. Recent observations have shown that besides inhibition of protein synthesis, programmed cell death or apoptosis may play a role in the VT mediated cell cytotoxity (67-69).

It is believed that the presence of Gb3 is necessary to mediate cytotoxicity. In concert with this theory is a study performed with rabbits. These animals were challenged with $^{125}$I- VT-1. Highest uptake of $^{125}$I- VT-1 was correlated with those areas (spinal cord, brain, cecum, colon and small bowel) that had the highest amount of the classical Gb3 receptor and corresponded with the thrombotic microangiopathy observed (70). Interestingly, the rabbit lacks VT receptors in the kidney and can therefore not be used as an animal model for the renal failure as seen in HUS. Interesting in regard to this receptor related cytotoxicity, is the observation of Lingwood that Gb3 is more pronouncedly present in the glomeruli of the kidney of children under 2 years old but not in adults (53). However, van Setten et al. was not able to confirm these results and suggested that inflammatory mediators are necessary for the induction of VT susceptibility in glomerular endothelial cells. The finding of strong VT binding in renal tissue of an adult patient with sepsis in her study strengthens this hypothesis (71).

**Target cells**

*The systemic circulation*

After passage of the intestine VT is transported through the systemic circulation to the target organs It is thought, that once VT has entered the circulation, it rapidly binds to Gb3 receptors on endothelial cells in the target organs The way of transport of the toxin through the circulation was not known at the start of our studies Three candidates for binding VT in the blood had been suggested P blood group antigens on erythrocytes, platelets and lipoproteins On the basis of *in vitro* studies, binding of VT to erythrocytes as function of their P blood group has been suggested (54) The antigens of the P blood group system include the Pk-, P- and $P_1$ -antigen, representing Gb3, Gb4 and pentaosylceramide, respectively Binding of VT to $P_k$- and $P_1$ -antigen, both having a terminal gal 1-4gal residue, have been found. Having the $P_k$- and $P_1$ antigen might protect target organs for the effects of the toxin by catching the toxin out of the circulation (72,73) Erythrocytes have no nucleus and are therefore not susceptible for the protein synthesis inhibitory effect of VT It cannot be excluded that VT affect membrane stability of the erythrocyte or maybe sensitize them to mechanical or oxidative stress In theory, VT might bind to erythrocytes in the circulation and once bound, is transported through the systemic circulation However, VT bound to erythrocytes has never been detected in patients with HUS

Besides the binding of VT to erythrocytes, interaction of VT with platelets has been reported Cooling et al (74) showed that human platelets express two different glycolipids that can bind VT (= SLT), the first one is Gb3, and the second one galabiosylceramide or P1, a novel glycosphingolipid that immunological is different from the Gb3 However, Yagi et al showed that leukocyte-depleted normal platelets lack the Gb3 receptor and VT not directly induced platelet aggregation Furthermore, he demonstrated that under low shear stress VT was capable of inducing platelet aggregation presumable by the release of cytokines or chemical compounds from target cells (75) Plasma from six out of seven HUS patients had activity similar to Stx-1 (75) Further investigation is needed to determine which source produces the cytokines or other chemical compounds that might play a role in this One of the sources of these cytokines might be monocytes *In vitro* studies have shown that LPS-stimulated monocytes are capable of binding VT-1 to a receptor that is different from that found on endothelial cells After binding, VT-1 can induce the synthesis of cytokines like TNFα, IL-6 and IL-8 (76,77) The induction of cytokine release is accompanied by an increase in cellular mRNA concentrations Elevated levels of IL-8, IL-6 and TNFα in plasma and serum of HUS patients and elevated concentrations of TNFα, IL-6 and IL-8 in urine of HUS patients have been found suggesting that the release of inflammatory mediators indeed play a pivotal role in the pathogenesis of

HUS. Activation of neutrophils and neutrophil mediated endothelial cell injury plays probably an important function in the pathogenesis of HUS. A significant correlation has been found between neutrophil count and outcome of the disease. Furthermore, polymorphonuclear leukocytes, isolated from patients with HUS adhere more avidly to the endothelium than controls and are capable to damage the endothelium (78-81). That neutrophils have an important role in the pathogeneses in HUS is supported by the presence of increased numbers of neutrophils in the kidney of HUS patients (82). However, it has to be considered that the elevated levels of IL-8 found in HUS patients also may just reflect inflammatory response in the intestine. Elevated levels of IL-8 have also been reported in patients with Crohn's disease and colitis ulcerosa (83). Therefore, a critical evaluation of the role of PMN in HUS is required.

*The Kidney*

The main organ involved in HUS is the kidney, but in severe cases other organs like brain and pancreas can become involved (4). The pathogenesis of HUS is characterized by endothelial damage of glomeruli and kidney arterioles (2). Histopathological studies of kidney material of D+ HUS patients showed swollen and detached endothelial cells and deposits of fibrin all in the glomerulus. It is generally believed that VT, after entering the systemic circulation, is transported through the systemic circulation until VT attaches to endothelial cells that express VT binding receptors. After binding, VT can cause inhibition of protein synthesis finally leading to cell death. *In vitro* studies have shown that VT is only cytotoxic for endothelial cells when they have been previously stimulated with inflammatory mediators, such as tumor necrosis factor alpha (TNFα) and interleukin-1. It is thought that the increase of galactosyl transferase activity by TNFα in these cells leads to the induction of the Gb3-receptor (55). Although it is believed that endothelial damage is one of the primary events in the pathogenesis of HUS, damage to other cell types, like tubular epithelial cells and mesangial cells have been suggested (84-87). Several *in vitro* studies performed with tubular epithelial cells have shown that VT is cytotoxic for these cells and can induce apoptosis (88,89). The finding of apoptosis in the renal epithelium of a HUS patient and the results found *in vitro* are suggestive for a contribution of renal epithelium in the pathogenesis of HUS (89). Another cell type that might play an important role in the pathogenesis of HUS is the mesangium. Mesangial cells, under normal conditions, regulate glomerular hemodynamics (90). The mesangium has a central anatomical position within the glomerulus and only a single layer of fenestrated endothelium is separating the circulation from these mesangial cells. If this single layer is damaged as in HUS, substances like VT can easily reach the mesangium. *In vitro* studies have shown that mesangial cells possess the classical receptor for VT, the neutral glycolipid Gb3. After binding VT was

able to inhibit protein synthesis by 75% but did not cause cell death Furthermore, it was shown that proliferation of human mesangial cells was inhibited even more than protein synthesis was inhibited (86,87,91) The B-subunit of VT, which did not inhibit protein synthesis, was able to inhibit cell proliferation indicating that two different pathways are involved (87) The interaction of VT with the mesangium may interfere with normal mesangial function and in this way with renal function For this reason, it might be necessary to study the effects of VT on mesangial cells further

**Outline of the thesis**

In the last decade, a lot of progress in understanding the pathogenesis of D+ HUS has been made The pathogenesis of the syndrome is hallmarked by endothelial damage of predominantly glomeruli and kidney arterioles Several in vitro studies have shown that verocytotoxin (VT) is able to inhibit protein synthesis leading to cell death in several cell types that express the classical receptor for VT, globotriaosylceramide Furthermore, it was demonstrated that *in vitro* stimulation of endothelial cells with inflammatory mediators appears to be required for VT cytotoxicity It has been suggested that monocytes might be the source of these inflammatory mediators Although all these data give more insight in the pathogenesis of HUS, we still do not know exactly how the infection with VTEC leads to D+ HUS in especially children Important questions considering the pathogenesis of D+ HUS can be asked For example, how is VT passing the intestine wall and gains access to the systemic circulation? How is the VT transported through the systemic circulation? How develop patients renal failure and why is the kidney the main organ involved in this syndrome? And finally, why are mainly children affected and not adults?

The aim of this thesis was to provide some of the lacking knowledge about the pathogenesis of the hemolytic uremic syndrome First of all, the possible route of transport of VT through the systemic circulation of D+ HUS patients was evaluated in this study (**Chapter 2, 3, 4 and 5**) In addition, the role of nitric oxide and endothelin-1 were studied (**Chapter 6**) Nitric oxide may play a role in the localization of the damaging effects that are mainly seen in the kidney These damaging effects could lead to disturbances in the production of endothelin-1 or may induce apoptosis in the kidney For this reason, the presence of apoptosis in renal biopsy material of HUS patients was evaluated (**Chapter 8**) As mentioned before, not every infected child will develop HUS and differences in severity of the disease are observed It might be that genetic variability plays a role in this Therefore, polymorphisms of the TNFα and plasminogen activator inhibitor-I (PAI-I) gene were investigated Both play a role in the pathogenesis of HUS High levels of TNFα and PAI-I are associated with a more severe form

of HUS (**Chapter 7**). Finally, the role of vascular endothelial growth factor as possible regulator of the restoration of damaged glomerular capillaries has been studied (**Chapter 9**). In the last chapter, **Chapter 10,** the results found in this study are being discussed, summarized and put into perspective.

# BINDING AND TRANSFER OF VEROCYTOTOXIN BY POLYMORPHONUCLEAR LEUKOCYTES IN HEMOLYTIC UREMIC SYNDROME

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***Blood 2000; 95: 3396-3402***

## ABSTRACT

The hemolytic uremic syndrome (HUS) is the most common cause of acute renal failure in children The role of a verocytotoxin- (VT-) producing *E coli* has been strongly implicated in the epidemic form of HUS Although direct toxicity of VT on glomerular endothelial cells has been demonstrated, it remained still unclear how the VT is transported from the intestine to the target organs

In this study we demonstrate that VT, when incubated in whole blood, binds rapidly and completely to human polymorphonuclear leukocytes (PMN) and not to other components of blood Binding studies with $^{125}$I-VT-1 showed a single class of binding sites on freshly isolated, non-stimulated human PMN The Kd of VT-binding to PMN was $10^{8}$ mol/L, a 100-fold less than that of the VT-receptor globotriaosylceramide On incubation of VT-preloaded PMN with human glomerular microvascular endothelial cells (GMVEC), transfer of VT-1 to the endothelial cells occurred Incubation of non-stimulated GMVEC with VT-preloaded PMN, but not with PMN or VT-1 alone, caused inhibition of protein synthesis and cell death Our data are in concert with a role of PMN in the transfer of VT from the intestine to the kidney endothelium This occurs by selective binding to a specific receptor on PMN and subsequent passing of the ligand VT to the VT-receptor on GMVEC, which causes cell damage This new mechanism further underpins the important role of PMN in HUS

## INTRODUCTION

The hemolytic uremic syndrome (HUS) is the most frequent cause of acute renal failure in children The traditional diagnostic criteria for this syndrome include hemolytic anemia with fragmented erythrocytes, thrombocytopenia and renal failure (2) The endothelium of kidney arterioles and glomeruli plays a central role in the pathogenesis of HUS Histopathological studies of the kidney of HUS patients show characteristic lesions consisting of swelling and detachment of the endothelial cells of glomeruli and deposits of fibrin in glomeruli and arterioles (3, 92) In severe cases, the cell damage is not limited to the kidney, but other organs such as brain and pancreas, are also involved

The epidemic form of HUS, or (D +) HUS, occurs mostly following a prodromal phase of bloody diarrhea In 90% of the cases with (D+) HUS, an infection with a verocytotoxin (VT) producing *Escherichia coli* is strongly implicated (3,4,5,43,92) Strains of the VT producing *E coli* associated with HUS can produce VT-1 or VT-2, or both The structure of the VT is formed by a biologically active A subunit and five B subunits, by which the toxin binds to

specific glycolipid receptors. The VT producing *E. coli* is transmitted by contaminated food or water or from person-to-person. After ingestion, the *E. coli* binds to specific receptors to the intestinal wall and VT enters the circulation via a still unknown mechanism (3,94,95). VT is transported to the target-organs and can bind specifically to its receptor globotriaosylceramide, also called Gb3. This receptor has been demonstrated in human renal tissue and in human endothelial cells (53,102). After binding to Gb3, the active subunit of the VT enters the cell and causes inhibition of protein synthesis (60,61,85,88,89, 96). The route of transport of the VT from intestine to the kidney or other target organs is not solved yet. Although epidemiological studies have pointed to a role of VT in (D+) HUS, no VT has been encountered thus far in the plasma of HUS-patients. *In vitro* experiments showed that VT can bind to erythrocytes, depending on the P-blood group glycolipids, that are structurally related to the known VT receptor Gb3. It has been reported that VT can bind to the P1-phenotype (Pk, P1, P2 antigens) and, to a less extent also to the P2 phenotype (Pk and P antigens) but not to the P-phenotype (lacking antigens)(93,54,97,98). *In vitro* experiments also showed binding to activated human monocytes (76). Other possible candidates for transporting the VT are platelets and lipoproteins (57,74,58). In this study we evaluate which fractions of the blood contribute to VT binding and transfer. Our data suggest that polymorphonuclear leukocytes (PMN) are responsible for transporting the VT in blood and that the receptor responsible for binding VT to PMN is different from that found on endothelial cells. Furthermore, we demonstrate that VT is transferred from PMN to human glomerular endothelial cells.

## MATERIALS & METHODS

### *Materials.*

Purified VT-1 was kindly provided by Dr. M.A. Karmali (Toronto, ON, Canada). Ficoll was purchased from Pharmacia (Uppsula, Sweden). Plastic coated silica gel F1500 Thin layer chromatography (TLC) plates were obtained from Schleicher and Schuell (Dassel, Germany). A standard mixture of pure neutral glycolipids was obtained from Biocarb AB (Lund, Sweden). VT-1 labeled with FITC (VT-FITC) was kindly donated by Dr. Lingwood (Hospital for Sick Children, Toronto, Ontario, Canada). FACS $^{TM}$ lysing solution was purchased from Becton Dickinson Immunocytometry Systems (San Jose, USA). CD13-PE, CD14-PE and CD45-TRITC were purchased from DAKO (Glostrup, Denmark). All other reagents were of analytical grade or as described previously (18,56,76).

***Flow activated cell sorter (FACS) analysis and immunohistological study of whole blood***

VT-FITC was used to determine binding of VT in whole blood. Blood was obtained from 10 different healthy donors, 8 adults and 2 children (age ten and six) and blood group of the adults was determined according to established procedures. Two donors had bloodgroup O, four bloodgroup A and two had bloodgroup B. There were two donors with the P1-bloodgroup. Blood was immediately put on ice. 100 µl of blood was incubated for 20 minutes on ice with VT-FITC, followed by addition of FACS™ lysing solution to remove erythrocytes. The solution was centrifuged 200x g for 5 minutes at 4°C. The cells were washed twice with phosphate buffered saline (PBS) containing 1 % albumin. Cells were resuspended in 500 µl of a 0.5% paraformaldehyde solution for fixation. Flow cytometry was used to determine binding of VT-FITC. Fluorescence was measured in a histogram using a log scale. The different cell types were characterized using monoclonal antibodies; CD13-PE and CD16-PE for PMN, CD14-PE for monocytes and CD45-TRITC for lymphocytes. To exclude non-specific binding whole blood was previously incubated with unlabeled VT for 20 minutes followed by incubation with VT-FITC. The same procedure as above was used for direct immunofluorescence studies. Briefly, cells were incubated for 20 minutes with VT-FITC and a monoclonal antibody to indicate the cell type. Cells were washed twice with PBS and were then resuspended in 500 µl of 0.5% paraformaldehyde. Cells were centrifuged by 200x g. Subsequently, cells were analyzed by a Zeiss fluorescence microscope (Aksioscope) with standard FITC filter (09) and PE-filter (014) (excluding the possibility of interference of FITC and PE-staining), for VT-FITC, CD13-PE, CD14-PE and CD45-TRITC staining, respectively.

***Isolation of PMN***

Twenty milliliters of EDTA or heparin blood of eight healthy adult donors and of two children was obtained for the isolation of PMN. Blood was directly put on ice. Blood was mixed with 15 mL of PBS and put into a 50-mL tube. The blood was underlayed using Ficoll 1.077 g/mL. The cells were centrifuged 20 minutes 200x g at 4 °C in a Sorvall centrifuge. The pellet contained PMN and erythrocytes. Erythrocytes were lysed using ammoniumchloride or FACS lysing solution and PMN were washed twice with PBS containing 1% bovine serum albumin (BSA). PMN were resuspended in PBS or RPMI-medium containing 1% of human serum and stored at 4°C till use for < 1 hour until use. The population PMN was over 95% pure as measured by an $H_3$-analyser (Technicon, Bayer).

***Flow-cytometric analysis after isolation of different cell types***

Pooled PMN as described above were used. Blood was mixed with PBS and cells were separated using Ficoll. The interphase was collected for studying binding to lymphocytes and monocytes separately. The pellet contained PMN and erythrocytes. The pellet was resuspended in 20 ml PBS. Subsequently, Ficoll 1.077 g/mL was used to separate PMN from erythrocytes. To make sure that the different cell populations were more than 95% pure, cell populations were analyzed by a $H_3$ -analyzer. Different cell types were incubated with 0,5 µL VT-FITC ( 1mg/mL) on ice for twenty minutes. Cells were then washed twice with PBS/1%BSA and centrifuged at 200*g* for 5 minutes. Cells were resuspended in 500 µL of 0.5% paraformaldehyde. Binding of VT-FITC was measured using flow cytometry. Experiments were repeated for the different cell types using an incubation time of three hours.

Results were confirmed by using direct immunofluorescence and incubation of the different cell populations separately with $^{125}$I-VT-1 (see below).

***Binding of VT-FITC to lipoproteins***

To examine the binding between VT and lipoproteins, lipoproteins were isolated by ultracentrifugation and a precipitation method (99). Very low-density lipoproteins (VLDL), high-density lipoproteins (HDL), or low-density lipoproteins (LDL) were incubated with VT-FITC during three hours on ice. After the incubation lipoprotein-depleted serum was added. The solution was mixed thoroughly, and incubated for ten minutes. Subsequently, a precipitation solution was added followed by centrifugation. After centrifugation binding of VT-FITC was determined by fluorescence spectrometry. Experiments were repeated using an incubation time of one hour or twenty minutes to determine whether binding of VT to lipoproteins occurred after a similar incubation period as needed for PMN.

***Binding of 125I-VT-1 to human PMN***

VT-1 was labeled with Na-$^{125}$I according to the Iodogen procedure (53). After isolation of PMN from EDTA blood, PMN were washed twice with PBS and were then resuspended in Hanks Balanced Salt Solution (HBSS) containing 1% Human Serum Albumin (HSA; Central Laboratory of the Red Cross, Amsterdam) at 0 °C. Subsequently, 0.5 x $10^6$ PMN/250 µL were incubated for three hours with $^{125}$I-VT-1 in different concentrations ranging from 0.3 up to 70 nmol/L. To determine non-specific binding unlabeled VT-1 in 25-fold excess was added parallel with $^{125}$ I-VT-1. After incubation the free fraction of $^{125}$I-VT-1 was separated from the fraction of $^{125}$I-VT-1 bound to the PMN using Ficoll 1.077 g/mL. PMN were then washed with

HBSS/1% HSA and centrifuged at 200 x g for 10 minutes. Cell-associated $^{125}$I-VT-1 was determined in a gamma-counter. All determinations were done in duplicate. Binding data were analyzed, using the method of Scatchard (100).

***Thin layer chromatography of neutral glycolipids extracted from PMN.***

PMN were isolated as described before. They were washed twice with PBS and subsequently resuspended in 0.5 ml of PBS. Next, glycolipids of the cells were extracted and separated as described by Lingwood et al. (101). For the extraction of neutral glycolipids 1 mol/L NaCl was used instead of water to receive maximal yield. After separation of the neutral glycolipids, the TLC-plate was coated with polyisobutylmetacrylate, blocked overnight with 1% PBS/Tween and incubated with $^{125}$I-VT-1 in 1% albumin and 0.05% Tween-20 in PBS (102). After washing, the binding of $^{125}$I-VT-1 was analyzed using a Fuji BAS 1000 phosphor-imager.

***Human glomerular microvascular endothelial cells***

Human glomerular microvascular endothelial cells (GMVEC) were isolated and cultured on gelatin-coated dishes as described by van Setten et al (56). Cells were characterized by indirect immunofluorescence microscopy using antibodies against von Willebrand factor, PECAM-1 and VE-cadherin. No immunoreactivity was observed with antigens against α-smooth muscle actin or cytokeratin 20, indicating that there was no contamination with mesangial or epithelial cells. GMVEC were cultured in 24-well plates to perform experiments and used between passages six and ten. They were used five days after reaching confluence and stimulated for 24 h with 10 ng/mL tumor necrosis factor alpha (TNF-α), if indicated.

***Transfer of VT from PMN to endothelium in vitro***

After isolation, PMN were washed twice with PBS and were then resuspended in medium M199 containing 20% fetal calf serum. PMN were incubated with a monoclonal antibody, CD16-PE, to exclude the possibility that PMN interfere with FACS analysis. Subsequently, different amounts of PMN, varying between 0.5 and 2.0 x $10^6$ PMN were incubated with 0.5 µl VT-FITC on ice during 30 minutes. After that, PMN were washed twice with medium M199. Non-stimulated GMVEC and TNF-α-stimulated GMVEC were incubated on ice, for four hours, with 0.5 - 2.0 x $10^6$ PMN loaded with VT-FITC. As control, GMVEC were also incubated alone with VT-FITC, PMN or with medium containing 10% fetal calf serum. After the incubation period, GMVEC were washed three times with M199. GMVEC were detached with trypsin treatment and centrifuged for 5 minutes 200g at 4°C. Cells were washed with PBS

and then resuspended in 500 µl 0 5% paraformaldehyde, and transferred into a tube suitable for FACS-analysis

***Measurement of protein synthesis***

GMVEC were cultured in 24-wells plates, and were used five days after reaching confluence GMVEC stimulated by TNF-α (10 ng/ml) and non-stimulated GMVEC were incubated with $10^6$ PMN at 37°C for 24 hours PMN were preloaded with VT in different concentrations, ranging from 0 1 nmol/L to 10 nmol/L VT and washed two times before adding to the GMVEC PMN not preincubated with VT were used as control Protein synthesis was determined by assaying the incorporation of $^3$H-leucine in newly synthesized proteins as described previously (18,56,76)

***Statistics***

All results of measuring protein synthesis are expressed as mean ± S E M Changes with respect to control values were analyzed by using an unpaired Student's t-test Values where P was equal or less than 0 05 were regarded as significant

**RESULTS**

***Binding of VT in blood***

Incubation of whole blood with VT-FITC showed after 20 minutes almost complete binding of VT to PMN (Figure 1C and 1D), independent of blood group or age of the donor Binding was completely blocked by pre-incubation of blood with unlabeled VT-1 for 20 minutes (data not shown) More than 90% of PMN were positive and all other cell types were negative Control blood, incubated with an IgG antibody conjugated with FITC, showed no staining (Figure 1A and 1B) Selective binding of VT-FITC was also demonstrated by direct immunofluorescence studies (Figure 2) Only PMN bound VT-FITC

To substantiate that PMN were the only cells binding VT in blood, VT-FITC binding to purified erythrocytes, monocytes and platelets was also studied Erythrocytes were incubated and binding was analyzed using FACS-analysis and direct immunofluorescence No binding of VT-FITC to erythrocytes was observed independent of the P-bloodgroup (8 donors) No significant binding of VT-FITC to non-stimulated monocytes was observed (6 donors) Lymphocytes and thrombocytes (3 different donors) also did not bind VT-FITC in our conditions Lingwood (57,58) suggested that lipoproteins may bind glycolipids such as Gb3,

and that VT may be cotransported by lipoproteins in a piggyback way. LDL and HDL preparations were incubated separately with VT-FITC on ice for three hours. No significant binding was observed.

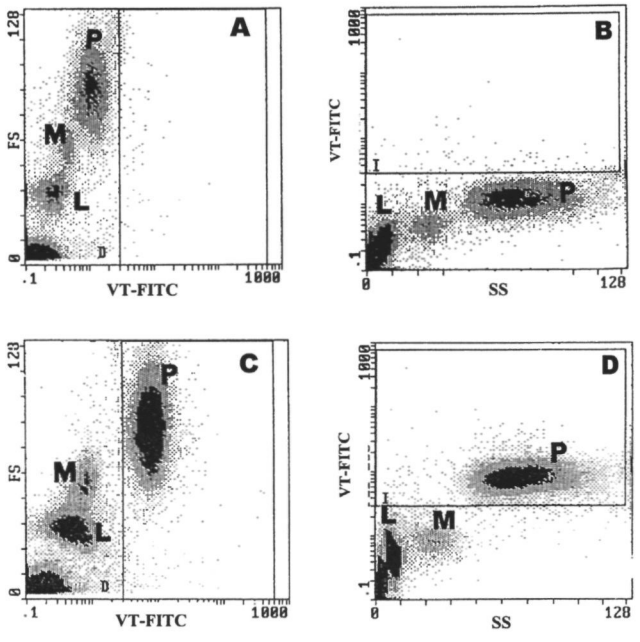



**Figure 1**. Binding of VT-FITC to PMN in whole blood was demonstrated by flow cytometric analysis. A,B: Flow cytometric analysis of control blood (incubated with IgG-FITC alone) before addition of VT-FITC. C,D Analysis after 20 minutes incubation with 0,5 µl VT-FITC (1mg/ml) exclusive binding to PMN was found. L: lymphocytes; M; monocytes; P: PMN. The experiment is representative for duplicate determinations in 10 experiments with blood of different donors.

This experiment was repeated with incubation times of 20 minutes and 1 hour. Again no binding was found (data not shown). Similarly, no binding of $^{125}$I-VT to these preparations was found. From these data we conclude that VT only significantly binds to PMN in whole blood and not to other components such as erythrocytes, lymphocytes, monocytes and lipoproteins.

***Analysis VT-binding to PMN***

To evaluate whether high-affinity binding sites were involved in the binding of VT to PMN, the binding of $^{125}$I-VT-1 to PMN was determined. Figure 3A shows that the binding of $^{125}$I-VT-1 is saturable and specific. After incubation with a 25-fold excess of unlabeled VT-1, binding

with $^{125}$I-VT-1 decreased more than 95%. Scatchard plot analysis (Figure 3B) showed that non-stimulated PMN have 2.1 x $10^5$ binding sites with a high affinity for VT (Kd = $10^{-8}$ mol/L). This affinity is 100-fold less than those others and we consistently found for VT-1 binding to Gb3 in human vein and GMVEC (56,102). To confirm that VT remained exposed on the surface, $^{125}$I-VT-1 was bound to PMN (30 minutes, 37°C), and after 3 washings, the VT-loaded PMN were incubated for 2.5 h at 37 °C in medium supplemented with 10% fetal calf serum and 100 U/mL aprotinin. After removal of the serum-containing medium, treatment of these PMN for 10 min by trypsin/EDTA released 95% of the $^{125}$I-VT-1 associated with the cells. This indicates that the VT-1 remained available at the cell surface.

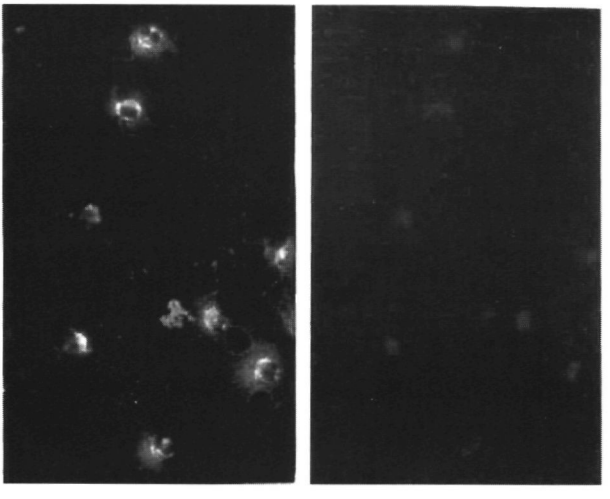
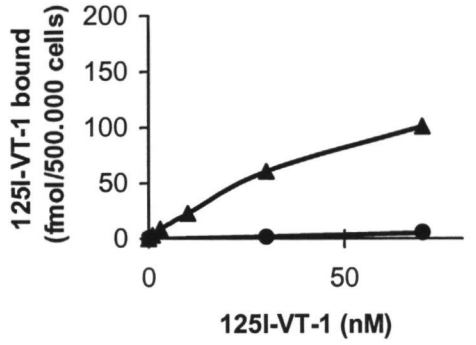


**Figure 2**: Direct immunofluorescence of binding of VT-FITC to PMN. Whole blood was incubated with CD13-PE (A), a specific marker for PMN, and with VT-FITC (B) on ice for twenty minutes. PMN were the cells that had bound VT-FITC. No other components of blood were positive for binding VT-FITC.

To investigate whether Gb3 or a different neutral glycolipid was responsible for binding of VT, thin layer chromatography (TLC) of neutral glycolipids extracted from non-stimulated PMN was performed and compared with those of TNF-α-stimulated GMVEC and LPS-stimulated monocytes.



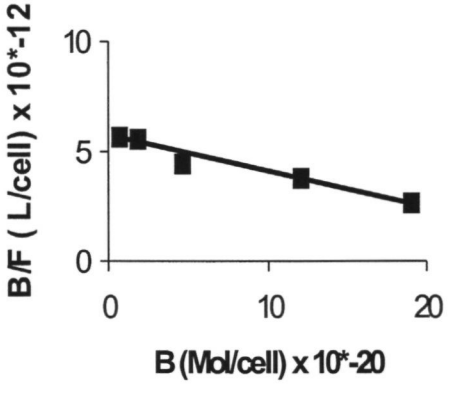




**Figure 3:** PMN of two different donors were used to determine binding of $^{125}$I-VT-1 to PMN. Figure **A**: PMN were incubated with increasing concentrations of $^{125}$I-VT-1 (0.3 to 70 nmol/l) at 4° C for three hours ( ). Non-specific binding ( ) was determined in the presence of 25-fold excess of unlabeled VT-1. Figure **B** shows the result of Scatchard plot analysis.

Thin layer chromatograms were incubated with radiolabeled VT-1 and washed thoroughly. The bound $^{125}$I-VT-1 was detected with the use of a phosphor imager (Figure 4). $^{125}$ I-VT-1 strongly bound to the Gb3 in the standard neutral glycolipid preparation (lane D) and the TNF-α-treated neutral glycolipid extract of GMVEC (lane B). Two other bands binding $^{125}$I-VT-1 just below the globotetraosylceramide (Gb4) were found in glycolipid extractions of monocytes (lane C)

and in smaller amounts in those of GMVEC. Non-stimulated PMN showed two small bands with an Rf value just below the Gb4 but distinct from that observed for monocytes. This pattern was consistently found with 4 different neutral glycolipid extracts of PMN obtained from four different donors.

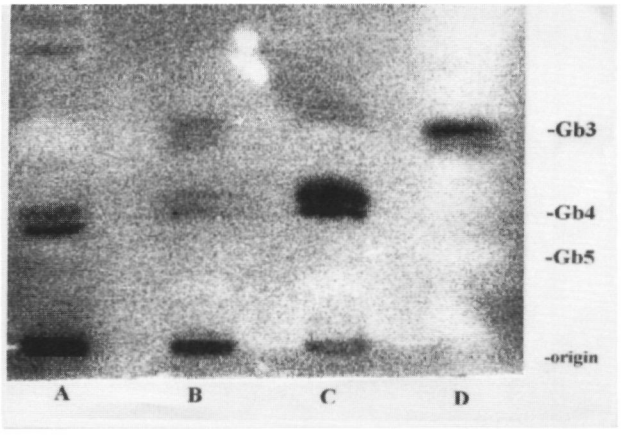



**Figure 4:** $^{125}$I-VT-1 binding to neutral glycolipid extracts from human PMN, GMVEC and monocytes. Glycolipids were extracted and separated as described in Materials and Methods. Binding of $^{125}$I-VT-1 was visualized using a phosphor imager. Lane **A**: Glycolipid extract of 30 million PMN of one representative donor. Lane **B**: Neutral glycolipid extraction of TNFα-treated GMVEC. Lane **C**: glycolipid extract of monocytes stimulated with LPS (1 ng/ml). Lane **D**: Standard mixture of neutral glycolipids, 2 μg of each glycolipid. Standard of neutral glycolipids was stained with orcinol. (Gb3, globotriaosylceramide: Gb4, globotetraosylceramide and Gb5, Forssman pentasaccharide).

***Transfer of VT from PMN to GMVEC***

In order to investigate if VT-1 could be transferred from PMN to GMVEC, we incubated non-stimulated and TNFα-stimulated GMVEC with PMN that had bound VT-FITC at 0°C.

PMN were previously incubated on ice with CD16-PE, a specific monoclonal antibody for PMN, to prevent interference of PMN by FACS-analysis. After an incubation time of three hours on ice, GMVEC were washed carefully three times with PBS. Subsequently, GMVEC were detached and analyzed by flow cytometry. No PMN were retained by non-stimulated GMVEC (as revealed, by FACS-analysis after staining with CD16-PE) (Figure 5A and C).

GMVEC were negative for CD16-PE staining (Figure 5C). However, TNF-α stimulated GMVEC retained a small percentage of the PMN added (ranging between 2.8% and 3.6%) (Figure 5B).

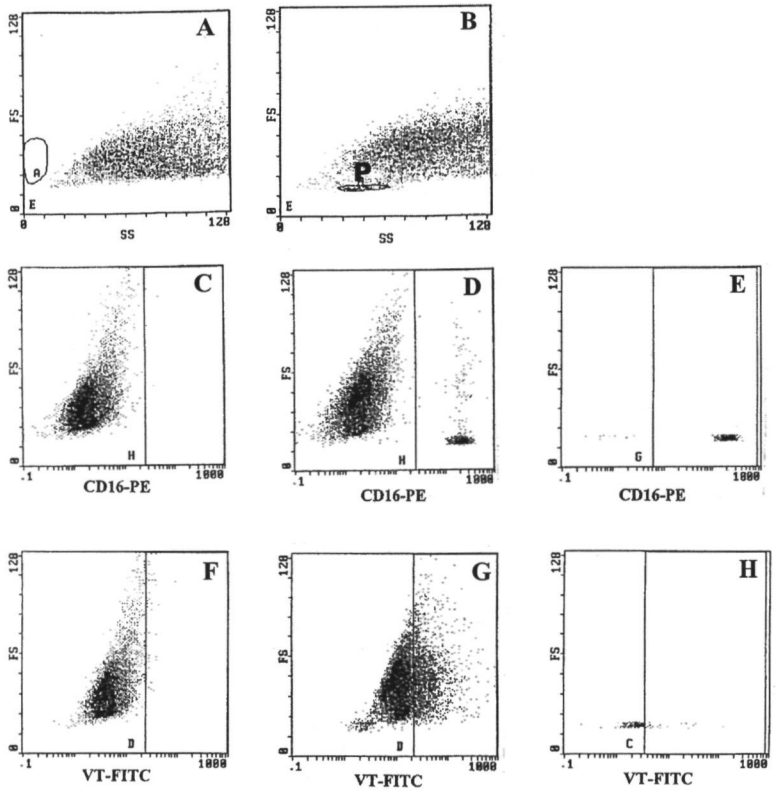



**Figure 5:** The transfer of VT-1-FITC bound to PMN to human GMVEC was studied by flow cytometry. Panels A ,C and F: FACS analysis of non-stimulated GMVEC incubated for 4 h with PMN loaded with VT-FITC on ice. (A) Forward scatter and side scatter of non-stimulated GMVEC are shown. (C) No positive staining for CD16-PE was observed, indicating that all PMN were removed by washing the monolayers. (F) Non-stimulated GMVEC did not bind any VT-FITC after incubation with VT-FITC-loaded PMN. Panels B, D, E, G, H: TNFα-stimulated GMVEC retained 2.8-3.6% of PMN (present in the gated area P in panel B) after 4 h incubation and washing of the monolayers. (D, E) PMN were distinguished from GMVEC using CD16-PE; panel D represents all cells, while panel E reflects the gated area. (G) TNFα-treated GMVEC incubated with VT-FITC-loaded PMN were able to bind 30-50% of VT-FITC after the incubation period of 4 hours (all cells minus the gated area P). (H) PMN showed no positive staining for VT-FITC indicating that ligand passing of VT-FITC from PMN to GMVEC had occurred.

The population of PMN was gated (indicated by the area P in Figure 5B), which_allowed simultaneous analysis of PMN and GMVEC. The population of gated PMN was positive for CD-16-PE (Figure 5E) and distinct from the CD-16-PE-negative GMVEC (Figure 5D). The differentiation between PMN and GMVEC by gating permitted us to investigate if a transfer of

VT-FITC from PMN to GMVEC had occurred. After the 3 hours incubation period 30% of the stimulated GMVEC had bound VT-FITC (figure 5G). Non-stimulated GMVEC did not bind any VT-FITC (Figure 5F). When IgG-FITC was incubated with GMVEC, no binding occurred. This excludes nonspecific binding of FITC to these cells. Ligand passing of VT-FITC from PMN to GMVEC was further confirmed by the fact that no VT-FITC could be demonstrated on PMN that were removed after the 3h incubation period on GMVEC on ice. Furthermore, PMN that were CD16-PE positive and still adhering to endothelial cells (2.8-3.6% of total PMN added) were negative for VT-FITC binding (Figure 5H). No uptake of VT-FITC by PMN occurred during the incubation period.

***GMVEC damage by ligand passing of VT from PMN***

Similar experiments were performed at 37 °C. Unlabeled VT-1 bound to PMN was used to study whether the transfer of VT-1 from PMN to GMVEC caused a biological effect. In TNF-α-stimulated GMVEC both VT-binding PMN and VT alone caused severe cytotoxity during a 24h period, while PMN alone had no effect (Figure 6). The exposure of non-stimulated GMVEC to VT-binding PMN also caused considerable cell death (30-40%), while exposure of VT-1 or PMN alone had no effect. Comparable toxicities were observed when the VT-binding PMN were washed 3 times and first incubated for 2.5 h at 37 °C (to mimic a circulation time before reaching the target tissue), before they were washed again and transferred to the endothelial cells (data not shown).

Because VT exerts its cytotoxic effects by inhibiting protein synthesis, we quantified the effect of VT-preloaded PMN on the viability of GMVEC by determining the overall protein synthesis (from the incorporation of $^{3}$H-leucine in newly synthesized proteins).

No effect on protein synthesis was observed when stimulated or non-stimulated GMVEC were incubated with PMN. However, when non-stimulated and TNFα-stimulated GMVEC were incubated with VT-preloaded PMN inhibition of protein synthesis (30 ±15 % and 60 ± 25 %, respectively) was observed (Figure 7).

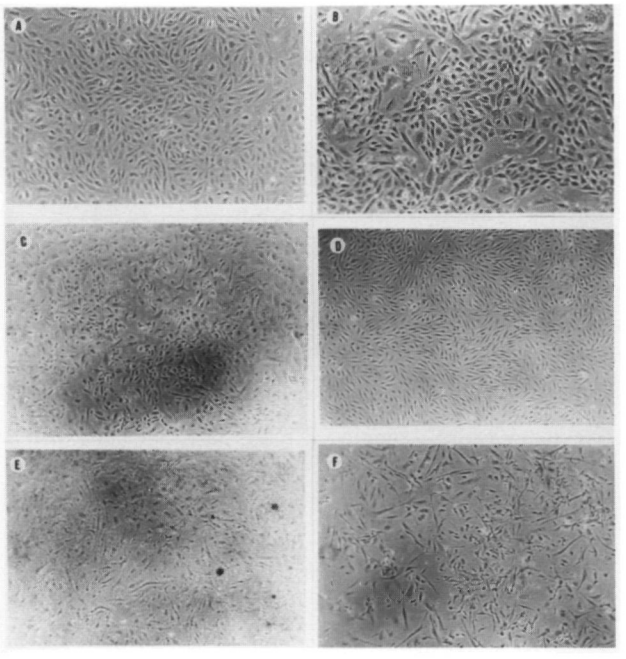


**Figure 6:** Phase-contrast microscopy of human GMVEC. Magnification used x 50, except for figure B (Magnification x 100). (A) Represents control GMVEC. (B) Shows the result of GMVEC incubated with PMN for 24h. No change of morphology was observed. When cells were incubated with PMN loaded with VT-1 cell death was observed after 24h incubation (C). (D) Represents control TNF-α-treated GMVEC. The effect of PMN loaded with VT-1 was also studied on TNF-α-treated GMVEC (E) and compared to the effect of VT-1 on TNF-α-treated GMVEC (F). In both conditions equal amounts of cell death were observed.

## DISCUSSION

In this study, we demonstrate that VT-1 binds almost exclusively to non-stimulated PMN in whole blood. Only 20 minutes of incubation was sufficient to bind VT-1 to PMN for more than 90%. No binding to other components of blood was observed, not even when incubation time was prolonged to 3 hours. The $K_d$ of the high affinity receptor for VT on PMN is a 100-fold less than that found for the functional receptor for VT, Gb3 on GMVEC. In line with this difference in affinity of VT, we found transfer of VT from PMN to GMVEC, and subsequent inhibition of protein synthesis and cell death. Several suggestions have been made to explain the transfer of VT from the intestine to the kidney. Bitzan et al. (54) described that erythrocytes could bind VT depending on their P-blood group phenotype.

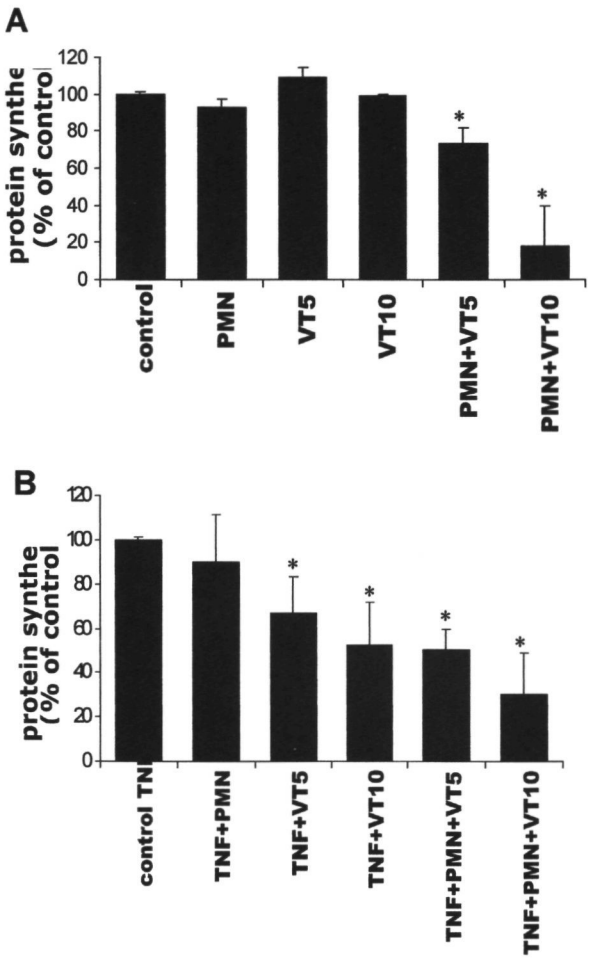



**Figure 7:** Measurement of protein synthesis by incorporation of $^{3}$H-leucine in newly synthesized proteins. **(A)** Non-stimulated GMVEC incubated with VT-1 loaded PMN showed during 24h incubation a reduction of protein synthesis. **(B)** GMVEC pre-stimulated for 24h with TNF-α (10ng/mL) showed strong inhibition during incubation with VT-1 alone or after incubation with VT-1 preloaded PMN. Similar results were obtained from five different donors for GMVEC. Results are expressed as ± S.E.M. Statistical analysis was performed using unpaired Student's t-test. P-values smaller than 0.05 were considered to be significant. * $P < 0.05$ as compared to non-stimulated GMVEC (**A**) and TNFα-prestimulated GMVEC ( **B**).

Robson et al as well as Taylor et al suggested even that there was an association between the outcome of the epidemic form of HUS and P blood group (97,98). However, others investigators could not demonstrate a protective effect of the P1 blood group in HUS. We also could not find VT binding to erythrocytes in whole blood of the two donors with P1 blood

group in our study. However, we cannot exclude yet that local variations exist in P1-blood group-binding proteins causing VT-binding in some people. Cooling et al. (74) indicated that in particular small and older platelets, obtained by apheresis, contain small amounts of Gb3 and can bind VT. In a whole blood environment we did not observe any significant binding of $^{125}$I-VT-1 and VT-FITC to platelets (8 subjects were studied). Therefore, the relative contribution of platelets to the transport of VT in plasma appears insignificant as compared to PMN. Lingwood suggested that lipoproteins might be responsible for transporting VT (57,58). However, in the present study we could not find binding of VT to human LDL, VLDL or HDL, thus excluding this possibility. In line with our previous observation that purified human monocytes only bind VT significantly after activation by LPS (76), we found no VT binding to monocytes in whole blood. On the contrary, VT binding occurred exclusively to PMN, independent of blood groups or age of the donor. These data leads to the conclusion that PMN are a good candidate for transporting VT in the systemic circulation to target organs in adults as well as in children.

The rapid binding of VT to PMN can explain why VT is usually not detectable in blood plasma (103). After binding the VT, PMN are able to transport the VT to target organs and transfer the VT to endothelial cells *in vitro*. Stimulating the GMVEC with inflammatory mediators, such as TNF-α or LPS, induces the VT receptor Gb3 on the cell surface (55,56). As expected transmission of VT-1 from PMN to endothelium increased when GMVEC were previous stimulated with TNF-α. We demonstrated in this study that one type of binding sites is involved in VT-1 binding on PMN, and that this binding site is different from the Gb3 receptor found on endothelial cells. It is uncertain whether the VT receptor on PMN represents a glycolipid, because VT-1 binding to PMN was highly sensitive to trypsin treatment. In contrast to the classical VT receptor, Gb3, the VT receptor found on PMN does not result in VT internalization. The lower Kd for the receptor on PMN than for Gb3 allows transfer of VT from PMN to endothelial cells. Within 24 h this transfer was accompanied by biological effects, especially inhibition of protein synthesis and cell death. Interestingly, we observed that such biological effects not only affected cells that were prestimulated with TNFα but also in non-stimulated GMVEC that were incubated with VT-binding PMN. These data indicate that PMN not only can bind VT in the circulation, but also can transfer it to target cells that express the VT-receptor Gb3.

That PMN may play a seminal role in pathogenesis of HUS has been suggested many times. Firstly, the number of PMN is elevated in HUS. It has been suggested that the number of PMN is a predictive factor for the outcome of the disease (11,105,106). In addition, Taylor and colleagues showed that there was an increased number of PMN in autopsy material of diarrhea

associated HUS patients (82,107). Furthermore, Fitzpatrick et al. described that PMN of HUS patients were activated and that, in HUS patients, levels of elastase and IL-8 were elevated (79,80) These results were confirmed by other investigators (108), and suggested that PMN may damage the endothelium through release of the intracellular components, such as elastase (79-81). Finally, Morigi and colleagues described that VT-1 increased PMN adhesion to the endothelium under flow conditions by up-regulating adhesive proteins (78). The binding of PMN to the endothelium was reduced by blocking of E-selectin, ICAM-1 and VCAM-1 with respective antibodies. The adhesion of PMN to the endothelium was enhanced by pre-exposure of the endothelial cells by TNF-α. These investigators found that VT-1 was able to inhibit the process of rolling that normally proceeds adhesion of cells to the endothelium. In line with these findings is that PMN of HUS patients adhere more avidly to endothelial cells than PMN of healthy control subjects (81). All these results together indicate that PMN play an important role in pathogenesis of hemolytic uremic syndrome. Our data show a new and crucial aspect of the involvement of PMN in the epidemic form of HUS, namely the specific binding and transport of VT to PMN in whole blood. Additional studies are needed to evaluate whether the binding of VT to PMN causes a metabolic effect in PMN themselves. In this respect, it is of interest to note that in a recent study, the induction of superoxide production in PMN by Shiga toxin-1 was described (110).

In conclusion, our study demonstrates an additional role of PMN in the pathogenesis of HUS and strongly suggests that PMN are the cells that transport the verocytotoxin form intestine to endothelium. This transport is facilitated by a receptor that has a 100-fold lower affinity than the high-affinity receptor (Gb3) that is expressed on glomerular microvascular endothelial cells after exposure to TNF-α. PMN loaded with VT display a direct cytotoxic effect to the endothelium of the kidney *in vitro* by inhibition of protein synthesis. The occurrence of VT-1 binding to PMN in vivo and ligand passing of VT and PMN has to be demonstrated in future studies. We believe that this observation is important in understanding the pathogenesis of (D+) HUS and opens perspectives for future treatment.

# DETECTION OF VEROCYTOTOXIN BOUND TO CIRCULATING POLYMORPHONUCLEAR LEUKOCYTES OF PATIENTS WITH HEMOLYTIC UREMIC SYNDROME

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**ABSTRACT**

The epidemic form of hemolytic uremic syndrome is the most common cause of acute renal failure in children and is characterized by a prodromal phase of some times bloody diarrhea. The role of verocytotoxin (VT)-producing *Escherichae coli* has been strongly implicated. Although antibodies against VT have been found in serum of HUS patients, VT itself has never been detected in circulating blood. In this study, VT-2 was detected in the systemic circulation in 9 out of 10 patients with the epidemic form of HUS. In those cases, VT-2 was bound exclusively to polymorphonuclear leukocytes (PMN). The detection of VT-2 bound to PMN was associated with the presence of (bloody) diarrhea at the time the blood samples were obtained. The one patient, for whom VT was not detected, presented with atypical HUS. For 5 of the 10 patients with HUS who were studied, the time course of VT binding was analyzed and decreased in four patients. The finding of VT bound to PMN in the systemic circulation of HUS patients is important for a clearer understanding of the pathogenesis of HUS and suggests new approaches for treatment in the future.

**INTRODUCTION**

The epidemic form of hemolytic uremic syndrome (HUS) is the most common cause of acute renal failure in children (2,3). Endothelial damage of predominantly glomeruli and to lesser extent arterioles of the kidney appears to play an important role in pathogenesis of HUS. In severe cases, however, endothelial cell damage is not limited to the kidney, but extends to other organs such as the brain and pancreas (4). In 90% of HUS cases, infection with a verocytotoxin (also termed "Shiga" or "Shiga-like toxin") producing *Escherichae coli (E. coli)* has been strongly implicated, especially serotype O157: H7. *E. coli* can produce verocytotoxin-1 (VT-1), verocytotoxin-2 (VT-2) or both. Most common among patients with HUS are infections caused by a VT-2 producing *E. coli* (10,92). VT can cause inhibition of overall protein synthesis after binding to globotriaosylceramide (Gb3) receptor, which is found on endothelial cells after stimulation with inflammatory mediators (55,56). Serological testing of HUS patients for antibodies to the lipopolysaccharide of *E. coli* O157: H7 is often used to detect infection by VT-producing *E. coli* O157 (18,111,112). However, VT itself has never been detected in blood of patients with HUS. Recently, we investigated the binding of VT-1 and VT-2 in whole blood (113). In that study, we demonstrated that VT binds rapidly and exclusively to polymorphonuclear leukocytes (PMN) when incubated in whole blood *in vitro*. The binding occurs via selective binding to a specific receptor on PMN. Thin layer chromatography demonstrated that the receptor exhibited an Rf value between Gb4 and Gb5. In addition, the receptor on PMN exhibited a 100-fold lower affinity compared with that of the Gb3-receptor,

which can be found on endothelial cells. PMN previously loaded with VT were able to pass the ligand VT to tumor necrosis factor alpha (TNF-α) -stimulated glomerular microvascular endothelial cells *in vitro* (GMVEC) which then causes inhibition of protein synthesis (113). In this study, we demonstrate for the first time, to our knowledge, the presence of VT-2 in the systemic circulation of patients in the acute phase HUS. Here we confirm that VT binds to PMN, as suggested by our *in vitro* studies, supporting our theory that PMN are responsible for transferring the ligand VT from the intestine to target organs.

## MATERIALS & METHODS

### *Patients and controls*

Ethylenediaminetetraacetate-treated blood was collected from 11 patients with HUS, during the acute phase of the disease. Ten of eleven patients exhibited a prodromal phase of sometimes bloody diarrhea, and antibodies against the *E. coli* O157: H7 were detected for seven patients (Table 1: patients 2 to 11). The patient without a prodromal phase had an atypical form of HUS. No *E. coli* (O157 or other types) was present in stool, and no antibodies against lipopolysaccharide could be detected. All patients exhibited thrombocytopenia, hemolytic anemia with fragmented erythrocytes, and renal failure.

Clinical characteristics of the patients are presented in Table 1. Blood samples were analyzed for the presence of VT-2 within two hours after withdrawal. Ethylenediaminetetraacetate-treated blood of 11 healthy volunteers and six patients with infectious disease (one with peritonitis, two with influenza virus, one with otitis media, one with toxic shock syndrome and one patient with an upper respiratory tract infection) were used as a negative control for the presence of VT.

### *Detection of verocytotoxin by indirect immunofluorescence and flow cytometry*

PMN were isolated as described previously (113). Briefly, whole blood of patients with HUS and control subjects was underlayed with an aliquot of Ficoll 1.077 g/mL (Pharmacia, Uppsula Sweden) and centrifuged at 200-x *g* for 20 minutes at 4 °C in a Sorvall centrifuge (Meyvis and Co., Bergen op Zoom, the Netherlands). The interphase, containing lymphocytes, monocytes and a few PMN, was collected and washed with phosphate-buffered saline (PBS). The pellet contained PMN and erythrocytes. The pellet was resuspended and erythrocytes were lysed in

| *Patient Nr:* | *Prodromal phase* | *Anti-O157* | *Hb mmol/L* | *Platelets X $10^9$/L* | *WBC x $10^9$/L* | *Creatinine µmol/L* | *P.D.* | *Days (*)* | *% PMN positive* |
|---|---|---|---|---|---|---|---|---|---|
| 1 | No | Neg. | 4.6 | 31 | 9.2 | 118 | No | 1,3,5,10 | 0 |
| 2 | Yes | Neg. | 7 | 68 | 13.8 | 452 | No | 10 | 1% |
| 3 | Yes | ND | 6.1 | 23 | 50 | 108 | Yes | 14 | 1-5% |
| 4 | Yes | Neg. | 5.2 | 169 | 21.2 | 355 | Yes | 10 | 0% |
| 5 | Yes | Pos. | 5.1 | 21 | 28 | 94 | No | 6 | 40-50% |
| | | | 6.5 | 169 | 18.1 | 206 | No | 11 | 5-10% |
| 6 | Yes | Pos. | 4.8 | 97 | 15.8 | 423 | Yes | 5 | 20% |
| 7 | Yes | Pos. | 5.6 | 45 | 10 | 859 | Yes | 9 | 22% |
| 8 | Yes | Pos. | 6.8 | 36 | 40 | 360 | Yes | 3 | 90% |
| | | | 4.5 | 60 | 42 | 340 | Yes | 8 | 15% |
| 9 | Yes | Pos. | 3.9 | 32 | 42.5 | 157 | Yes | 5 | 96% |
| | | | | | | | Yes | 10 | 92% (†) |
| 10 | Yes | Pos. | 2,2 | 52 | N.D. | 600 | No | 8 | 14% |
| | | | | | | | No | 13 | 0% |
| 11 | Yes | Pos | 4.2 | 56 | 18 | 306 | No | 9 | 80% |
| | | | 4.4 | 252 | 9.5 | 93 | No | 14 | 20% |

**Table 1**: Clinical characteristics of HUS patients and percentage of PMN positive for VT-2 binding. (*) = Number of days after start of the diarrhea that had passed before receiving the blood sample. Patients 1,2,3 and 4 had no (bloody) diarrhea at the time the blood sample was collected. Patient 5 and 8 and 10 had no diarrhea anymore at the time the second sample was taken. Patient 1 was diagnosed having atypical HUS. Results of Hb, platelet count, and white blood cell count (WBC) and serum creatinine presented here, are the values measured at the time of blood sampling for the detection of verocytotoxin on PMN. Patient nine died on the same day the second sample was taken and patient 10 was dismissed on the day the second sample was taken and for this reason no values indicating WBC, Hb, etc. are present for these patients. N.D.= not determined P.D.= peritoneal dialysis.

ammoniumchloride or fluorescence activated cell sorting (FACS)-lysing solution. The remaining PMN were washed twice with PBS. Total number of cells was counted and $1x10^6$ cells were used in each experiment. PMN ($1x10^6$ cells) and interphase samples ($1x10^6$ cells) from patients and control subjects were incubated for 1 hour, on ice, with a monoclonal antibody against VT-2B subunits (concentration 0.1 µg/µL; Toxin Technology; Kordia, Leiden, The Netherlands) in PBS with 10% fetal calf serum. Subsequently, cells were washed three times with PBS and incubated for 30 minutes, on ice, with FITC-goat anti-mouse (1:1000;

DAKO, Glostrup, Denmark). Cells were washed again three times with PBS followed by an incubation for 1 h on ice with CD13-phycoerythrin, CD14- phycoerythrin, or CD45-tetrarhodamine isothiocyanate (DAKO) to differentiate PMN, monocytes, and lymphocytes, respectively. Subsequently, cells were washed three times with PBS and resuspended in 0.5% paraformaldehyde for fixation. Cells were analyzed either by using a Zeiss microscope (Aksioscope; Bakker and Co., Zwijndrecht, the Netherlands) with standard equipment or by FACS. FACS analysis made it possible to quantify the percentage of positive cells.

**RESULTS**

On the basis of the results of our *in vitro* work (113) we focused in this study on the binding of VT-2 to PMN. Indirect immunofluorescence assays demonstrated positive staining for VT-2 on PMN in nine of 10 patients in the acute phase of HUS (Table 1). The result of an indirect immunofluorescence assay for one representative patient are presented in Figure 1. VT-2 binding was observed only to PMN. To confirm that PMN were the only cells binding VT in blood, VT-binding to purified blood cells of patients with HUS was studied. As suggested by our previous findings from healthy donors (113), VT-2 bound only to PMN and not to lymphocytes, monocytes or erythrocytes from patients with diarrhea associated (D+) HUS in the acute phase of the disease.

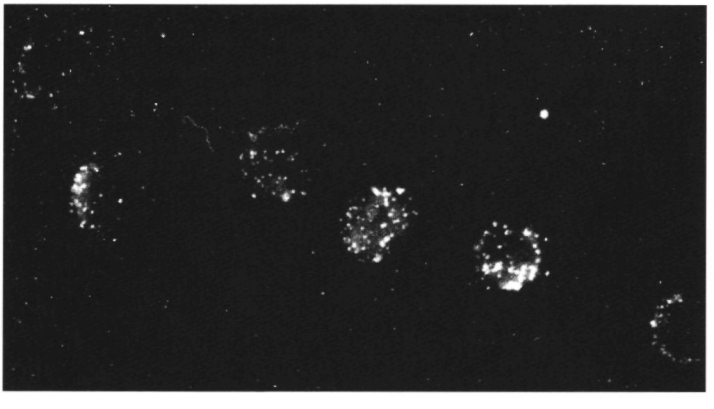

**Figure 1:** Indirect immunofluorescence of VT-2 bound to PMN in one representative patient (patient 9, respectively) in the acute phase of HUS. Magnification used 1000x.

FACS-analysis was used to quantify the indirect immunofluorescence results. No VT-2 binding was detected in control blood from any of the healthy volunteers (Figure 2, A,C and E). In addition, no binding of VT-2 was observed for six patients with infectious diseases (see above).

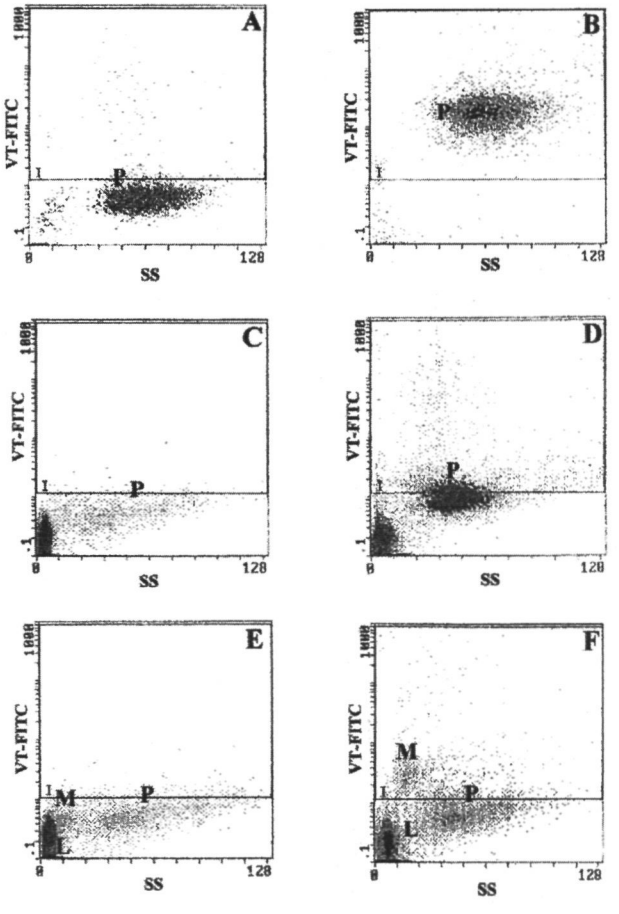


**Figure 2:** Flow cytometric analysis for detection of VT-2 bound to PMN. (A and C) PMN isolated form control blood and incubated with a monoclonal antibody against VT-2, followed by incubation with FITC-conjugated goat anti-mouse IgG. No positive binding was observed. (E) Interphase sample from the same control subject, containing lymphocytes (L), monocytes (M) and PMN (P), incubated with a monoclonal antibody against VT-2. No binding of VT-2 was observed. (B) results of the first sample of blood from a patient with HUS (patient 8), using the same incubation steps as used for the control sample. Of all PMN, 90% were positive for VT-2 binding. (D) Results showing that, five days later, VT-2 binding to PMN was reduced to 15%. (F) Results showing that, interestingly, monocytes were positive for VT-2 binding on day 5, whereas no binding was observed on day 1.

Figure 2 B presents the results of VT binding to PMN from one patient (patient 8) on the first day after admission to the hospital. On the first day 90% of the PMN were positive for VT-2 staining. Five days later only 15% of PMN were still positive (Figure 2D) suggesting that VT was transferred to target cells (data not shown). Interestingly, at that time not only PMN but also monocytes (Figure 2E) were positive for VT-binding which is highly indicative of activated monocytes as described by van Setten et al (76). For patient 9, VT-2 binding to monocytes was also observed 5 d after collection of the first sample. No VT-2 binding to monocytes was observed in the other patients, and patient 10 exhibited no binding to PMN anymore 5 d after collection of the first sample.

Time course of VT-2 binding to PMN was studied only in five patients with the greatest numbers of VT-2 positive cells (patients 5, 8, 9,10 and 11, respectively) (Figure 3). For patients 5,8, 10 and 11, large decreases in the numbers of PMN positive for VT-2 were observed in the 5 d after collection of the first sample. The last sample of patient 9 was obtained 5 d after the first sample, and no significant decrease of VT-2 was observed. A possible explanation for this may be that additional toxin was absorbed from the circulation and transferred to the white blood cells as fast as they could transfer the toxin to the Gb3 containing glomerular cells. Patient 9 had a very severe form of HUS, with neurological involvement and died on the day the last sample was obtained.

No positive staining for VT-2 was found in patient 1 and in patient 4. All patients who exhibited positive results for VT-2 binding to PMN were still experiencing diarrhea at the time the blood samples were obtained. Patient 1 exhibited no prodromal phase of (bloody) diarrhea and exhibited a relapse of HUS just a few weeks later. He responded to treatment with plasmapheresis. Therefore, he was considered to have an atypical form of HUS.

## DISCUSSION

In this study, we demonstrate for the first time the presence of VT-2 in the systemic circulation in nine of ten patients in the acute phase of the epidemic form of HUS. In 85% of all cases of D+ HUS in Western Europe, infections with VT-2 producing *E. coli* are found (21). Therefore, we investigated the presence of VT-2 in the systemic circulation of patients with D+ HUS in the acute phase of the disease.

VT-2 bound to PMN and not to erythrocytes, monocytes or lymphocytes during the period of diarrhea. There was a strong association between the detection of VT-2 bound to PMN and the presence of (bloody) diarrhea at the same time. One patient with atypical HUS showed repeatedly exhibited no positive staining in the acute phase of the disease. In the other

case were no VT-2 binding was detected (patient 4) the result was probably negative due to the late admission to the hospital This patient was without diarrhea for seven days at the time the blood sample was taken A reason for this may be that the toxin had already been transferred from intestine to the kidney Alternatively, this patient may have had an infection with a VT-1 producing *E coli, which* we did not detect with the specific monoclonal antibody against VT-2 used in this study

It has often been suggested that PMN may play a critical role in pathogeneses of D+ HUS PMN levels are elevated in HUS, and it has been suggested that the number of PMN is a predictive factor for the outcome of the disease (12,105) In addition, increased numbers of PMN were found in glomeruli of kidney autopsy material from patients with D+ HUS (82,107) Furthermore, PMN in patients with HUS are activated and elevated levels of IL-8 and elastase are found (80) It has been suggested that PMN of HUS patients may damage the endothelium through release of intracellular components such as elastase, or formation of superoxide (81,110) In our *in vitro* experiments, we indeed demonstrated that PMN loaded with VT-1 were able to induce endothelial cell death whereas VT-1 or PMN alone had no effect (113) In addition, a recently published study showed that apoptosis of PMN was inhibited by VT, those authors concluded that longer survival of neutrophils might aggravate neutrophil-mediated tissue damage (114) Finally, Zoja and colleagues (78) reported that VT-1 can cause an increased adhesion of PMN to the endothelium under flow conditions, by up-regulating adhesive proteins Administration of antibodies against E-selectin, ICAM-1 and VCAM-1 reduced the adhesion of PMN to the endothelium

Karmali and colleagues investigated the distribution of VT-1 in serum of rabbits (70) and demonstrated a short serum half-life (2 minutes) for VT-1 From those results, the authors concluded that VT is probably rapidly cleared from the systemic circulation and for thus is not detectable in the circulation of patients with HUS They did not consider a role for PMN in transporting VT, however In our *in vitro* experiments, we demonstrated that VT binds rapidly and exclusively to PMN (113) No binding to other components of blood was observed Interestingly, it has been suggested that PMN play also a very important role in the pathogenesis of Kawasaki disease, and it has been reported that lipopolysaccharide is bound and transported by PMN (115)

In line with our in *vitro* data is the finding of VT-2 bound to PMN *in vivo* in nine of ten HUS patients with the epidemic form of HUS We think that VT has never been detected in serum of HUS-patients, because VT binds rapidly to PMN after entering the systemic circulation and thus is absent in plasma or serum of patients with HUS

The finding of VT-2 bound to PMN in D+ HUS patients not only represents the missing link between the intestinal infection and damage to the target organs but also provides new approaches for therapy. Two patients with severe HUS whom we have studied exhibited high percentages of PMN positive for VT-2 binding; in a later phase. binding to monocytes was also observed. We think that, during the phase of bloody diarrhea, VT traverses the intestine-blood barrier, binds to PMN and thus is transferred to target organs.

Synsorb Pk, a synthetic analogue of the Gb3 receptor, can bind VT *in vitro* and can neutralize VT when mixed *in vitro* with VT-positive stools from children with HUS (116,117). When Synsorb Pk is administered orally to patients with the epidemic form of HUS while they have still diarrhea, it reduces the amount of VT available; this reduction might prevent and/or decreases the binding of VT to PMN and the subsequent transfer of VT from the intestine to target organs. However, additional therapy is required in severe cases. We think that, on the basis of the finding that VT is bound to PMN in D+ HUS patients, leukopheresis would be a possible solution in such cases. The presence of VT bound to PMN can be assessed rapidly, using a simple, reproducible and quick method, as we have described in this report. PMN loaded with VT can be removed using leukopheresis, thus preventing the transfer of VT to target organs or protecting target organs from severe damage.

In conclusion, our data demonstrate a new and crucial aspect in the pathogenesis of HUS, namely the specific binding and transfer of VT by PMN in the systemic circulation. We think that this observation is important for clarification of the pathogenesis of HUS and for treatment of HUS patients. We suggest that in severe cases of D+ HUS an effective therapy with leukopheresis should be considered.

**Acknowledgement:** We want to thank all the University Hospitals that have collaborated by collecting material of HUS patients. We especially want to thank Prof. Dr. C. Schröder, University Hospital Utrecht; Dr. Davin, Amsterdam Medical Centrum; Dr. J. van de Walle, University Hospital Gent, Belgium; Dr. J. Nauta, Sophia Kinderziekenhuis, Rotterdam and Dr. van Daal; University Hospital Groningen.

# VEROCYTOTOXIN BINDING TO POLYMORPHONUCLEAR LEUKOCYTES IN HOUSEHOLD MEMBERS OF CHILDREN WITH HEMOLYTIC UREMIC SYNDROME

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**ABSTRACT**

The hemolytic uremic syndrome (D+ HUS) is the leading cause of acute renal failure in childhood and can be caused by different serotypes of verocytotoxin- (= Shiga toxin-) producing Escherichia coli (VTEC). Recently we have shown that verocytotoxin (VT) is bound to polymorphonuclear leukocytes (PMN) in the systemic circulation of patients with D+ HUS. In this study we investigated whether VT bound to PMN can be detected in household members of D+ HUS patients. Serum antibodies against Escherichia coli O157 and when available feces from D+ HUS patients and household members were investigated to establish the presence of VTEC infection. The circulating PMN of 82% of the household members were positive for VT, whereas based on examination of their stool and/or serum only 21% were positive. This study shows that with the methods currently used, the total number of infected people in the surrounding of D+ HUS patients is underestimated.

**INTRODUCTION**

The hemolytic uremic syndrome (HUS) is clinically characterized by hemolytic anemia, thrombocytopenia and acute renal failure (2). The syndrome is preceded in a majority of the patients by an episode of diarrhea with or without blood in the stools and has therefore often been termed D+ HUS (4). It has been proven that infection with verocytotoxin- (= Shiga toxin-) producing Escherichia coli (VTEC) plays a central role in the pathogenesis of disease, finally leading to HUS in 10% of the children infected (3,118,119). VTEC strains involved in D+HUS may belong to different serological groups but infection with strains belonging to serotype O157: H7 is most frequently detected (10). VTEC strains can produce verocytotoxin-1 (VT-1), verocytotoxin-2 (VT-2) or both, but in 80% of the human cases in Western Europe VTEC strains producing only VT-2 are identified (21,120). Several approaches can be used to demonstrate an infection with VTEC, including the examination of patients' stool for free VT and/or VTEC organisms and investigating the patients' serum for the presence of antibodies binding to lipopolysaccharide (LPS), especially O157 LPS (70% of the cases is caused by VTEC belonging to this serogroup (121). Household members of children with D+ HUS are often asymptomatically infected with VTEC. Examination of stool samples showed evidence of an infection with VTEC in about 30% of the parents and in 45% siblings (18). To cause its systemic effects, the toxin must gain access to the circulation. Acheson et al. showed that VT is capable of passing across epithelial cells without any apparent cellular disruption (39,122). Other possible routes include the translocation of the toxin through lesions of the mucosal barrier.

Recently, we have demonstrated that in the acute phase of patients with D+ HUS, VT is bound to circulating polymorphonuclear leukocytes (PMN) (123). The binding of VT to PMN occurs by selective binding to a specific receptor that is different from the classical VT receptor globotriaosylceramide (Gb3) found on endothelial cells (113). The discovery of VT bound to PMN not only explains the question of how VT is transported from the intestine to target organs but also offers a new detection method to provide evidence for infection with VTEC. Furthermore, this detection method enables us to evaluate whether VT is also circulating in the blood of the parents and siblings of D+ HUS patients, who did not develop HUS themselves. This evaluation provides further insight into the question whether the entry of VT into the circulation is the only key factor that determines the development of D+ HUS, or whether additional promoting or protective factors also play a role. To provide extra evidence of VTEC infection serum samples of D+ HUS patients and their household members were investigated for the presence of antibodies to LPS. Additionally, when available fecal samples were analyzed for the presence of free VT and VTEC strains causing the infection. Finally, the different detection methods were compared and combined to find the true frequency of VTEC infection in household members.

## MATERIALS & METHODS

### *Patients, household members and controls*

Samples of whole blood (EDTA), serum, and feces were collected from 13 D+ HUS patients as well as from one or more of their respective household members (n=28) to find evidence of infection with VTEC. Patients represented in this study were collected during the year 2000 and are non-related individual cases of D+ HUS in the Netherlands and Belgium. All D+ HUS patients had thrombocytopenia, hemolytic anemia with fragmented erythrocytes and renal failure. In the majority (75 %) of the household members enrolled in this study no symptoms of diarrhea did occur at the time of admission or 1 week before admission of the D+ HUS patient to the hospital. Whole blood (EDTA) of twelve healthy controls and three patients with atypical HUS were studied as negative controls for the presence of VT-1 and/or VT-2.

### *Analysis of whole blood*

Within 2 hours after withdrawal, samples of whole blood of the HUS patients, and household members (collected in the acute phase of the disease of the child), and controls were tested for the presence of VT-1 or VT-2 bound to PMN. PMN were isolated as described previously (113). The total number of cells was counted and in each experiment $1x10^6$ cells were used. PMN ($1x10^6$ cells) from patients, household members and controls were incubated with a

monoclonal antibody against the B subunit of VT-1 or VT-2 (concentration 1 μg/μL; Toxin Technology; Kordia, Leiden, The Netherlands: diluted 1:50) in phosphate buffered saline (PBS) with 10% fetal calf serum for 1 h on ice. Subsequently, the cells were washed three times with PBS and incubated with phycoerythrin (PE) or fluorescein isothiocyanate isomer (FITC)-labeled goat anti-mouse IgG antibody (1:1000; DAKO, Glostrup, Denmark) for 30 minutes on ice. Subsequently, the cells were washed three times with PBS and then were resuspended in 0.5% paraformaldehyde for fixation. Cells were analyzed by flow activated cell sorting (FACS, Coulter). FACS-analysis made it possible to quantify the percentage of cells positive for VT-binding.

***Serological examination***

Serum samples (collected as soon as possible after admission of the patient to the hospital) were tested for the presence of antibodies of the IgM-class binding to the LPS of *E. coli* O157 by ELISA and immunoblotting (120,121). When tested negative, serum samples of HUS-patients were additionally examined for the presence of antibodies to the LPS of *E. coli* of serogroups: O26, O104, O111, O115 and O145.

***Stool examination***

Stool samples (collected as soon as possible after admission of the patient to the hospital) of D+ HUS patients and household members were streaked onto sorbitol MacConkey (SMAC) agar and/or SMAC agar supplemented with cefixime (0.05 mg/L) and tellurite (2.5 mg/L) to isolate VTEC O157 strains. To detect VTEC strains of other serogroups, stools were enriched in modified tryptone soy broth (18-20 h at 37°C) and DNA extracts from the enrichment cultures were used as templates for a VT-PCR. Furthermore, the cytotoxic activity of both stool extracts (free VT) and culture broth filtrates was assayed on Vero cells.

**RESULTS:**

***VTEC infection in D+HUS patients***

All D+ HUS patients (100 %) included in this study were found positive for VT binding to PMN in the systemic circulation (Table 1). All controls investigated were negative for the presence of VT confirming that the detection of VT bound to PMN is specific. Sixty-six percent of the patients had IgM-class serum antibodies to O157 LPS whereas in 50% of the patients evidence for infection with VTEC of serogroup O157 was found based on examination of patients' stool. In most hospitals only the presence of *E. coli* O157 is investigated (in feces

and serum) and therefore patients that are infected with a different strain will be missed. When the binding of VT to PMN is determined in the acute phase of the disease it will be possible to identify all patients infected with VTEC (Table 1).

Table 1: Detection of VTEC infection in D+ HUS patients

| *Patient Nr* | *Diarrhea* | *Feces (VTEC O157, VTEC non-O157, free VT)* | *Serum antibodies to LPS* | *% PMNs with VT (bound / type of VT)* |
|---|---|---|---|---|
| 1 | Yes | Negative ([a]) | O26 | 10% / VT-1 |
| 2 | Yes | VTEC O157 positive ([b]) | O157 | 9% / VT-2 |
| 3 | Yes | VTEC O157 positive ([a]) | O157 | 18% / VT-2 |
| 4 | Yes | Negative ([a]) | O157 | 12% / VT-2 |
| 5 | Yes | Negative ([a]) | O111 | 95% / VT-2 |
| 6 | Yes | Negative ([a]) | Negative | 40% / VT-2 |
| 7 | Yes | VTEC O157 Positive([c]) | O157 | 45% / VT-2 |
| 8 | Yes | Negative([c]) | Negative | 79% / VT-2 |
| 9 | Yes | VTEC O157 Positive ([a]) | O157 | 47% / VT-2 |
| 10 | Yes | VTEC O157 Positive ([a]) | O157 | 77% / VT-2 |
| 11 | Yes | Negative ([b]) | O145 and O115 | 88% / VT-2 |
| 12 | Yes | VTEC O157 positive ([a]) | O157 (*) | 10% / VT-2 |
| 13 | Yes | Negative ([a]) | O157 | 98% / VT-2 |

(*) Serum antibodies to O157 LPS were weak positive in this patient.

*(a) examined only by directly streaking feces onto sorbitol MacConkey (SMAC) agar*

*(b) examined by directly streaking feces onto SMAC agar, SMAC agar supplemented with cefixime and tellurite (CT-SMAC) and VT-PCR examined by directly streaking feces onto SMAC agar, CT-SMAC agar, VT-PCR and performing verocelcytotoxicity tests*

***VTEC infection in household members of D+HUS patients***

In 82% (n=28) of the household members of the D+ HUS patients VT bound to PMN was found (Table 2). The percentage of positive cells ranged between 8 and 90 % similar to the percentages found in D+ HUS patients.

Table 2: VTEC infection in household members of D+ HUS patients

| *Patient* | *Household members* | *Diarrhea* | *Feces ( VTEC O157, VTEC non-O157, free VT )* | *Serum antibodies to LPSO157* | *% PMNs with VT bound / type of VT* |
|---|---|---|---|---|---|
| 1. | Mother | No | Negative[a] | Negative | Negative |
| 2. | Mother | No | Positive O157[b] | Negative | 10% / VT-2 |
| | Father | No | Negative[b] | Negative | Negative |
| 3. | Mother | No | ND | Negative | 22% / VT-2 |
| 4. | Mother | No | ND | Negative | 12% / VT-2 |
| | Father | No | ND | Negative | 13% / VT-2 |
| 5. | Mother | No | ND | Negative | 80% / VT-2 |
| 6. | Mother | No | Negative[a] | Negative | 33% / VT-2 |
| | Father | No | Negative[a] | Negative | 8% / VT-2 |
| | Grandfather | Yes | Negative[a] | Negative | 70% / VT-2 |
| 7. | Mother | No | Negative[c] | Negative | 50% / VT-2 |
| | Father | No | Negative[c] | Negative | 85% / VT-2 |
| 8. | Mother | No | Negative[c] | Negative | Negative |
| | Father | No | Negative[c] | Negative | 22% / VT-2 |
| 9. | Mother | No | Negative[a] | Negative | 24% / VT-2 |
| | Father | No | Negative[a] | Negative | 49% / VT-2 |
| | Brother | No | Negative | Negative | Negative |
| | Sister | Yes | VTEC O157 Positive | Positive | 42% / VT-2 |
| 10. | Mother | No | Negative[a] | Negative | 85% / VT-2 |
| | Father | Yes | Negative[a] | Negative | 90% / VT-2 |
| | Brother | Yes | Negative[a] | Weak response | 51% / VT-2 |
| | Sister | Yes | VTEC O157 positive | Positive | 47% / VT-2 |
| 11 | Mother | No | ND | Negative | 53% / VT-2 |
| | Grandmother | No | ND | Negative | 71% / VT-2 |
| 12 | Father | No | ND | Negative | Negative |
| 13 | Mother | No | Negative | Negative | 60% / VT-2 |
| | Father | Yes | Negative | Negative | 96% / VT-2 |
| | Sister | Yes | Negative | Negative | 44% / VT-2 |

*(a) examined only by directly streaking feces onto sorbitol MacConkey (SMAC) agar*

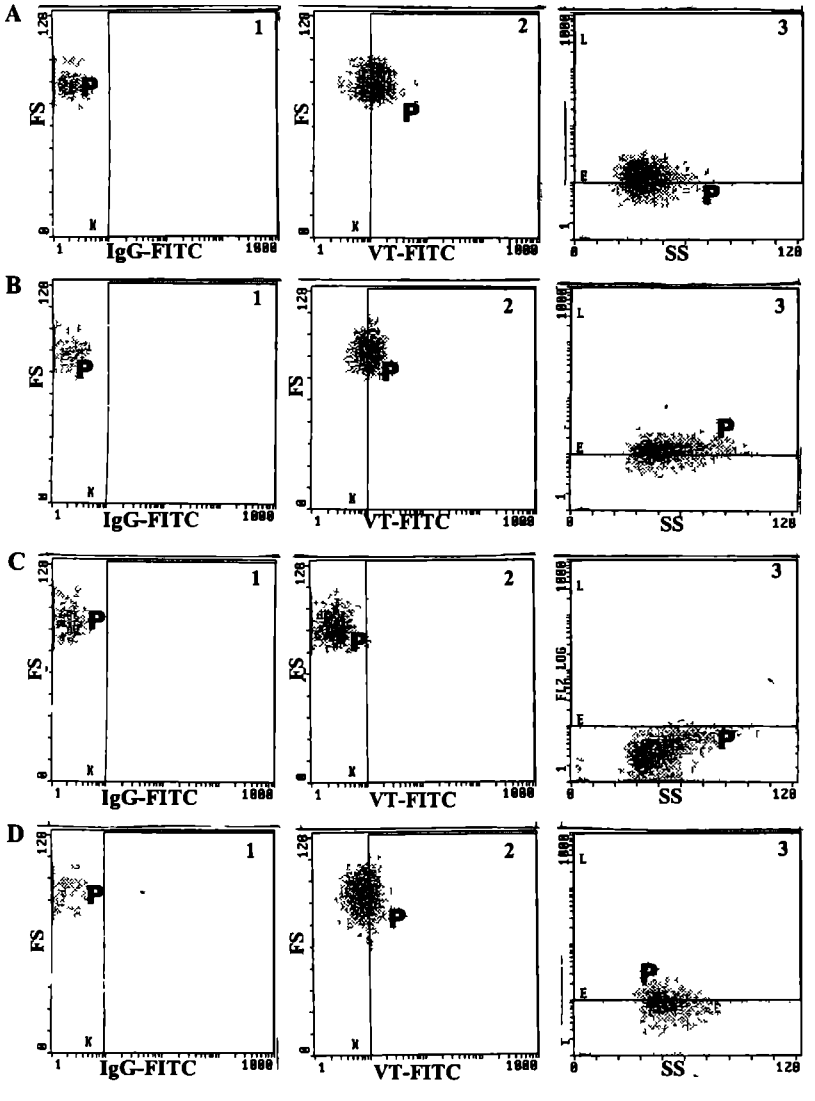
*(b) examined by directly streaking feces onto SMAC agar, SMAC agar supplemented with cefixime and tellurite (CT-SMAC) and VT-PCR*

*(c) examined by directly streaking feces onto SMAC agar, CT-SMAC agar, VT-PCR and performing verocelcytotoxicity tests*

ND, not done

The experimental data of FACS-analyses of one HUS patient and the respective household members are demonstrated in Figure 1. All were positive for VT-2 binding to PMN except for the brother of the HUS patient. The brother had been absent for two weeks, and therefore had probably not been exposed to the source of the VTEC infection. VT bound to PMN was determined one week later in four household members of patient 8 and patient 9 that were

positive on the first day. All four household members were negative one week after admission of the HUS patient, whereas the two D+ HUS patients were still positive (37 and 45%, respectively) Serum antibodies to LPS were detected in 10% (n=28) of the household members examined and 10% (n=19) had positive stool samples for the presence of VTEC.



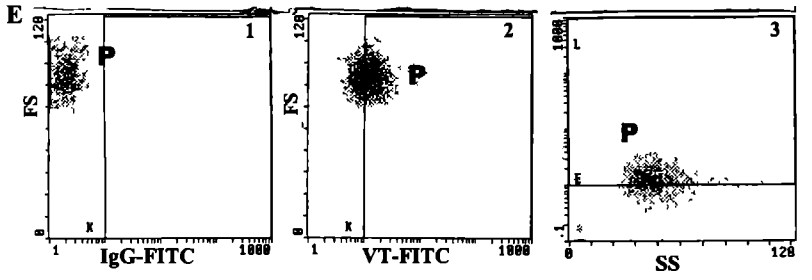



**Figure 1.** FACS-analysis of a D+ HUS patient (patient 9) and the respective household members. Cells are differentiated on the basis of size by a forward scatter (FS) or on the basis of granulation by a side scatter (SS). Binding of VT was visualized by a fluorescent signal (FITC). Flow-cytometric analysis of control blood (incubated with IgG-FITC alone) is shown in the first picture ($A_1$-$E_1$). The D+ HUS patient had 47% positive cells ($A_{2+3}$). The FACS analysis of the sister of the patient (42%) and brother of the patient (0%) are shown in Figure $B_{2+3}$ and $C_{2+3}$ respectively. The brother had been absent for two weeks and was probably not exposed to the source of the infection. The mother ($D_{2+3}$) and the father ($E_{2+3}$) had respectively 24% and 49% of PMNs positive for VT-binding.

## DISCUSSION

VT bound to PMN was found in 82 % of the household members. Based upon the combined examination of their stool and serum samples only 21% were positive. No relationship was found between the presence of diarrhea and the amount of VT bound to PMN. The finding of VT bound to PMN in the circulation of parents and siblings indicated that not only was there the entry of the toxin into the circulation of household members of D+ HUS patients, but also other additional factors, for example LPS, may play a role in the development of HUS.

The finding of parents of D+HUS patients being negative for the presence of serum antibodies to O157 LPS is confirmed by earlier reports and serological testing is therefore not a useful tool to provide evidence for VTEC infection in household members (18). Occasionally, siblings produce antibodies to LPS. Despite the low number of household members detected positive, LPS serology is still considered as one of the most applied methods used to provide evidence of VTEC infection in D+HUS patients (18,21,120,121).

However, we demonstrate in the current study that the detection of VT bound to PMN is more sensitive for providing evidence for VTEC infection both in D+HUS patients and in their household members.

In the literature, the percentage of household members found positive for VTEC infection based on the examination of their stools can be as high as about 40%, depending on the time of collection of the samples and the detection method used (18,25). In this study we have shown that 80% of the household members were positive for VT-2 binding to PMN indicating that they have been infected with VTEC recently. So it is very likely that at present the frequency of VTEC infection in household members is being underestimated using stool culture combined with serological analysis. Negative stool samples may be explained by a rapid clearance of the VTEC organisms from the gastrointestinal tract of humans and therefore the organisms and the toxins they produce are no longer detectable anymore in feces. It has been described that the median duration of shedding of VTEC O157 is 13 days in patients with hemorrhagic colitis and 21 days in patients with HUS (124). For household members no data are available on the length of shedding.

Although D+ HUS patients and their household members had similar numbers of PMN that had bound VT (one million of PMN being investigated) are found differences in the amount of toxin entering the circulation probably occur. In regard to this, it is interesting to know that although household members were infected they did not develop symptoms of hemolytic uremic syndrome (serum creatinine, blood smear and urine sediment were normal) even if more that 50% of PMN were positive for VT-2 binding. The detection of VT bound to PMN only provides evidence for the presence of a recent infection. It should be taken in account that it is not yet possible to determine the exact amount of VT bound by one individual polymorphonuclear leukocyte. Therefore it might be possible that, although high percentages of PMN binding VT are found in household members, the true amount of toxin present is much less than in D+HUS patients. Furthermore, life span of PMN is normally about 2-3 days, but an *in vitro* study showed that the life span of PMN was prolonged when incubated with VT-2 (114). As a consequence of the prolonged life span, toxin that entered the circulation can be detected for longer than 2-3 days so long as it is not transferred to target cells or eliminated by macrophages. In addition, it has to be considered that although equal percentages of positive cells are found in D+HUS patients and household members, the number of white blood cells is increased in patients with D+ HUS (12,105). Therefore relatively more VT might be present in the systemic circulation of HUS patients. To determine the amount of toxin that is present in or is entering the circulation, it would be necessary to quantify the toxin present on PMN.

The finding of family members positive for VT bound to PMN further underlines the importance of measure to prevent further transfer of VTEC infection, especially to children because they are more susceptible. Secondary spread of VTEC both during outbreaks and sporadic cases have been documented (125-127). It has been estimated that in a large hamburger-associated outbreak in the United States in 1993 about 11% of all cases resulted from secondary spread (128). The assay of VT binding to PMN provides an adaptive and sensitive tool to verify whether people carry a VT producing *E. coli* infection.

In conclusion, this study has shown that the number of household members of a D+HUS patient infected was much higher than expected before from the examination of feces and/or LPS antibodies in their serum alone. Examination of serum and feces remains important to determine the origin of the infection and to determine the serotype of the infecting VTEC strain. Based on the results of this study one have to assume that almost all household members of an HUS patients will be infected. This underlines the importance of advice on the risk of person-to-person spread and on necessary hygienic precautions in the home of a D+HUS patient to prevent further transmission within families as much as possible.

# LIPOPOLYSACCHARIDE IS BOUND TO POLYMORPHONUCLEAR LEUKOCYTES IN HEMOLYTIC UREMIC SYNDROME: ACTIVATION OF POLYMORPHONUCLEAR LEUKOCYTES

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***Submitted***

**Abstract**

The hemolytic uremic syndrome (HUS) is the most common cause of acute renal failure in childhood. The syndrome is in most cases caused by an infection with a verocytotoxin (VT) (= Shiga toxin) producing *E. coli* (VTEC). Recent studies demonstrated that VT is transported by polymorphonuclear leukocytes (PMN) through the systemic circulation in D+ HUS patients but also in non-symptomatic VTEC infected patients. Therefore, it is reasonable to assume that other additional factors are necessary for the development of HUS. Lipopolysaccharide (LPS) may be one of these factors.

In this study we investigated whether LPS could be detected in the systemic circulation of patients with HUS and in their household members. Seven out of nine patients with HUS were positive for the presence of LPS bound to PMN whereas 5 out of 18 household members were also positive.

In addition, the effects of VT and LPS on PMN were studied in whole blood of healthy volunteers. The expression of different activation markers such as CD63, CD66b and CD11b/CD18 (= anti-ICAM) increased after incubation with LPS whereas incubation with VT alone had no effect. LPS and VT together had similar effects compared to incubation with LPS alone. Furthermore, LPS was able to induce the release of elastase by PMN when incubated in whole blood. VT was able to induce a slight, although not significant, increase in elastase release. Incubation with LPS and VT together induced a release of elastase comparable to incubation with LPS alone.

This study shows for the first time, to our knowledge, the presence of LPS bound to PMN in the systemic circulation of patients with HUS. Furthermore, this study demonstrates that not VT but the presence of LPS bound to PMN causes activation of PMN *in vitro*. Therefore we postulate that the activation of PMN as observed in patients with D+ HUS is caused by the presence of LPS. In addition, we hypothesize that the amount of LPS together with the amount of VT entering the systemic circulation is essential for the development of HUS.

**Introduction**

The hemolytic uremic syndrome (HUS) is defined by the triad of hemolytic anemia, thrombocytopenia, and acute renal failure and occurs mainly in childhood (2,4). The pathogenesis of the syndrome is hallmarked by endothelial damage of mainly glomeruli and arterioles within the kidney (92). It is now generally accepted that the development of the diarrhea-associated form of HUS (D+ HUS) is caused by an infection with a verocytotoxin (= Shiga like toxin or Shiga toxin) producing *Escherichia coli* (VTEC)(10). Recently, we have demonstrated that verocytotoxin (VT) is transported by polymorphonuclear leukocytes (PMN) through the systemic circulation of D+ HUS patients (123). *In vitro* studies performed showed that PMN were able to transfer VT to endothelial cells (113). These data are suggestive for the conclusion that PMN form the missing link between intestinal infection and organ damage. However, VT bound to PMN is also present in household members of D+ HUS patients with only VTEC infection (129). Therefore we hypothesized that not only the entry of VT into the systemic circulation of D+ HUS patients but also other additional factors are required for the development of HUS.

One of these additional factors might be lipopolysaccharide (LPS). LPS is a major component of the outer surface of gram-negative bacteria. Picomolar concentrations of LPS are sufficient to elicit inflammatory responses in the host by activating PMN, monocytes, and endothelial cells (130). This initiation of cellular response is necessary for the host defense against gram negative bacteria. However, if large amounts of LPS enter the circulation an excessive response can be seen leading to severe complications like circulatory failure and organ damage (130). LPS binding to cells is facilitated by the presence of lipopolysaccharide binding protein (LBP) and of CD14, a glycosylphosphatidyl inositol-anchored species on the surface of monocytes and PMN and also present as soluble protein in plasma. It has recently been shown that after binding of LPS to the cell, a signal is transferred through Toll-like receptors leading to the activation of NF-κβ (131-133). LBP is a 60-kd serum glycoprotein that is synthesized hepatically (134). The concentration of circulating LBP is increased in response to LPS and other inflammatory cytokines (135). Levels of LBP in plasma of HUS patients were significantly higher when compared to patients with uncomplicated VTEC infection (136). Furthermore, serum antibodies against LPS can be found in D+ HUS patients whereas this is rarely being detected in patients with only VTEC infection (137,138). These data suggest that LPS have a role in the pathogenesis of D+ HUS. Although serum antibodies against LPS can be detected in D+ HUS patients, LPS itself has never been detected.

Because PMN transport VT through the systemic circulation of D+ HUS patients and have a CD14-binding domain, we investigated whether LPS might be present on PMN of HUS

patients. Furthermore, the presence of LPS on PMN of parents and siblings of D+ HUS patients was studied. In addition, the effects of LPS and VT on PMN in whole blood of healthy volunteers was evaluated. Our data suggests that the presence of LPS has a decisive role for the development of full-blown HUS.

## Materials & Methods

### Reagents and antibodies

Hanks Balanced Salt solution without phenol red (HBSS) was obtained from Life Technologies, Paisley, Scotland. Human serum albumin was obtained from Behringwerke, Marburg, Germany). Luminol, phorbol 12-myristate 13-acetate and horseradisch peroxidase (hrp) were from Sigma, St Louis, MO, USA. VT-2 was ordered from Kordia (Toxin Technol.), Leiden, The Netherlands. Monoclonal antibody against the 2-keto-3-deoxyoctonic acid, that is a part of the inner core of LPS (KdO, clone 20) was purchased from HyCult biotechnology Bv, Uden, The Netherlands. Monoclonal antibodies against CD66b (fluorescein isothiocyanate (FITC)-labeled), CD63 (-phycoerythrin conjugated (PE)-labeled) were ordered from Immunotec, Marseille, France. Antibodies against L-selectin, and CD11b/ CD18 complex (both FITC-labeled) were purchased from Dako A/S, Denmark as also the secondary antibody goat-anti-mouse FITC.

### *Patients and control subjects*

Ethylenediaminetetraacetate-treated (EDTA) blood was collected from 9 patients with D+ HUS immediately after submission, 18 household members of HUS patients on the day of admission of the HUS patient, and 5 healthy volunteers. All HUS patients exhibited a prodromal phase of bloody diarrhea and presented with thrombocytopenia, hemolytic anemia, and renal failure. Blood samples were used for the isolation of PMN as described previously (113). Contaminating erythrocytes were removed through lysis with ammoniumchloride. PMN were washed twice with PBS. Total number of cells was counted and 1 $x10^6$ cells were used for determination of the presence of LPS. PMN of patients and control subjects were incubated in PBS with 10% fetal calf serum with a monoclonal antibody against the KdO of endotoxin for 1 h, on ice. Subsequently, cells were washed three times with PBS, followed by incubation with goat-anti-mouse IgM-FITC. Cells were analyzed using flow-activating cell sorting (FACS). FACS analysis made it possible to quantify the percentage of positive cells.

***Superoxide production***

PMN of eight different healthy donors were isolated as described above. PMN were incubated with VT-2 0.1 nM, VT-2 10 nM, LPS 1 μM alone, or LPS 1 μM in combination with VT-2 0.1 and 10 nM for 10 min, 1 h, and 4h, respectively. Immediately, after the incubation period, luminol-enhanced chemilumniscence of proteose-peptone-elecided PMN was measured on a Victor 1420 counter (Wallac, Turku, Finland) at 37 °C using white 96-well microplates as described previously (139). Each well-contained 2 x $10^5$ PMN, 50 μM luminol, 4.5 U/ml horseradish peroxidase and 50 ng/mL PMA in 200 μL of HBSS without phenol red, supplemented with 0.25% human albumin. Phorbol 12-myristate 13-acetate (PMA) was used as a positive control.

***Detection of activation markers on PMN***

100 μL Whole blood of four healthy volunteers was incubated with either LPS 1 μg/ml, VT 0.1 nM, VT 10 nM alone or LPS (1 μg/mL) in combination with VT 0.1 nM or VT 10 nM for 2 h at room temperature. Subsequently, erythrocytes were lysed using ammoniumchloride. The solution was centrifuged at 200*g* for 5 minutes at room temperature. PMN were identified by flow cytometry (Coulter® Epics® XL-MCL) using their morphological criteria and using CD13-PE as specific marker for PMN. Monoclonal antibodies against CD63 (-phycoerythrin conjugated) and CD66b (-FITC) were used as degranulation markers of PMN and monoclonal antibodies against L-selectin and CD11b/CD18 (= Mac-1 = anti-ICAM-1) were used to study the expression of adhesion molecules. Antigen expression was measured as mean fluorescence intensity of 5000 cells by flow cytometry. Data acquisition and analysis were performed using XL-2 software (Coulter).

***Statistical analysis***

The Mann-Whitney U test was used to assess the significance of differences between mean baseline values of the HUS patients versus controls. Wilcoxen signed rank test was used to test significance of within-group differences. Differences were considered significant at $P < 0.05$.

**Results**

***Detection of LPS bound to PMN in HUS***

FACS analysis of PMN isolated from D+ HUS patients showed that seven out of nine HUS patients were positive for the presence of LPS bound to PMN. All HUS patients were positive for the binding of VT-2 to PMN (Table 1).

Table 1. Detection of VT-2 and LPS-bound to PMN of D+ HUS patients

| Patient No. | Diarrhea | Anti-O157 antibodies | % VT-2 positive | % LPS positive |
|---|---|---|---|---|
| 1 | Yes | Negative* | 92 | 79 |
| 2 | Yes | Positive | 76 | 13 |
| 3 | Yes | Positive | 47 | 0 |
| 4 | Yes | Positive | 69 | 0 |
| 5 | Yes | Negative | 79 | 43 |
| 6 | Yes | Positive | 46 | 42 |
| 7 | Yes | Negative | 21 | 77 |
| 8 | Yes | Positive | 95 | 25 |
| 9 | Yes | Positive | 52 | 34 |

In patient 1 antibodies against *E. coli* O115 and O145 were found.

Five out of 17 household members investigated in this study were positive for the presence of LPS whereas 15 were positive for the presence of VT bound to PMN (Table 2). Only three of them, all three children, had serum antibodies against *E. coli* O157. No LPS or VT-2 was detected in any of the healthy controls.

Table 2 Detection of LPS and VT-2 in household members of D+ HUS patients

| Patient no | Household Members | Diarrhea | Presence of VTEC infection feces | Anti-O157 antibodies | %PMN VT-2 positive | % PMN LPS positive |
|---|---|---|---|---|---|---|
| 1 | Mother | No | ND | Negative | 67 % | 0 % |
| 2 | Mother | No | Negative | Negative | 86 % | 0 % |
| | Father | No | Negative | Negative | 90 % | 0 % |
| | Brother | No | Positive O157 | Weak posit | 47 % | 0 % |
| | Sister | No | Positive O157 | Weak posit | 76 % | 0 % |
| 3 | Mother | No | Negative | Negative | 24 % | 0 % |
| | Father | No | Negative | Negative | 50 % | 3 % |
| | Brother | No | Negative | Negative | 0 % | 0 % |
| | Sister | Yes | Positive O157 | Positive | 41 % | 0 % |
| 4 | N D | | | | | |
| 5 | Mother | No | Negative | Negative | 0 % | 0 % |
| | Father | No | Negative | Negative | 16 % | 5 % |
| 6 | Father | No | N D | Negative | 20% | 10% |
| 7 | Mother | No | Negative | Negative | 12% | 0 % |
| 8 | Mother * | No | Negative | Negative | 60 % | 25 % |
| | Father | Yes | Negative | Negative | 60 % | 35 % |
| | Sister | Yes | Positive O157 | Negative | 40% | 0 % |
| 9 | Mother | No | N.D | Negative | 22% | 0 % |

N.D. = not determined

* Fragmented erythrocytes were detected in the blood smear of the mother of patient 8

Figure 1 shows the results of the detection of LPS in a healthy control, and patient 1. The control was negative for the presence of LPS (Figure 1A) whereas in-patient 1 more than 75% of the PMN were positive for the presence of LPS (Figure 1B). At the same time, more than 90% binding of VT-2 to PMN was found. No positive staining for LPS was observed for patient 3 and 4 whereas in both patients VT-2 bound to PMN was found (47 and 69%, respectively) and serum antibodies against *E. coli* O157 were present. Unfortunately, it was not possible to follow LPS binding in course of the disease in HUS patients because of logistic reasons.

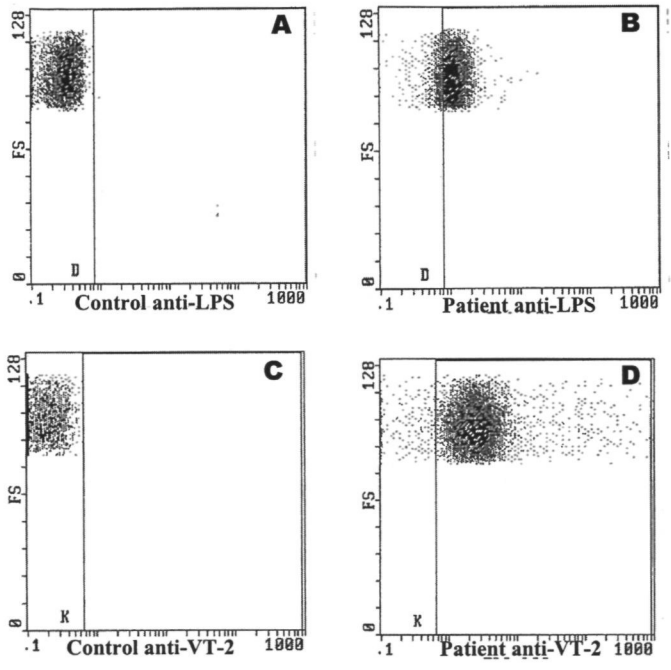



**Figure 1**: Detection of lipopolysaccharide (LPS) and verocytotoxin (VT-2) in D+ HUS patient (patient 1, respectively)

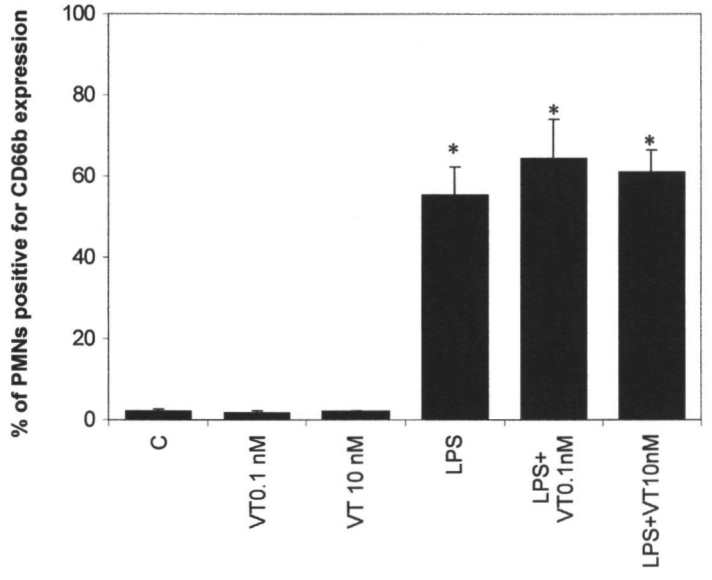
**Superoxide production**

Spontaneous superoxide production as measured by chemilumniscence activity was very low. Upon stimulation with PMA, as has been well documented, a peak in chemilumniscence activity was observed that was maximal between 5 and 10 minutes after adding the stimulus (data not shown). Incubation of the PMN of eight different healthy donors with VT-2 as stimulus did not show any difference with the control situation whereas LPS induced a small increase in superoxide production as described previously (110) (data not shown). PMN, previously incubated with VT-2 0.1 nM, VT-2 10 nM, LPS 1 μM alone, or LPS 1 μM in combination with VT-2 0.1 and 10 nM for 10 min, 1 h, and 4h respectively showed no significant difference in superoxide production as compared with control PMN. No significant differences in superoxide production was observed in response to PMA in cells previously incubated with LPS 1 μM alone, or LPS 1 μM in combination with VT-2 0.1 and 10 nM, compared to control situation (data not shown).

***Activation of PMN***

*Degranulation markers:* Incubation of PMN with different concentrations VT-2 did not cause any changes in the expression of CD66b on PMN (Figure 2A). However, a significant increase in the expression of CD66b was found when PMN were incubated with LPS (1 μg/mL).

**Figure 2A.** Expression of CD66b, a degranulation marker on the surface of PMN.




LPS caused an upregulation of the expression of CD66b whereas VT-2 did not have any effect. $P < 0.05$ is considered to be significant (*).

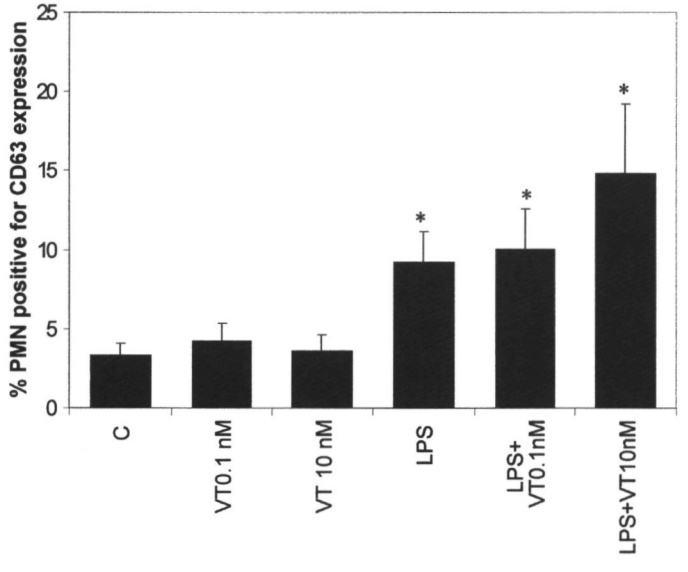



**Figure 2B.** Expression of CD63 another degranulation marker on the surface of PMN. LPS caused an increase of CD63 expression although this increase was less extensive compared to that of CD66b. Again VT-2 did not influence this expression. $P < 0.05$ is considered to be significant (*).

Similar results were obtained by incubation with LPS + VT0.1 nM or LPS + VT 10 nM. In addition, the expression of CD63 increased significantly after the incubation with LPS alone (Figure 2B). Incubation of PMN with LPS and VT together showed a similar pattern to the incubation with LPS alone. Again, VT itself had no effect.

*Adhesion-molecules:* The expression of CD11b/CD18 complex and L-selectin on PMN were studied with FACS analysis to evaluate whether VT, LPS or both could induce changes in expression of these adhesion molecules. Figure 4 shows the results of these experiments. As described previously, LPS (1 μg/mL) induced the expression of CD11b/CD18 complex (Figure 4A) and at the same time reduced the expression of L-selectin compared to non-stimulated PMN (Figure 4B). VT was not able to induce any significant changes in this expression. Incubation of PMN with LPS and VT together showed a similar expression pattern compared to incubation with LPS alone. Apparently, VT had no additional effect.

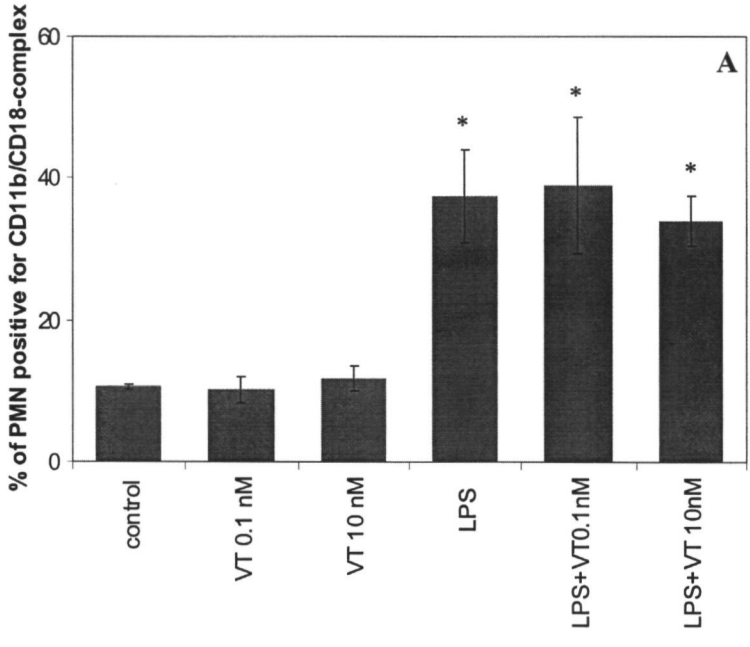



**Figure 4A** Expression of CD11b/CD18 complex and L-selectin. CD11b/CD18 complex is upregulated after incubation with LPS. VT did not influence this expression.

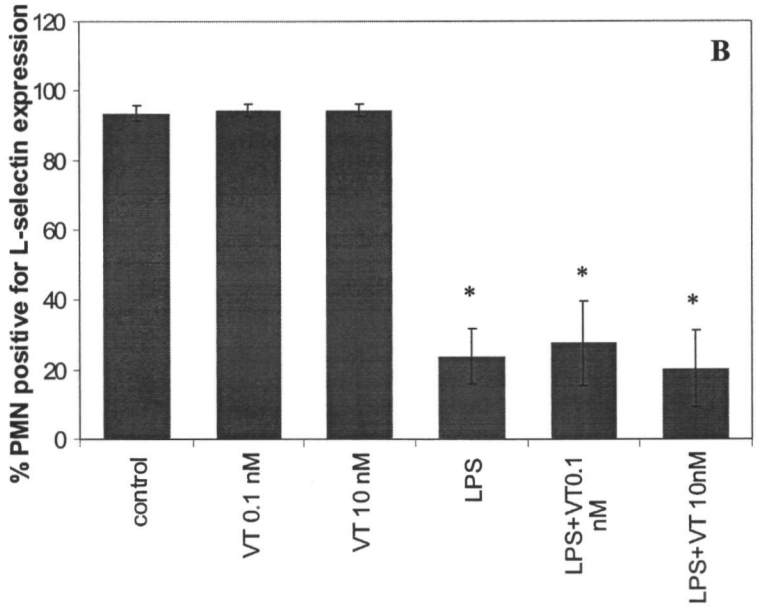



**Figure 4B** L-selectin expression decreased after incubation with LPS (Figure 4B) and again VT-2 had no effect. $P < 0.05$ is considered to be significant (*).

**Discussion**

Recent studies indicate that PMN play a critical role in the pathogenesis of HUS by transporting VT from the intestine to target organs (113,114). In this study we investigated whether LPS is also bound to PMN in D+ HUS patients and determined the role of VT and LPS in activation of PMN. All D+ HUS patients investigated were positive for the binding of VT to PMN in the acute phase of the disease.

The presence of LPS has been demonstrated before only in patients with HUS due to shigellosis using the Limulus assay (141). The presence of LPS in the circulation of HUS patients due to VTEC infection could not be detected using the same assay (142). The presence of LPS bound to CD14 positive cells like PMN and monocytes might explain the negative result obtained with this assay. The results of this study show that LPS was bound to PMN in seven out of nine D+ HUS patients with proven VTEC infection. This is the first time, as far as we know, that LPS in the circulation of D+ HUS patients has been found. Two patients in our

study were negative for the presence of LPS bound to PMN although they were both positive for the presence of serum antibodies against *E. coli* O157.

An explanation for this may be the transfer of LPS to other cells and plasma components or internalization of LPS into PMN (143). Interestingly, only five of the 17 household members investigated were positive for the presence of LPS bound to PMN. Two of the household members positive for the presence of LPS had less than 5% positive cells and in a third household member 10 % of PMN were positive for LPS binding. The other two household members (of patient 8 respectively) had higher amounts of LPS bound to PMN (25 and 35%, respectively). However, no antibodies against *E. coli* O157 were found suggesting that although LPS was present bound to PMN, the amount of LPS present in the circulation was not enough to induce an immunological response by producing antibodies. In three household members, all three children, antibodies against *E. coli* O157 were found. Apparently, enough LPS had entered the circulation to induce an immunological response although the presence of LPS bound to PMN could not be demonstrated in these three children. In addition, these three children did not develop HUS suggesting that not only the amount of LPS attached to the PMN but probably also the amount of VT bound to the PMN determines what is happening.

It has been suggested several times that PMN play a pivotal role in the pathogenesis of D+ HUS. The number of PMN on admission is a predictive factor for the outcome of the disease (11). In addition, ultrastructural studies on PMN of D+ HUS patients revealed a reduction of granules in some patients. Furthermore, elevated levels of α1-antytrypsin complexed elastase are found at presentation indicating activation of PMN (79,80). The mechanism leading to the activation of PMN in HUS is not fully understood. The finding of both VT and LPS bound to PMN in D+ HUS patients may be an explanation. For this reason we investigated whether VT or LPS or maybe both are capable to activate PMN *in vitro*.

Interestingly, only LPS was able to induce the expression of markers for activation on PMN namely CD66b and CD63, whereas VT had no effect. Furthermore, LPS reduced the expression of L-selectin and at the same time caused an increase of the CD11/CD18-complex (= anti-ICAM). Again, VT did not influence the expression levels of either one. Similar expression patterns compared to LPS alone were observed when PMN were incubated with LPS and VT together. Apparently, VT had no additional effect. In addition, LPS caused a slight increase in superoxide production whereas VT had no effect (data not shown). These data seem to be in contrast with a report of King et al. who demonstrated that VT could induce superoxide production in PMN but did not find any changes in the expression of CD11b/CD18 as a consequence of incubation with VT or LPS (110). However, the induction of CD11b/CD18 on the membrane of PMN after incubation with LPS has been described several times before

(144) Furthermore, it has been reported that LPS can induce the expression of CD66b and reduces the expression of L-selection (145) as also observed in our study The finding that VT alone did not lead to up-regulation of adhesion molecules or induced superoxide production suggests that the present of LPS is necessary to activate PMN and not VT Only very small amounts of LPS can already lead to activation of PMN Therefore, the superoxide production induced by Stx1 in the assay of King et al might be caused by the presence of very small amounts of LPS although they had pretreated toxin to remove LPS (110)

Our study demonstrates that the presence of LPS in the circulation is probably an essential factor in the development of HUS Only five household members (= 29 %) of the 17 investigated were positive for the presence of LPS bound to PMN whereas 7 out of 9 (= 78 %) HUS patients had LPS bound to PMN In addition, the finding of serum IgM -antibodies against LPS in serum of D+ HUS patients and only in a few cases of non-symptomatic VTEC infected patients suggests that there might be a difference in amount or distribution of LPS in the circulation

After entering the circulation, approximately 80-90% of LPS will interact with lipoproteins in plasma leading to inactivation of LPS Only a minor part of the LPS is cell-associated (152) However, large amounts of LPS in the circulation will lead to relative more LPS bound to CD14 positive cells (130) Monocytes and PMN are the two main targets for LPS in the circulation In this study we investigated whether LPS could be detected bound to PMN but it has to be considered that LPS can also be present bound to monocytes in the circulation Previous studies have shown that monocytes stimulated with LPS can bind VT leading to the production of inflammatory mediators The detection of LPS bound to PMN in D+ HUS patients indicates that LPS plays a role in HUS and opens therefore possibilities for treatment of patients Treatment with recombinant bactericidal/permeability-increasing proteins might be such a possibility In a randomized, double blind trial in children with severe meningococcal sepsis it has been suggested that this recombinant is beneficial by decreasing the amount of complication (154) However, more research is necessary before this can be indeed used in patients with HUS

In conclusion, this study demonstrates for the first time, to our knowledge, the presence of LPS bound to PMN in the circulation of D+ HUS patients Furthermore, *in vitro* experiments have shown that not VT but LPS bound to PMN is necessary to activate these cells Previous studies have shown that not only the presence of VT determines whether someone develops HUS or not Although this study does not provide direct evidence for the role of LPS, we suggest that the entry of high amounts of VT together with LPS has a decisive role for the development of full-blown HUS

# VEROCYTOTOXIN-1 INFLUENCES BASAL NITRIC OXIDE PRODUCTION OF HUMAN GLOMERULAR ENDOTHELIAL AND MESANGIAL CELLS BUT HAS NO EFFECT ON BASAL ENDOTHELIN-1 SYNTHESIS

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***Submitted***

**ABSTRACT**

Acute renal failure hallmarks the pathogenesis of hemolytic uremic syndrome (D+HUS). Endothelial damage of glomeruli and/or arterioles of the kidney play a crucial role in the pathogenesis of HUS. Endothelial damage will lead to a disturbance of factors, like endothelin (ET-1) and nitric oxide (NO) that normally are secreted to maintain vascular tone and an antithrombotic surface. It has been suggested that mesangial cells also play a role in the pathogenesis of HUS. Mesangial cells also produce NO but they do not make ET-1.

In this study, we investigated the influence of VT-1 on nitric oxide production of mesangial cells. Furthermore, the influence of VT-1 on NO and ET-1 synthesis of human glomerular microvascular endothelial cells (GMVEC) was evaluated.

NO production of mesangial cells was measured using the classical Griess-method (detection limit 3 μmol/L) whereas NO production of endothelial cells was measured using a highly sensitive technique (detection limit 30 nmol/L). Indirect immunofluorescence, Western-blot analysis and L-citrulline assay were used to confirm the results found by these methods. The effects of VT-1 on ET-1 production of GMVEC were studied using a radio- immunoassay for ET detection. In addition, prepro-ET-1 transcript levels were analyzed using reverse polymerase chain reaction (r-PCR).

NO synthesis by human mesangial cells is in the micromolar range and that of GMVEC in the picomolar range. VT-1 reduced NO production of non-stimulated and TNFα-pretreated human mesangial cells and to a lesser extent of non-stimulated GMVEC. A maximal reduction of NO production of human mesangial cells was found after an incubation of 10 fmol/L VT-1 whereas a concentration of five nmol/L VT-1 was necessary to find a significant reduction of NO production by GMVEC. Interestingly, NO synthesis of GMVEC previously stimulated with TNFα decreased as did the expression of endothelial NOS (eNOS). Pretreated GMVEC incubated with VT-1 had a NO synthesis that was comparable to controls. Western blot analysis and L-citrulline assay of human mesangial cell showed that VT-1 decreased the expression of inducible NOS (iNOS) in a dose-dependent manner, confirming the results found by the Griess-method. No effects of VT-1 on ET-1 synthesis were observed in non-stimulated GMVEC. In addition, no changes were observed on prepre-ET-1 transcript levels. However, TNF pretreated GMVEC showed a dose-depended reduction in ET-1 synthesis after incubation with VT-1.

Although we are aware of the limitations of our results found *in vitro*, we postulate that the reduction of NO synthesis caused by VT-1 might play an important role in the acute renal failure and thrombotic microangiopathy seen in patients with D+ HUS.

The hemolytic uremic syndrome (HUS) is the most common cause of acute renal failure in children (2,155) Endothelial damage of glomeruli and/or arterioles of the kidney hallmark the pathogenesis of HUS Histopathological studies of the kidney of HUS patients revealed swollen and detached endothelial cells and fibrin deposits in the glomerulus (3,82) The epidemic form of HUS, in most cases characterized by a prodromal phase of (bloody) diarrhea, is strongly associated with an infection by a verocytotoxin (also termed Shiga-like toxin) producing *Escherichiae coli* (*E coli*), especially serotype O157 H7 (10) *In vitro* studies performed with human umbilical vein endothelial cells and glomerular endothelial cells (GMVEC) have shown that verocytotoxin (VT) can bind to globotriaosylceramide (Gb3) found on the surface of these cells after stimulation with inflammatory mediators (55,56) After binding, verocytotoxin is internalized The active part of the toxin undergoes partial proteolysis and disulfide bond reduction in the target cell, generating an active intracellular enzyme This enzyme can cause inhibition of overall protein synthesis through the enzymatic inactivation of the elongation factor dependent binding of aminoacyl transfer RNA molecules to the 60S ribosomal unit leading to cell death (60,61) The binding of VT to human mesangial cells has also been described and leads to inhibition of mitogenesis and protein synthesis without affecting cell viability (86) It is still unknown whether VT-1 affects endothelin and /or nitric oxide (NO) production in the human kidney

Endothelin is an important vasoconstrictor Three different isoforms of endothelin have been described, endothelin-1, 2 and 3 Endothelin-1 (ET-1) is the only one produced by endothelial cells (154) Endothelial cells are able to synthesize the pro-hormone big-endothelin and express endothelin-converting enzymes to generate ET-1 This conversion is necessary to obtain full vascular activity (155) ET-1 functions primarily in an autocrine or paracrine way as also nitric oxide (NO) functions NO was for the first time described as an endothelium derived relaxing factor (156) that caused an acetylcholine-dependent relaxation of vascular smooth muscle cells Further investigations showed that EDRF-mediated vascular relaxation was associated with increased levels of cyclic guanosine monophosphate, resembling the action of nitrovasodilators It is now generally accepted that the substance previously called EDRF is nitric oxide (NO) Elevated levels of NO can cause vasodilatation, but also inhibit platelet aggregation and adhesion, inhibits activation and adhesion of leukocytes and inhibits proliferation of the underlying vascular smooth muscle (157-159) Besides this, NO can react with oxide and form peroxynitrite, which in low concentrations will have the same effect as NO

but in high concentrations will be cytotoxic. NO is formed by the enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthases (NOS). At least three different isoforms of NOS have been identified so far (160,161), namely neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3).

The purpose of this study was to investigate whether VT-1 affects NO and ET-1 production by glomerular endothelial cells. Furthermore, the effects of VT-1 on NO production by human mesangial cells was evaluated knowing that NO counteracts vasoconstriction, platelet aggregation and the interaction of leukocytes with the vessel wall (162). We used an improved technique for the detection of NO synthesis of human GMVEC and demonstrate that VT-1 reduces NO production of but fails to modify ET-1 synthesis.

## MATERIAL & METHODS

Purified VT-1 was kindly donated by Dr. MA Karmali (Center for Vaccine Development, University of Maryland School of Medicine, USA). The endotoxin content of VT-1 preparation was less than 0.05 EU/ml by Limulus amebocyte lysate assay. EBM-medium containing hEGF, bovine brain extract, hydrocortisone, gentamicin sulfate and amphotericin B were purchased from Bio Whittaker (Walkersville, MD). Radioimmunoassay kit for quantitative determination of endothelin levels was from Nichols Institute Diagnostics (Wijchen, The Netherlands). 6-keto-prostaglandin F1$\alpha$ [$^{125}$I] was purchased from Amersham (Buckinghamshire, England). Sulfanic acid, N (1-naphthyl)-ethylenediamine, sodium nitrate, aprotinin from bovine lung, Nonidet P-40, sodium orthovanadate, aminoguanidine were purchased from Sigma (St. Louis, MO). $N^G$-monomethyl-L-arginine (L-NMMA) was from Calbiochem (La Jolla, CA). Nitrate reductase from *Aspergillus spp.*, Flavin-adenin-dinucleotid (FAD), L-lactate dehydrogenase (L-LDH) from rabbit muscle and nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Roche Diagnostics, (The Netherlands). Polyclonal antibodies against iNOS (NOS2) and eNOS (NOS3) were purchased from Santa Cruz Biotechnology (CA). A monoclonal antibody against iNOS was obtained from Transduction Laboratories (Lexington). Streptavidin conjugated with FITC was from Dako (Denmark). Nitrite/Nitrate fluorimetric assay kit was obtained from Cayman Chemicals (Alexis Corporation, Switzerland). ECL Western blotting analysis system was purchased from Amersham, England. L-Citrulline assay was performed using the NOSdetect™ assay kit of the Alexis corporation (Switzerland). All other reagents used were of analytic grade or described previously (55,56).

***Human mesangial cells***

Human mesangial cells were cultured in complete medium as described previously (86). Briefly, glomeruli from the cortex of normal human kidney tissue were isolated by gradual sieving procedure. Glomeruli were plated on gelatin-coated wells and attached in a few days. Outgrowth of predominantly epithelial cells and a limited number of endothelial cells were noted. Within 10 to 30 days after the glomeruli were seeded, outgrowth of mesangial cells was observed. Mesangial cells were selectively collected by scrapping them of the tissue culture plate using the tip of a plastic pipette and then expanded on gelatin-coated wells. To purify the mesangial cell populations further, an immunomagnetic separation technique was used (86). The cultured mesangial cells showed no immunoreactivity to anti-PECAM-1 antibody or the anti-cytokeratin 8 antibody excluding the presence of endothelial or epithelial cells. Cultured human mesangial cells were used between passages 5 and 10.

***Human glomerular microvascular endothelial cells***

The isolation and purification of glomerular microvascular endothelial cells (GMVEC) was performed as described previously (56). Cells were cultured in complete medium (containing M199, 10% human serum, 10% newborn calf serum, 100 IU/ mL penicillin/0.1 mg/mL streptomycin, 2 mmol/L L-glutamine, 5 U/mL heparin and 150 μg/mL crude preparation of endothelial cell growth factor). The purity of GMVEC cultures was assessed using morphological and immunological criteria. Indirect immunofluorescence revealed the presence of von Willebrand factor and other endothelial cell specific antigens (56). No positive staining was observed with anticytokeratin 8 or the α-smooth-muscle actin monoclonal antibodies excluding contamination of the GMVEC with epithelial- and mesangial cells, respectively. GMVEC were used between passages 6 and 10.

***Indirect immunofluorescence studies of eNOS and iNOS***

Indirect immunofluorescence studies were performed to demonstrate the presence of eNOS in GMVEC and iNOS in human mesangial cells. Cells were cultured on glass cover slips until they had reached confluence. GMVEC and mesangial cells were incubated with VT-1, TNFα or both, and compared to cells that were cultured in medium. Cells were fixed using 80% acetone for ten minutes at room temperature. Subsequently, cells were washed three times with phosphate buffer saline (PBS) and incubated in PBS containing 10% fetal calf serum for twenty minutes to suppress non-specific binding of IgG. GMVEC and mesangial cells were washed again three times with PBS followed by incubation for 60 minutes with a polyclonal antibody against eNOS and iNOS (1:1000), respectively. Subsequently, cells were washed and incubated

for 45 minutes with biotin conjugated anti-goat IgG antibody, followed by incubation with streptavidin conjugated with FITC. Cells were washed extensively with PBS and the presence of eNOS or iNOS was studied using a Zeiss microscope (Aksioscope) with standard equipment. As control α-actin was used, a housekeeping protein to exclude the possibility that effects observed was due to protein synthesis inhibition.

***Western blot analysis for eNOS and iNOS***

GMVEC and human mesangial cells were cultured in six-well plates. Five days after reaching confluence, non-stimulated and TNFα (10ng/mL)-prestimulated cells were incubated with various concentrations of VT-1 for 24 hours. After the incubation, cells were washed with PBS. Subsequently, RIPA-buffer, containing PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/mL PMSF, aprotinin and 100mM sodium orthovanadate, was added to the cells and the cells were detached from the cell culture plates using a scraper. Cells were passed through a 21-gauge needle and transferred to microcentrifuge tubes. 10 µl of 10 mg/mL PMSF stock solution was added to the lysate followed by incubation for 60 minutes on ice. Subsequently, the cell lysates were centrifuged at 15000-x *g* for 20 minutes at 4° C. The supernatant fluid was used to perform Western blot analysis. Equal amounts of lysate proteins were separated by 5% SDS-PAGE and subsequently transferred to a nitrocellulose membrane. The blots were incubated with either a polyclonal antibody against eNOS or a monoclonal antibody against iNOS and washed afterwards. Immunoreactive bands were visualized using enhanced chemilumniscence. The amount of actin presence in the extracts was determined with α-actin to exclude the possibility that changes in eNOS or iNOS expression was a consequence of changes in protein synthesis activity.

To quantify the relative amount of eNOS antigen, the spots were scanned and the product of intensity and surface area was determined. Serial dilutions of an eNOS-containing preparation were used to make a calibration curve. Only spots from the same blot were compared.

***Measuring NO production by human mesangial cells***

NO production of human mesangial cells was measured using the Griess method (163). Mesangial cells were cultured in 24-wells to perform experiments. Complete medium, in which cells were cultured until they reached confluence, was replaced by EBM-medium, lacking phenol red and fetal bovine serum. Non-stimulated and TNFα-pretreated (24 h; 10 ng/mL) mesangial cells were incubated with VT-1 in concentrations ranging between 10 fmol/L till 10

nmol/L for 24 hours at 37°C. Subsequently, culture medium (total 250 µL) was collected and incubated with nitrate reductase from *Aspergillus spp.* in combination with NADPH and FAD for 30 minutes at 37°C to convert nitrate to nitrite. By adding 2 µL lactate dehydrogenase (final concentration 10 µg/reaction) and 8 µL of pyruvate dehydrogenase (final concentration 10 mmol/L) the reaction was stopped. Subsequently, 100 µL of the samples and of standard mixtures of nitrate and nitrite were mixed with an equal volume of Griess-reagents (0.01% sulfanilamide, 0.01% N- (1-naphthyl) ethylenediamine, 2.5% phosphoric acid) (163). Absorbency was measured in an ELISA plate reader at 540 nm and final NO-concentration was determined from standard nitrite/nitrate curves. Aminoguanidine (AMG), an irreversible inhibitor of iNOS, and L-NMMA were used to determine specificity of NO-measurements by the Griess-method.

***L-citrulline assay***

The enzymatic conversion of L-arginine to L-citrulline in cultured human mesangial cells was determined using a commercial NOSdetect™ assay kit. Briefly, human mesangial cells were cultured on six-well gelatin-coated plates. Mesangial cells were exposed either to VT-1 (0.1 nmol/L) or to TNFα (10 ng/mL) alone or previously stimulated with TNFα followed by incubation with VT-1 (0.1 nmol/L) for 24 hours at 37°C. Subsequently, cells were washed with PBS and harvested with PBS/ 1 mmol/L EDTA followed by centrifugation in a microcentrifuge at full speed for 2 minutes. Supernatant of the cells was removed and the pellet was resuspended in homogenization buffer (250mmol/L Tris-HCl, pH 7.4, 10 mmol/L EDTA, 10 mmol/L ethyleneglycol-bis (β-aminoethylether)-N, N, N', N'-tetra-acetic acid (EGTA)) and centrifuged again for 5 minutes at full speed in a microcentrifuge. The supernatant was separated from the homogenate and the resulting protein sample was adjusted to a concentration of 5 µg/µL. Subsequently, 10 µL of each sample was incubated with a reaction mixture (50 mmol/L Tris-HCl, pH 7.4, 6 µmol/L tetrahydrobiopterin, 2 µmol/L FAD,
10 mol/L NADPH, arginine (1 µCi/µL, $H_2O$) for 10 minutes on room temperature. The reaction was stopped by adding 50 mmol/L N-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES), pH 5.5 and 5 mmol/L EDTA. One hundred µL of resin was added to each sample and the samples were transferred to spin cups. Spin cups were placed into cup holders and centrifuged at full speed for 30 seconds. The eluate was transferred to scintillation vials and radioactivity was measured. As a positive control for the reaction rat cerebellum extract was used.

***Measuring NO production by GMVEC***

NO production by GMVEC was determined by measuring the $NO_2^-$ using a highly sensitive nitrate/nitrite fluorimetric assay kit (Caymann). Briefly, cells were cultured in 24-wells using complete medium. Five days after reaching confluence cells were used for experiments and complete medium was replaced by EBM medium lacking fetal bovine serum and phenol red to exclude the possibility of interfering in the measurement of NO production. Non-stimulated and TNFα-pretreated (24 h; 10 ng/mL) GMVEC were exposed to VT-1 in a concentration of one pmol/L - 5 nmol/L for 24 hours. An improved technique, a nitrate/nitrite fluorimetric assay, kit was used to detect NO production. In this assay nitrate is first converted to nitrite by nitrate reductase as also in the Griess-method (163). However, this assay differs from the Griess method in that 2,3-diaminonaphtalene is added followed by incubation with NaOH, which converts nitrite into a fluorescent compound (1(H)-naphthotriazole). Measurement of this component at an excitation wavelength of 365 nm and an emission of 450 nm in a fluorometer was used to determine $NO_2$ formation in culture medium. Using this assay $NO_2$ concentrations as low as 30 nmol/L could be detected which is not possible using the conventional Griess method. To substantiate that NO-measurements were specific, similar experiments were performed using L-NMMA, a reversible inhibitor of the NO production.

***Determination of Endothelin-1 production***

Non-stimulated and TNFα-pretreated (24 h: 10 ng/mL) GMVEC were exposed to VT-1 in a concentration of 0.1 nmol/L - 10 nmol/L for 24 hours or to PMNs (1 $x10^6$ cells) loaded with VT 10 nmol/L and PMNs (1 $x10^6$ cells) alone. After exposure, supernatant was removed and used to determine the amount of endothelin-1 produced by the cells. Samples were acidified and extracted on C18 columns (Nichols Institute Diagnostics) for endothelin-1 determination. After extraction, samples were dried using nitrogen gas at 37°C. After 45 minutes, 0.5 mL 100% ethanol was added, and drying was continued until the smell of acetic acid had disappeared. Subsequently, extracts were resuspended in 0.5 mL assay buffer (lyophilized borate buffer, pH 8.4). Subsequently, each sample was incubated in duplicate with 100 µL $^{125}$I-Endothelin and 100 µL anti-endothelin for 18 h at 4°C. After the incubation period, anti-rabbit (donkey) coated cellulose was added and mixed thoroughly. After 30 minutes, 1 mL deionized water was added and samples were centrifuged for 15 minutes at 2000-x g. Supernatant was removed and radioactivity was determined in a gamma scintillation counter for each sample. Endothelin standard curves were prepared at the same time of the assay and made it possible to determine the exact amount of endothelin present in each sample. Interassay variation of this assay is 6.8%.

***Quantification of mRNA levels of prepro-Endothelin-1***

GMVEC were cultured in six well plates until they reached confluence. Non-stimulated and TNFα-pretreated GMVEC were exposed to VT-1 in a concentration of 0.1 nmol/L - 5 nmol/L for 24 hours. Subsequently, total RNA was extracted after the incubation period followed by reverse transcriptase reaction (RT) to form cDNA. RT reaction was performed with 200 U Superscript II reverse transcriptase and random hexamer primers for 1 h at 42° C. Amplification of cDNA was performed by polymerase chain reaction (PCR) with specific primers for preproEndothelin-1 (155).

Forward primer preproEndothelin-1 TCTACTTCTGCCACCTGGACAT and reverse primer CTCGGTTGTGGGTCACATAA (Genebank accession no Y00749). The PCR program consisted of 38 cycli of 60 seconds 95°C, 61°C and 72°C. The cycles were preceded by an initial denaturation step at 95° C and were followed by a final extension at 72°C. The PCR products were visualized by ultraviolet light on a 1.5% (w/v) agarose gel electrophoresis and ethidium bromide staining. The quantity for mRNA spotted for each sample was normalized using the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT).

***Inhibition of protein synthesis***

To investigate whether changes in NO and ET-1 generation were a consequence of inhibition of overall protein synthesis, protein synthesis of GMVEC and mesangial cells was determined by assaying the incorporation of $^{3}$H-leucine in newly synthesized proteins as described previously (56).

***Statistics***

All data represented are expressed as mean plus SEM. Significance of increase or decrease of NO production compared to controls was analyzed using Wilcoxen signed rank test. The statistical level of significance was defined as $P < 0.05$.

## Results

***eNOS and iNOS in endothelial and mesangial cells***

To investigate the presence of eNOS and iNOS in GMVEC and human mesangial cells immunofluorescence studies were performed. Expression of eNOS antigen was consistently found in GMVEC (Figure 1A). There was no cross-reactivity with the antibody against iNOS. Expression of iNOS antigen was detectable in mesangial cells (Figure 1B). It was present in

non-stimulated cells (Fig. 1B) and increased after stimulation with TNFα (see below). eNOS antigen was not detected in human mesangial cells.

***NO production by human mesangial cells***

Nitric oxide production of human mesangial cells was measured by the Griess method (163). The aminoguanidine- inhibitable NO production, reflecting iNOS-derived NO, was six μmol/L on the average (Figure 2A). In addition, Griess reactivity equivalent to five μmol/L NO was found, which could not be inhibited by aminoguanidine and may reflect a non-NO-related reactivity. After a 24 h incubation of the cells with various concentrations of VT-1, a significant reduction of NO production was observed in non-stimulated mesangial cells (Figure 2A). A similar reduction was obtained by aminoguanidine indicating that the VT-1 reduced iNOS-dependent NO production. A maximal inhibition of NO production was already obtained at a concentration of 10 fmol/L VT-1. When mesangial cells were pre-stimulated by TNFα (10 ng/ml), NO production increased (Figure 2C), which was completely due to an increase in aminoguanidine-inhibitable NO (iNOS).

Incubation of TNFα-pretreated cells with VT-1 caused a dose-dependent decrease of NO production, which was significant at 10 fmol/L and maximal at one pmol/L. The high sensitivity of iNOS-depedent NO production was unexpected, because the amount of VT needed to inhibit protein synthesis was several orders of magnitude higher, both under basal and TNFα-stimulated cells (Figure 2B,D). Similar results were obtained when the reversible inhibitor L-NMMA (7 μmol/L) was used instead of aminoguanidine. The results obtained by the Griess-method were confirmed by measuring the enzymatic conversion of L-arginine to L-citrulline. A decrease of 78% of iNOS activity was found after the addition of VT-1 (0.1 nmol/L) to non-stimulated mesangial cells, while in TNFα-treated mesangial cells the reduction was even more than 95%.

**Figure 1** Indirect immunofluorescence of eNOS in GMVEC (A) and iNOS in human mesangial cells (B). No staining was observed when iNOS and eNOS were used as first antibody on endothelial cells and mesangial cells, respectively. Magnification used x 350.

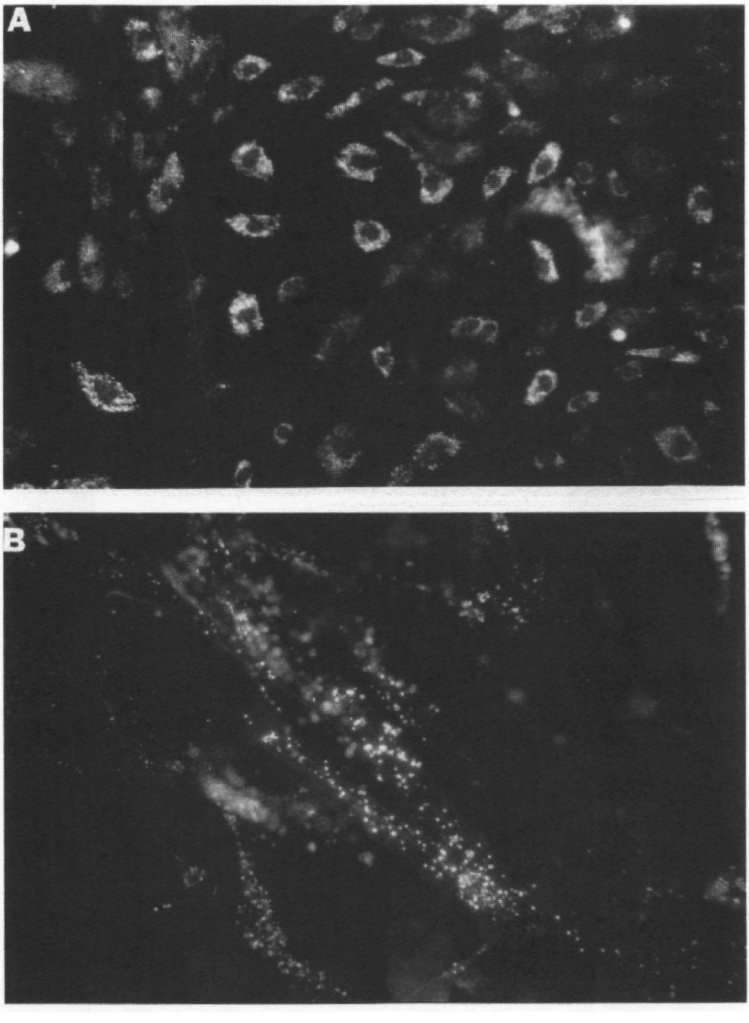
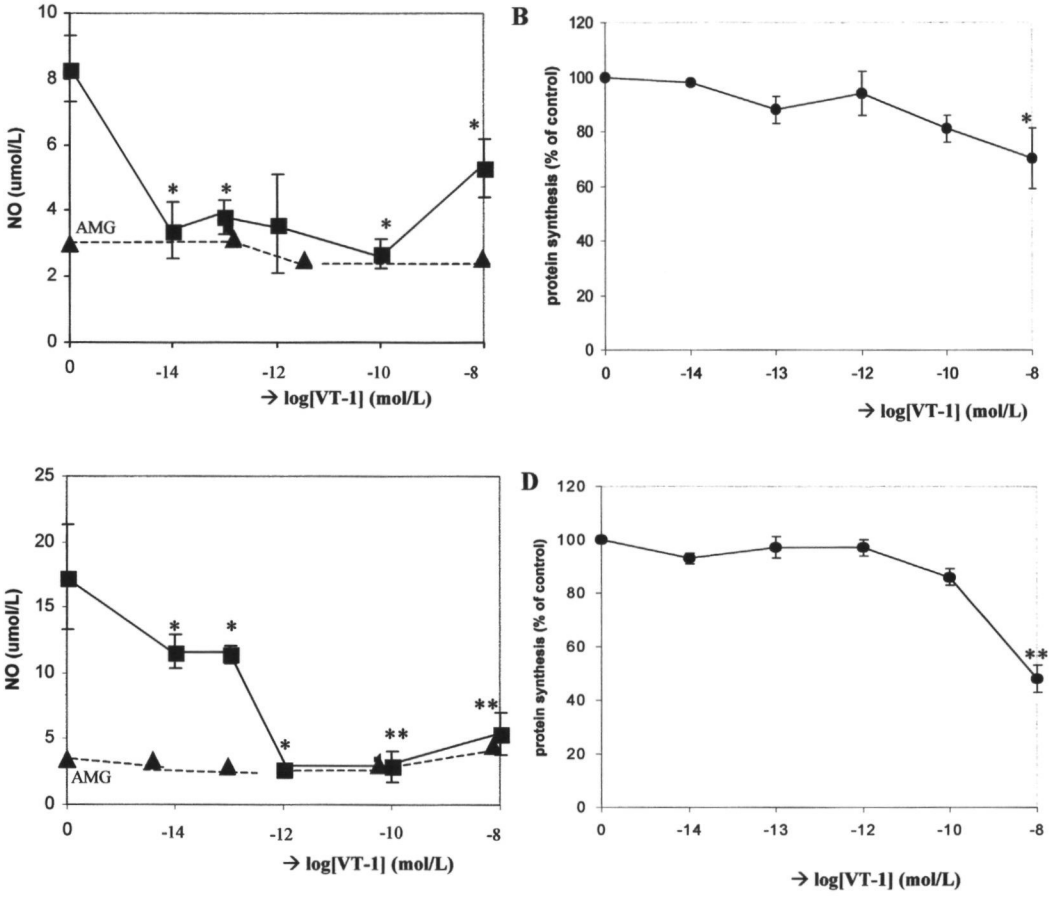


**Figure 2:** NO-production of human mesangial cells of four different donors. Non-stimulated mesangial cells were incubated with VT-1 in different concentrations ranging from 10 fmol/L to 10 nmol/L. A concentration VT-1 10 fmol/L was sufficient to induce maximal reduction of NO production and was similar as the reduction seen in cells incubated with aminoguanidine (AMG). Cells incubated with VT-1 and aminoguanidine together showed a similar reduction as AMG or VT-1 alone (▲). Protein synthesis was not inhibited (B). C: Mesangial cells previously stimulated with TNFα 10 ng/mL showed an increased NO production compared to basal NO production. Incubation of pretreated mesangial cells with VT-1 showed a dose-dependent reduction of NO production (■) to a level obtained with aminoguanidine (▲). D: Protein synthesis of mesangial cells was not inhibited after an incubation with VT-1 in the range of 10 fmol- 1pmol/L. Statistical significance was calculated using unpaired Student's t-test. Values below 0.05 were regarded to be significant ( * $P<0.05$ and ** $P< 0.001$).



*Effect of VT-1 on iNOS antigen levels of human mesangial cells*

To investigate whether the effect of VT-1 on iNOS activity was due to a reduced expression of iNOS protein or inactivation of iNOS, the cellular protein content was assayed. Western blot analysis of mesangial cells showed a characteristic band of 130 kD for iNOS. (Figure 3A). No reactivity with an antibody for eNOS was observed in the mesangial cell extracts (data not shown). The expression of iNOS antigen increased after stimulation with TNF but showed a strong reduction when mesangial cells were subsequently incubated with VT-1 (Figure 3B+C).

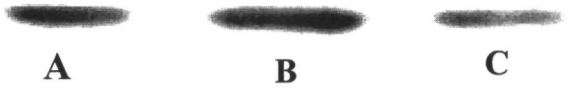



**Figure 3** western blot analyses of iNOS in mesangial cells. A. Basal iNOS expression in mesangial cells. B. TNFα (10ng/mL) caused an induction of iNOS. C. When TNFα pretreated cells were incubated with VT-1 (0.1 nmol/L) a strong reduced expression of iNOS was observed.

*NO production by human GMVEC*

Nitric oxide production by human GMVEC is in the picomolar to nanomolar range and was determined using a highly sensitive nitrite/nitrate fluorimetric assay kit (detection limit 30 nmol/L). Incubation of non-stimulated GMVEC with VT-1 for 24 hours caused a concentration-dependent decrease in NO production (Figure 4A). Protein synthesis was not inhibited after 24 h incubation of non-stimulated GMVEC with VT-1 at different concentrations (Figure 4B). In highly confluent GMVEC inhibition of protein synthesis by VT was only seen in TNFα-stimulated cells (Figure 4D), which is due to the fact that pre-incubation with TNFα increases the number of VT-receptors in GMVEC and enhances their sensitivity for VT-1 (Figure 4D)(43). While TNFα (10ng/mL) by itself reduced NO production over a 24 h period by 40% (Fig. 4A,C), incubation of TNFα-pretreated GMVEC with VT-1 increased NO production to the level of control cells (Figure 4C). This finding was consistently found in four different preparations of human mesangial cells obtained from five different donors. The restoration of NO production of TNFα-pretreated cells to control levels

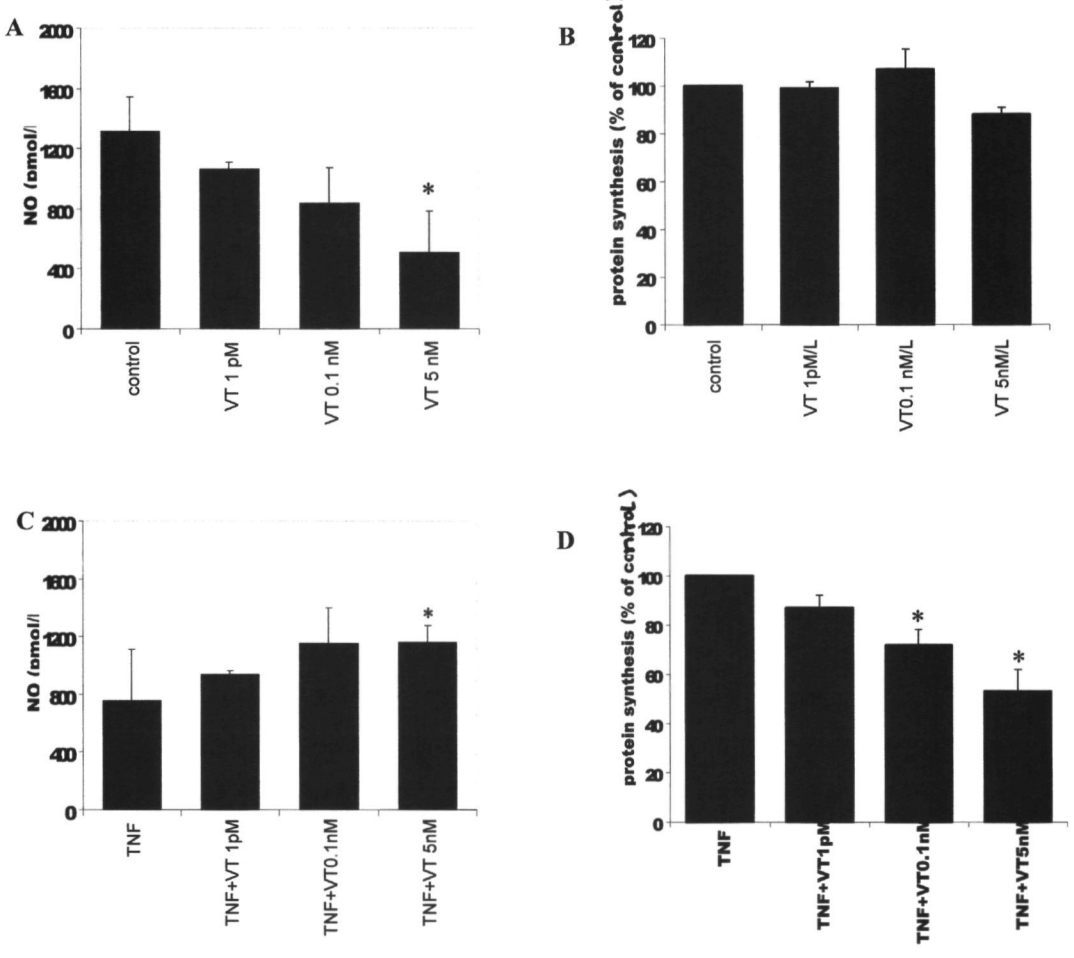



**Figure 4:** The influence of VT-1 on NO production of human GMVEC was measured in cells of five different donors. A. Non-stimulated GMVEC were incubated with VT-1. A significant decrease of NO production was observed when VT-1 was added at a concentration of 5 nmol/L, whereas the decrease in NO production caused by VT-1 0.1 nmol/L and 1 pmol/L was not significant (P= 0,06 and P= 0,05, respectively). B. No inhibition of protein synthesis was measured at the same time. C. TNFα-pretreated GMVEC showed a decrease in NO production. However, TNFα pretreated GMVEC incubated with VT-1 showed an increase of NO-production to control levels of non-stimulated cells. D. At the same time, VT-1 caused a dose-dependent inhibition of protein synthesis. * $P < 0.05$

by the subsequent incubation with VT-1 occurred despite the fact that the protein synthesis was reduced (Fig. 4C,D). The presence of L-NMMA (0.7 μmol/L) in the culture medium

caused a reduction of 30 % ± 6% (= SEM) in NO production in non-stimulated GMVEC and 38%± 10% (= SEM) in TNFα-pretreated GMVEC indicating that NO production was indeed eNOS-dependent.

***Effect of VT-1 on eNOS levels of human GMVEC***

Western blot analysis performed with human GMVEC showed a specific band of 130 kD for eNOS (Figure 5A). No band was detected when GMVEC were incubated with a monoclonal antibody against iNOS. The expression of eNOS was not visibly affected after incubation with VT-1 (Figure 5B). Incubation with TNFα (10 ng/mL) for 24 hours caused a reduced expression of eNOS of about 50% (Figure 5C). Subsequently, if TNFα pretreated cells were incubated with VT-1 expression of eNOS was similar to control cells (Figure 5D).

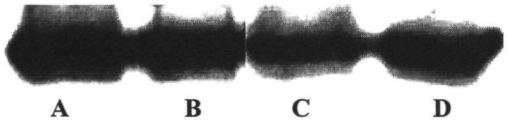



**Figure 5** Western blot analysis of eNOS in human GMVEC of a representative donor.
A. Basal expression of eNOS in GMVEC. B. Non-stimulated GMVEC incubated with VT-1 (5 nmol/L). C. TNFα-pretreated GMVEC. D. TNFα- pretreated GMVEC incubated with VT-1 (5 nmol/L).

***ET-1 production of human GMVEC***

Incubation of non-stimulated GMVEC with VT-1 for 24 hours had no effect on ET-1 production by non-stimulated human GMVEC (Figure 6A). However, in TNFα-pretreated GMVEC a dose-dependent inhibition of ET-1 synthesis caused by VT-1 was observed (Figure 6B). Quantification of mRNA levels of prepro-Endothelin-1, the precursor of ET-1, showed no significant changes in mRNA levels in non-stimulated GMVEC because of VT-1 incubation (data not shown). VT-1 5 nM caused a slight reduction in mRNA expression of prepro-ET-1 in TNFα-prestimulated GMVEC (data not shown). Therefore, it is likely that the reduction of ET-1 synthesis observed is a consequence of protein synthesis inhibition.

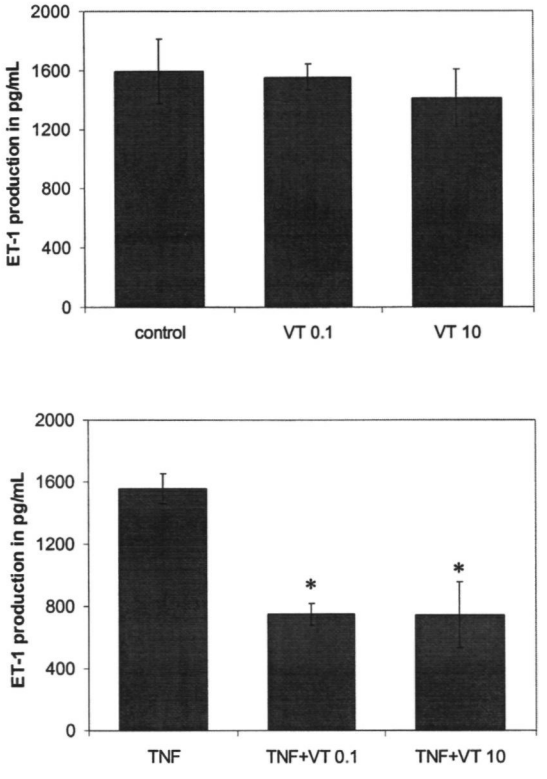



**Figure 6.** The influence of VT-1 on endothelin-1 production by non-stimulated GMVEC (A) and TNFα pretreated GMVEC (B). VT-1 had no effect on ET-1 production of non-stimulated cells. However, VT-1 reduced ET-1 synthesis significantly in TNFα pretreated cells.

**DISCUSSION**

In this study, we determined the effect of VT-1 on NO and ET-1 production by GMVEC and determined the role of VT-1 on NO production by human mesangial cells. The major finding was the ability of VT-1 to reduce *in vitro* basal NO production of human mesangial cells and to a lesser extent of GMVEC. The reduction of NO production by VT-1 found in human mesangial cells is in the micromolar range and that of GMVEC, in the nanomolar range. Besides this, a concentration of 10 fmol/L was sufficient to cause maximal reduction of non-stimulated mesangial cells whereas only a concentration of 5 nmol/L caused a significant reduction in NO synthesis by human GMVEC. For this reason, we postulate that the decrease of NO production of human mesangial cells, caused by VT-1, has more impact than the

decrease observed in basal NO-formation of GMVEC. TNFα pretreated GMVEC showed an increase of NO production after incubation with VT-1 in different concentrations despite an inhibition of protein synthesis. VT-1 caused in TNFα pretreated mesangial cells a decrease in NO synthesis comparable to the inhibition of aminoguanidine. VT-1 had no effect on ET-1 production or on prepre-ET-1 mRNA levels of non-stimulated GMVEC and caused a dose-dependent decrease of ET-1 production in TNFα- pretreated GMVEC.

Histopathological studies performed on autopsy material of the kidney of patients with D+HUS have shown that there is an increased influx of leukocytes and monocytes compared to controls (84). The reduction of NO synthesis as observed *in vitro* might contribute to increased presence of platelets and leukocytes as observed *in vivo* (162,16,165). Besides, mesangial cells are a major determinant in the regulation of glomerular filtration (90). The inhibition of NO synthesis by VT-1 might therefore be one of the factors contributing to the acute renal failure as seen in HUS.

The possibility that a VT-1 induced reduction of NO production by human mesangial is also partly dependent on inhibition of protein synthesis caused by VT cannot be completely excluded. However, protein synthesis of mesangial cells was not inhibited after incubation with VT-1 in the range of 10 fmol/L – 1 pmol/L, but at the same time a maximal reduction of NO production was found, comparable to the inhibition of aminoguanidine. This, in combination with the results found on non-stimulated GMVEC is suggestive for the conclusion that the influence of VT-1 on protein synthesis and NO formation are two at least partly independent processes.

Basal NO production of endothelial cells is normally in the range of picomolar or nanomolar and is therefore not detectable in medium originating from GMVEC culture using the Griess method. Therefore, a highly sensitive nitrite/nitrate fluorimetric assay, a 100-fold more sensitive than the Griess method, was used. Surprisingly, VT-1 caused a decrease of basal NO production in non-stimulated GMVEC without affecting protein synthesis suggesting that VT-1 can activate endothelial cells directly. TNFα- pretreated GMVEC also showed a decrease of NO synthesis. This is partly explained by a decrease in eNOS antigen (as demonstrated by Western blot analysis). An explanation might be that TNFα induces the synthesis of an enzyme that inhibits the production or capacity of eNOS and in this way reduces NO production. Interestingly, incubation of the cells with TNFα and VT-1 simultaneously caused a NO production that is comparable to control cells. Incubation with VT-1 can cause an inhibition of protein synthesis and might inhibit the production of this enzyme that leads to a normal capacity of eNOS and normal NO production again.

Incubation of TNFα pretreated GMVEC with VT-1 caused a dose-dependent inhibition in ET-1 production whereas VT-1 had no effect on ET-1 synthesis in non-stimulated cells. The finding that VT-1 failed to modify the amount of ET-1 produced in non-stimulated cells indicates that ET-1 produced by GMVEC probably not has a role in the development of renal failure observed in HUS. Previous investigation performed by Bitzan and colleagues showed that VT was able to induce the expression of preproET-1 mRNA in quiescent bovine aorta endothelial cells without having an effect on protein synthesis (166). Their results and the results we found on GMVEC implicate that activation of endothelial cells by VT, without the presence of inflammatory mediators, may have an important role in the pathogenesis of HUS. However, in our study no changes in the expression of prepro-ET-1 mRNA in non-stimulated GMVEC were found. Bitzan and colleagues also investigated the influence of verocytotoxin on nitric oxide production in bovine aorta endothelial cells but did not observe any significant changes (166). However, it cannot be excluded that bovine aorta endothelial cells react differently after exposure to VT as compared to GMVEC.

In patients with recurrent thrombotic microangiopathy, a disease with similar clinical features compared to HUS, it has been suggested that increased NO formation can be a mediator of injury of the endothelium (167). Serum of patients with thrombotic microangiopathy induced NO synthesis in cultured endothelial cells more than serum of healthy individuals. Moreover, the formation of large amounts of NO has been implicated in cell and organ damage as observed in patients with endotoxemic shock (168,169). On the other hand, NO measurements performed in the urine of a child with D+ HUS in course of the disease showed an increased excretion of NO metabolites where at the same time an improvement of renal function was observed (170). They suggested that NO production was decreased in the beginning of the disease and that therapy with NO analogs might have beneficial effects. A similar suggestion was made by Jaradat et al. (171). He proposed that L-arginine should be given to patients with the epidemic form of HUS and to patients with thrombotic thrombocytopenic purpera in high amounts through parental nutrition. L-arginine would generate high amounts of nitric oxide, leading to a decrease of platelet aggregation and an increase of vasodilatation. Based on our *in vitro* data we suggest that this might be a good approach in the treatment of patients with epidemic HUS although we believe that experiments with an animal model of HUS have to substantiate this hypothesis.

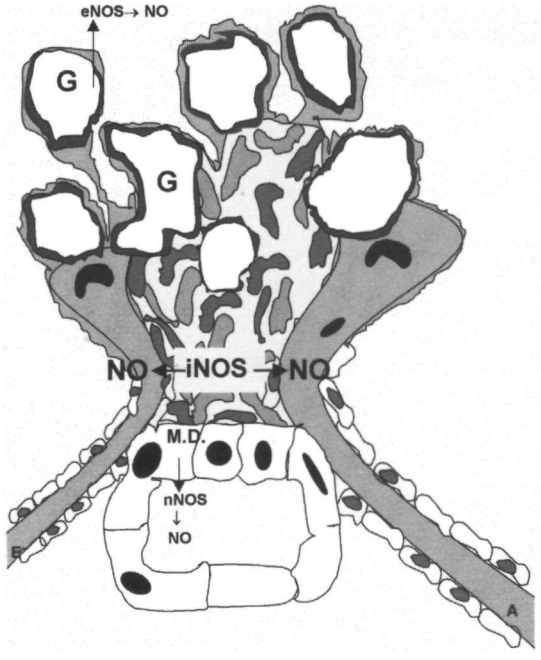



**Figure 6.** Schematic representation of the juxtaglomerular apparatus. A= afferent arteriole E=efferent arteriole MD= macula densa G= glomerulus. iNOS is expressed in mesangial cells. NO produced by these cells can directly act on the arterioles. Because of the presence of haem and oxide in the circulation NO produced in the mesangial cells will not reach completely the peripheral capillaries of the glomerulus. Local NO production by eNOS in de glomerular endothelial cells prevents adhesion of leukocytes and platelets. nNOS is formed in the macula densa and modulate the renin secretion in the juxtaglomerular apparatus. It may also effect the renal secretion (175).

Previous investigations performed by our group have shown that activated monocytes are able to produce inflammatory cytokines such as TNFα after binding VT-1 (76). TNFα can lead to upregulation of Gb3, the classical receptor of VT (55,56). Subsequently, VT can bind to this receptor followed by internalization of the toxin causing inhibition of protein synthesis and serious damage of the endothelium. By damaging the glomerular endothelium, circulating nucleotides and vasoconstrictors as well as VT may gain access to the mesangial cells and promote vasoconstriction of the afferent arterioles of the glomerulus (see Figure 7). Vasoconstriction will lead to increased presence of leukocytes and platelets and a decreased renal blood flow (175). Interestingly, the kidney is the only organ in the human body that contains mesangial cells and therefore may be the only organ that will show vasoconstriction because of VT action. This might partly explain why the kidney is the main organ involved in the pathogenesis of HUS. Besides this, it has been reported that the developing renal

vasculature responds to stimulation and inhibition of NO synthesis in a different way from the adult renal vasculature (173). Solhaug et al. showed that in the 3-week-old developing piglet infusion with 3 μg/kg L-NAME caused a 3-times higher decrease of renal blood flow than compared to the adult (174). We hypothesize that VT might cause a stronger inhibition of NO synthesis in children than in adults. This, in combination with the fact that arterioles in childhood are smaller than in adulthood might partially explain why children develop HUS.

However, it is also possible that activated monocytes will reach the glomerular endothelial cells after the endothelial monolayer has already been damaged. TNFα then might have beneficial effects by stimulating iNOS in mesangial cells leading to enhanced NO production and inhibition of platelet aggregation. Furthermore, the presence of TNFα and VT at the same time causes a reduction in ET-1 production by human GMVEC *in vitro*. More experiments will be needed to learn more about the influence of NO and ET-1 in HUS.

In conclusion, we observed a strong reduction *in vitro* of basal NO production in human mesangial cells and to a lesser extent of GMVEC because of incubation with VT-1. No effect of VT-1 on ET-1 synthesis in non-stimulated cells was observed. Although we are aware of the restriction of our results found *in vitro*, we postulate that a reduction of NO synthesis, and not changes in ET-1 production, caused by VT might play an important role in the acute renal failure and thrombotic microangiopathy seen in patients with D+ HUS.

# RELEVANCE OF POLYMORPHISMS IN THE TUMOR NECROSIS FACTOR α AND PLASMINOGEN ACTIVATOR INHIBITOR-1 GENES IN THE HEMOLYTIC UREMIC SYNDROME.

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***Submitted***

**ABSTRACT**

The hemolytic uremic syndrome (HUS) is characterized by a triad of symptoms namely hemolytic anemia, thrombocytopenia and acute renal failure Hereditary factors may determine the occurrence and/or severity of the disease In this study PAI-I and TNFα levels were measured in former HUS patients and compared to healthy controls Besides this, the 4G/5G polymorphism in the PAI-I gene and two polymorphisms in the TNFα gene at position –308(G/A) and –863(C/A) were evaluated and compared to non-symptomatic VTEC infected patients and controls

Levels of PAI-I and TNFα were in the normal range in both former HUS patients and in controls Interestingly however, levels of PAI-I in HUS patients, that recovered several years ago of the disease, were significantly higher compared to age-matched healthy controls ($P<0.05$) For this reason we investigated the 4G/5G polymorphism in the PAI-I gene, which is associated with higher PAI-I levels, in HUS patients, patients with non-symptomatic VTEC infection, and healthy controls No significant difference in frequency of this polymorphism was found in patients versus healthy controls and between non-symptomatic VTEC infected patients and healthy controls However, the frequency of the 4G/5G polymorphism was significantly higher in non-symptomatic VTEC infected patients than in D+ HUS patients suggesting that this polymorphism has not directly a role in the development of HUS In addition, no relationship was found between the presence of the 4G/5G polymorphism and severity of the disease according the classification of Gianantonio Also the polymorphism in the TNFα gene at position –863 (C/A) and at position –308(G/A) were not associated with the severity of the disease according the classification of Gianantonio although a relationship between serum creatinine and the polymorphism at position –863 was found These results indicate that the 4G/5G polymorphism in the PAI-I gene and the two polymorphisms in the TNF gene probably do not have a role in the pathogenesis of HUS

**INTRODUCTION**

The hemolytic uremic syndrome is one of the leading causes of acute renal failure in childhood and is mostly caused by an infection with a verocytotoxin (= Shiga toxin) producing *Escherichae coli* (VTEC), especially serotype O157 H7 (4,118,120) Endothelial damage of glomeruli and in lesser extent kidney arterioles caused by the interaction of verocytotoxin (VT) with its classical receptor (globotriaosylceramide or Gb3) is believed to play a central role in the pathogenesis of D+ HUS (10,53) Histopathological studies of kidney material of HUS patients reveal a characteristic thrombotic microangiopathy with swollen and detached

monoclonal antibody against splicing factor (Sigma) used at a dilution of 1/200 and visualized by a goat anti-mouse alkaline phosphatase antibody. Fast blue was used as a chromogen.

## RESULTS

Renal biopsy material of seven HUS-patients seemed to show a significant amount of apoptotic cells (about 30%) after staining with TUNEL-technique alone (figure 1A). However, staining with RNA splicing factor showed that most of the cells positive with the TUNEL technique were also positive for RNA splicing activity, indicating that these cells were not in the execution phase of the apoptotic process. (Figure 1B). Number of apoptotic cells was counted in five different glomeruli and surrounding tubuli in each patient and total number of apoptotic cells are shown in table 2. Co-labeling showed that just 2.5% of the cells (1.5 % located in tubules) stained with the TUNEL technique were indeed apoptotic (Figure 1C-F) . However, even after co-labeling the sections (TUNEL and SC-35), renal biopsy material of HUS patients showed a significant increase (Wilcoxen test: $p < 0.05$) in apoptotic cells in glomeruli and tubuli compared to controls (Table 2). Interestingly, the amount of apoptotic cells detected in tubuli of HUS patients was significantly higher (30% of total apoptotic cells) than those detected in the glomeruli.

Furthermore, a much stronger positive staining for RNA splicing factor in the tubular epithelial cells was found in patients with HUS compared to controls (Figure 1) indicating that there was a higher transcription activity in HUS patients. Clinical characteristics of patients are shown in table 1. All patients had a prodromal phase of (bloody) diarrhea. Interestingly, those patients that were treated with dialysis (pts 3-6) had more apoptotic cells than patients that received no dialysis suggesting that there might be a relationship between severity of the disease and amount of apoptotic cells detected. Finding of apoptotic cells suggest that apoptosis plays a role in the pathogenesis of HUS.

**Table 2** Number of apoptotic cells detected in five different glomeruli and surrounding tubuli in each biopsy of HUS-patients and controls.

| *Patient no.* | *glomeruli (5)* | *tubuli* | *total:* |
|---|---|---|---|
| 1 | 0 | 3 | 3 |
| 2 | 1 | 0 | 1 |
| 3 | 1 | 7 | 8 |
| 4 | 2 | 4 | 6 |
| 5 | 2 | 6 | 8 |
| 6 | 0 | 7 | 7 |
| 7 | 0 | 1 | 1 |
| MLS | 0 | 0 | 0 |
| MLS | 0 | 0 | 0 |
| TBM | 0 | 0 | 0 |
| TBM | 0 | 0 | 0 |
| TBM | 0 | 0 | 0 |
| TBM | 0 | 1 | 1 |
| TBM | 0 | 0 | 0 |

For statistical analysis Wilcoxen test was used. There was a significant increase of apoptotic cells in patients with HUS compared to controls ($p< 0.05$). 80% of apoptotic cells observed in HUS-patients were localized in the tubuli and only 20% in glomeruli. MLS, minimal lesions nephrotic syndrome; TBM, thin basement membrane.

## DISCUSSION

In this study we used an improved and stringent TUNEL technique to detect apoptotic cells in renal biopsies. We investigated renal biopsy material of seven D+ HUS-patients and found an increased number of apoptotic cells in glomeruli and tubuli compared to control patients with minimal lesions nephrotic syndrome or thin basement syndrome. Endothelial damage of primarily glomeruli is a characteristic feature of HUS. Although a lot of the pathogenesis of HUS is still unclear, it has been postulated that VT plays an important role in the damage of endothelial cells of glomeruli and to a lesser extent renal arterioles (2,18). In several studies a role of verocytotoxin in inducing apoptosis has been suggested (19,20).

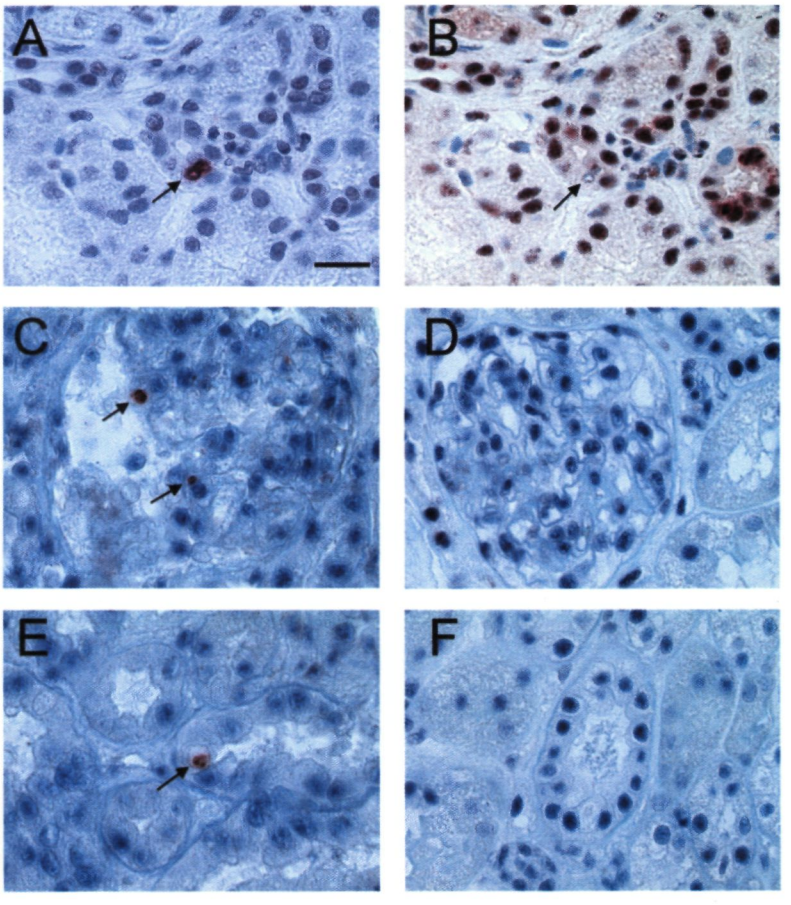


**Figure 1: Detection of apoptosis in renal biopsies in D+ HUS patients versus controls.**

**A)** Labeling with TUNEL technique and SC-35. Nuclei that show high levels of RNA synthesis/splicing are stained in blue which will mask the non-specific (brown) signal of the TUNEL technique. Only one cell is really apoptotic (indicated with an arrow). **B)** The same section of figure A is shown, however the material was washed out and labeled again with SC-35 alone (brown this time): A tubulus and a part of a glomerulus are shown. Numerous nuclei of the tubulus and the glomerulus are labeled (brown-labeled nuclei). The cell positive for apoptosis in figure A after staining with the TUNEL technique and labeling with SC-35, shows in figure B no positive staining at all indicating that this cell has no RNA synthesis/splicing activity and is really apoptotic. All the other cells have high RNA synthesis/splicing activity and are therefore not apoptotic. **C)** Double labeling of TUNEL and SC-35. A glomerulus of a HUS patient contains two TUNEL positive nuclei that are negative for SC-35, which indicates the presence of apoptosis in the glomerulus. **D)** Double labeling of TUNEL and SC-35. A glomerulus of a control patient is shown without apoptosis. The nuclei show signs of RNA synthesis/splicing. **E)** Double labeling of TUNEL and SC-35. A tubulus of a HUS patient that contains TUNEL positive nucleus that is negative for SC-35, which indicates apoptosis in the tubular epithelial cells of this HUS patient. **F)** Double labeling of TUNEL and SC-35. A tubulus of a control patient is demonstrated without apoptosis.

*In vitro* experiments have shown that VT binds to the Gb3 receptor found after stimulation on endothelial cells, enters the cell and can cause inhibition of overall protein synthesis (3,4). *In vitro* studies performed with Vero-, Burkitt's lymphoma and renal tubular epithelial cells have shown that VT can induce apoptosis (85,217-220). Experiments performed in our own group demonstrated that VT can also induce apoptosis in human glomerular endothelial cells in vitro by a mechanism that involves caspase 3 (211). Mitra et al described that plasma of patients with thrombotic thrombocytopenic purpera, a disease that is closely related to HUS, can induce apoptosis in restricted lineages of human microvascular endothelial cells (221). Furthermore, it has been described that endothelial cells undergoing apoptosis become procoagulant and proadhesive for platelets (222). The finding of apoptotic cells in HUS patients *in vivo* using a double labeling for RNA-syntheses splicing factor (SC-35) together with the TUNEL-technique has not been described before. In a recent study, the presence of apoptosis was studied in biopsy material of one child and in postmortem tissue of two children with D+ HUS (84). Apoptotic cells were detected in tubular structures and in lesser extent in glomeruli of the kidney. TUNEL-technique was used to identify apoptotic cells. However, it has been reported that different factors such as RNA synthesis/splicing and small calcium containing vesicles can interfere with TUNEL-labeling (212,216). In this study of biopsy material of seven HUS patients we demonstrate that there is indeed non-specific labeling of non-apoptotic cells in biopsy material by the TUNEL-reaction. Therefore we used a co-labeling for RNA synthesis splicing factor and incubated the material of patients and controls with citric acid to remove calcium-containing vesicles. RNA transcription and splicing requires intact DNA indicating that cells positive for both TUNEL and RNA-splicing factor probably are not apoptotic. Sections of renal biopsy material of HUS patients stained with TUNEL showed a high fraction of nuclei that were labeled (30%) mainly in tubuli and in lesser extent glomeruli. Correction for RNA splicing factor made it possible to differentiate between TUNEL false positive cells and to identify true apoptotic cells. Especially, non-apoptotic nuclei showing signs of active gene transcription can be labeled non-specific by the TUNEL technique. Renal epithelial cells show high levels of RNA synthesis and therefore are prone for this non-specific labeling in the TUNEL technique. However, we still found an increased number of apoptotic cells in HUS patients compared to controls indicating that apoptosis probably plays a role in HUS. It is even possible that there is an underestimation of the amount of apoptosis, because all biopsy material we used was taken in the second week of the disease or even later and patients might be already recovering at that time. Normally, apoptotic cells are removed by macrophages in just a few hours (208,223).

In addition of finding apoptosis, we observed an increased RNA splicing activity in biopsy material of HUS patients compared to controls. This indicates that cells were active and transcribing genes that might be related or completely unrelated to the apoptotic process. Because the induction of apoptosis normally does not require new protein synthesis it is more reasonable to think that high RNA synthesis and splicing activity is not related to the apoptotic process. It is even possible that the high RNA synthesis and splicing activity is related to a phase of repair and re-establishment (224). Furthermore, we found that most apoptotic cells were seen in those patients that were anuric or that received peritoneal dialysis suggesting that there is a relationship between seriousness of the disease and amount of apoptotic cells.

In conclusion, we demonstrated the presence of apoptotic cells in glomeruli and tubuli of renal biopsy material of HUS patients. The finding of apoptotic cells suggest that apoptosis plays a role in HUS. Probably more apoptosis will be seen at an early point of time in the course of the disease.

# ELEVATED LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN SERUM OF PATIENTS WITH D+ HEMOLYTIC UREMIC SYNDROME.

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***Submitted***

**ABSTRACT**

The epidemic form of hemolytic uremic syndrome (D+ HUS) is the most common cause of acute renal failure in children. The pathogenesis of this syndrome is characterized by endothelial damage of glomeruli and tubules all within the kidney. In several other diseases in which glomerular endothelial damage occurs, elevated serum levels of vascular endothelial growth factor (VEGF) have been described. VEGF is involved in angiogenesis, permeabilization of blood vessel endothelium and can have a role in wound repair. In this study we evaluated VEGF levels in serum of 40 D+ HUS patients in the acute phase and in course of the disease. VEGF levels were measured using a double sandwich ELISA technique with a detection limit of 12 pg/mL. Indirect immunohistochemistry was performed for the detection of VEGF in renal biopsy material of three HUS-patients. Significantly elevated VEGF levels were found in HUS patients compared to controls in both serum ($P<0.001$) and plasma ($P<0.05$). A significant relationship was found between VEGF levels and severity of the disease according to the classification of Gianantonio et al. ($p< 0.05$). Levels of VEGF in blood increased during the second and third week after HUS was diagnosed. Immunohistochemistry of renal biopsy material showed increased levels of the receptors for VEGF in the glomeruli and a disappearance of staining of VEGF in tubuli of patients compared to controls. During the course of HUS plasma VEGF levels increase depending on the severity of the disease. This is probably associated with the repair process. In the kidney glomerulus of HUS patients the VEGF receptors Flt-1 and KDR are elevated.

**INTRODUCTION**

Vascular endothelial growth factor (VEGF or VEGF-A), also known as vascular permeability factor, is related to platelet-derived growth factor and is a member of a growing family of closely related growth factors. VEGF is an endothelial cell mitogen, which can promote angiogenesis as well as permeabilization of blood vessels (225). It has been shown to modulate hemostatic properties of monocytes and macrophages by inducing tissue factor expression and exerting a chemotactic action on monocytes (226). VEGF plays a role in several physiological and pathological processes, including embryogenesis, diabetic complications, tumor growth, wound healing and tissue remodeling (227). It is encoded by a single gene, localized on chromosome 6p21.3, and exists in five different isoforms of 121, 145, 165,189 and 206 amino acids due to alternative mRNA splicing. These isoforms differ in biological properties such as their ability to bind to cell-surface heparan sulphate proteoglycans and neuropilin-1. The most

abundantly expressed splice variant is $VEGF_{165}$ It is associated with a variety of cells such as platelets, leukocytes and mesangial cells (225,228,229) The biological function of VEGF is mainly mediated by interaction with two tyrosine kinase receptors Flt-1 (fms-like tyrosine kinase), which is expressed on endothelial cells, monocytes and renal mesangial cells, and KDR/Flk-1 (kinase domain-containing receptor/fetal liver kinase), which is present on endothelial cells and several hematopoietic precursor cells (225, 230) Hemolytic uremic syndrome (D+HUS) is the most common cause of acute renal failure in childhood and its pathogenesis is characterized by endothelial damage of glomeruli and arterioles of the kidney (4,92) The syndrome is associated with an infection with a verocytotoxin producing *Escherichia coli (E coli)*(10) Verocytotoxin is believed to play an important role in the endothelial damage observed in D+ HUS (56) Elevated serum VEGF levels have been found in several other diseases, in which glomerular endothelial damage plays a role (231,232) To elucidate whether VEGF is involved in the pathogenesis and/or recovery phase of D+ HUS, VEGF levels in serum and plasma of D+ HUS patients were measured and compared to the levels in controls Furthermore, the relationship between VEGF levels and clinical parameters on the day of admission and in course of the disease were investigated Finally, the presence of $VEGF_{165}$ and the expression of Flt-1 and KDR, the main receptors for VEGF, were studied in renal biopsy material of three HUS patients and compared to controls

## MATERIALS & METHODS

### *Demographic and clinical data of HUS patients*

Blood samples of 40 patients presenting with D+ HUS (nineteen boys and twenty-one girls, age ranging between 4 months and 10 years) were collected during the time period 1985 till 1999 and evaluated in this study The criteria for HUS were defined as hemolytic anemia with fragmented erythrocytes, thrombocytopenia and acute renal failure All patients investigated presented with a prodromal phase of (bloody) diarrhea Fulfilling the criteria for HUS, the number of platelets, red blood cells and renal function were significantly decreased on admission Table 1 shows the clinical characteristics of D+ HUS patients presented in this study The severity of renal failure was classified according to Gianantonio et al (5) Thirteen children had a mild form of renal dysfunction, whereas the moderate and severe form was observed in eighteen and nine children respectively Control samples of children were collected during the time period 1989 till 1998

| | Total number of HUS patients (n=40) |
|---|---|
| Age (mean ± SE (yrs)) | 3.6 ± 2.6 |
| Gender (male/female; no.) | 19/21 |
| Peripheral blood cells on admission (mean ± SD) | |
| Hb (mmol/L) | 5.5 ± 1.5 |
| WBC (x $10^9$/L) | 17.9 ± 8.8 |
| Plt (x $10^9$/L) | 81.5 ± 89.3 |
| Renal function on admission (mean ± SD) | |
| ureum (mmol/L) | 32.5 ± 18.2 |
| creatinine (μmol/L) | 340 ± 259 |
| Anuria during the prodromal phase (yes/no; no.) | 5/38 |
| Duration anuria (mean ± SD (days); n=20) | 5.4 ± 4.1 |
| Duration oliguria (mean ± SD (days); n=32) | 6.1 ± 4.7 |
| Duration of dialysis (mean ± SD (days); n=28) | 9.0 ± 6.7 |
| Extra renal complications (no.) | |
| cerebrum | 5 |
| pancreas | 1 |
| Creatinine after 2 years (mean ± SD; n=30) | 48.8 ± 9.2 |

Table 1: Clinical and demographic characteristics of D+ HUS patients investigated in this study.

***Detection of VEGF in serum and plasma using a double sandwich ELISA***

Serum and citrate plasma samples of D+ HUS patients were collected starting on the day of admission followed by day seven and fourteen and evaluated for the presence of $VEGF_{121+165}$. To exclude the possibility that decreased renal function is a cause of changed levels of VEGF, material of eleven patients with end-stage renal failure receiving continuous ambulatory peritoneal dialysis (CAPD), were collected and studied. This group consisted of seven boys and four girls, age ranging between 1.5 and 16.5 years. To determine normal VEGF levels, serum samples of 39 control children were investigated (age ranging between 6 months and 11 years). VEGF levels in plasma of 15 D+ HUS patients were measured to assess the contribution of VEGF release by platelets in serum during clotting (233,234).

VEGF was detected using a double sandwich ELISA that detects $VEGF_{165}$ and $VEGF_{121}$, as described earlier (235). Sensitivity of this assay was 12 pg/ml. Intra-assay variation was 6 % and inter-assay variation was 12%.

***Histopathological studies***

Kidney specimens of three different D+ HUS patients were obtained from renal biopsies taken in the acute phase of the disease on days 9, 14 and 15, respectively, after HUS was diagnosed.

These renal biopsies were a kind gift of Dr MC Gubler (Hôspital Necker-Enfants Malades, Paris, France). Biopsy material had been stored at -80°C. All three children presented with diarrhea-associated HUS. The diagnosis HUS was established by combining clinical data and light microscopy of biopsy material. The presence of VEGF was studied in these biopsies and as control, biopsy material of three patients with thin basement syndrome (TBS) was used (stored at -80°C). VEGF was demonstrated histochemically on cryosections (4 μm) that were fixed in acetone at room temperature for ten minutes. The sections were incubated with the primary antibody (rabbit polyclonal anti-VEGF, dilution 1:25; Santa Cruz Biotechnology, CA, Santa Cruz, USA) for one hour in phosphate-buffered saline (PBS) at room temperature. Subsequently, sections were washed thoroughly with PBS followed by incubation with biotinylated affinity-purified anti-rabbit IgG antibody for 30 minutes (dilution 1:200: Vector Laboratories Inc., Burlinghame CA, USA). The sections were washed with PBS and incubated with peroxidase-labeled biotin avidin complex (Vector Laboratories Inc.).

Two successive sections of each biopsy specimen were also incubated with mouse anti-Flt-1 (Sigma, the Netherlands; dilution 1:100) and mouse anti-KDR (Sigma, the Netherlands; dilution 1:100) at 4° C overnight to determine the presence of VEGF receptors in glomeruli and tubules. After this incubation and all following steps, sections were rinsed with PBS. Subsequently, sections were incubated with biotinylated affinity-purified anti-mouse IgG (Vectastain, Vector Laboratories Inc.; dilution 1:200) for 30 minutes at room temperature followed by incubation for the Flt-1 and KDR staining with peroxidase-labeled biotin avidin complex (Vectastain, Vector Laboratories). After this, sections were incubated for 10 minutes with biotinylated tyramine (dilution 1:400) followed by incubation with ABC-peroxidase solution as described previously (238). All stainings were developed by a 10 minute incubation with 0.4 mg/mL amino-9-ethyl-carbazole solution (Aldrich, Steinheim, Germany). In control sections primary antibodies were omitted. All stainings were counterstained for 45 seconds with Harris' haematoxylin (Merck, Darmstadt, Germany) at room temperature. The stained sections were mounted in Imsol-mount medium.

***Determination of Platelet Factor - 4 (PF- 4)***

Citrated plasma samples of 11 HUS children and 10 healthy age-matched controls were used for the evaluation of PF-4. PF-4 is a marker for platelet activation that is not influenced by renal function. PF-4 levels were determined using a standard commercial ELISA-assay from Roche Diagnostics (Almere, The Netherlands).

***Statistical analysis***

All determinations were performed in duplicate. ANOVA was used to analyze statistical significance of differences found for VEGF levels between the control group and HUS-patients and HUS patients and CAPD patients. The correlation between VEGF levels and several clinical characteristics of HUS patients, including platelet count, hemoglobin, serum creatinine, and white blood cell count were calculated using Spearman's rank test. Multi-variance analysis was performed including all these factors. Statistical level of significance was defined as $P < 0.05$. All values are expressed as mean ± SEM.

## RESULTS

***VEGF- levels in serum and plasma of D+ HUS patients***

Levels of VEGF were measured in serum and plasma of patients with D+ HUS and compared to VEGF levels found in healthy controls.

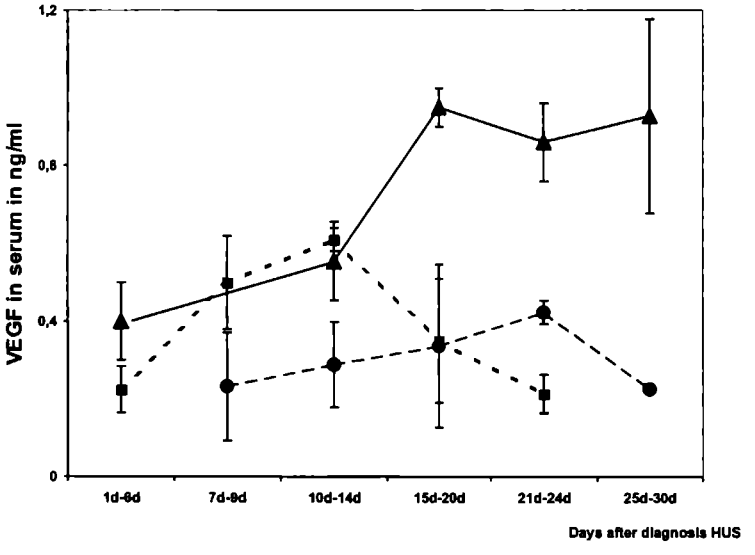



**Figure 1** VEGF levels in serum during the course of the disease. VEGF levels increased during the second and third week after patients were diagnosed having HUS. VEGF levels were calculated from three or more data obtained of different HUS patients at each time period except for the two last time points in the mild form of HUS (n=2 and 1, respectively). The graph shows the results of patients with the mild form (● ; n=13), moderate form of HUS (■; n= 18) the severe form (▲ ; n= 9) according the classification of Gianantonio (13).

Upon administration to the hospital, serum VEGF levels of almost all D+ HUS patients were comparable to those of controls (0.34 ± 0.04 ng/mL versus 0.29 ± 0.13 ng/mL). They increased during the second and third week after the onset of hemolytic uremic syndrome (Figure 1). Highest VEGF levels were found in those patients with the most severe form of HUS according to the classification of Gianantonio et al. (5). Figure 2 shows the results of highest values found for VEGF in serum of patients in the second or third week of the disease

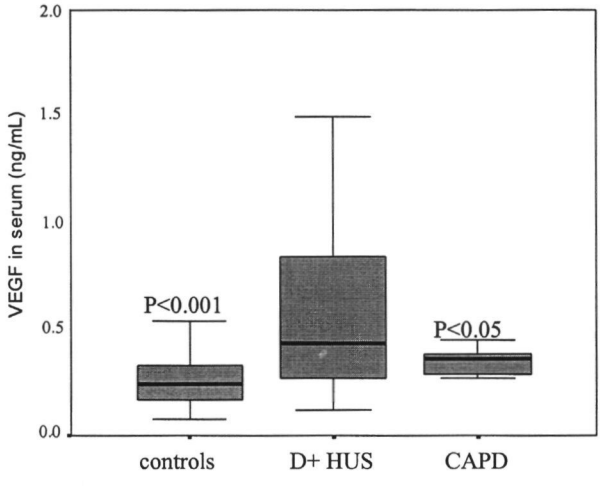



**Figure 1** Box-whisker plots of VEGF levels in HUS-patients versus CAPD-patients and healthy controls. The box-whisker plots show lowest values and highest values measured and medium of the values (black line). VEGF levels in HUS patients were significantly elevated compared to controls ($p<0.001$) and patients receiving CAPD ($p<0.05$). No significant difference was found between CAPD patients and controls.

and healthy controls. In D+ HUS patients significantly elevated VEGF levels were observed whereas only small amounts of VEGF were found in controls (0.55 ± 0.38 ng/mL versus 0.29 ± 0.13 ng/mL, $P<0.001$). Serum VEGF levels were measured in patients with end-stage renal failure to exclude that VEGF levels were changed due to renal failure. D+ HUS patients showed significantly higher VEGF levels in serum than patients with end-stage renal failure receiving CAPD (0.55 ±0.38 ng/mL versus 0.33 ± 0.09 ng/mL, $P<0.05$). VEGF levels in plasma of D+HUS patients were also significantly elevated when compared to control plasmas (0.15 ±0.01 ng/mL versus 0.10 ± 0.02 ng/mL, $P<0.05$).

Statistical analysis using Spearman's rank correlation test was used to determine whether raised VEGF levels were correlated with severity of the disease. Clinical parameters such as serum

creatinine, white blood cell count and platelet count, hemoglobin and duration of the anuric phase were included. A significant positive correlation was found between highest VEGF levels found in D+ HUS patients and number of white blood cells on the day of admission (data not shown, $P<0.05$). Multivariate analysis, including platelet count, white blood cell count and serum creatinine levels as indicators for severity of the disease, showed that there was a negative correlation in course of time between VEGF levels and these factors ($P<0.05$). Furthermore, a significant positive relationship was found between severity of the disease according the classification of Gianantonio et al. (5) and VEGF levels ($P <0.05$), indicating that the highest VEGF levels were found in those patients with the most severe form of HUS (Figure 2). Platelets are a main source of VEGF in blood and for this reason we investigated the relationship between number of platelets and VEGF but also platelet factor 4 (PF-4) and VEGF levels in plasma. In both cases no significant relationship was found ($P= 0.46$ and $P=0.56$, respectively). PF-4 levels were significantly (negative) correlated with number of circulating platelets as expected ($P<0.05$).

***Presence of VEGF-A and VEGF receptors in renal biopsy material of HUS patients***

To investigate whether an increase in VEGF-A production in the kidney was associated with the increased plasma VEGF-A level, the presence of VEGF-A in renal biopsy material of D+ HUS children and controls was immunohistochemically investigated using a polyclonal antibody against VEGF. In concert with previous reports (18; 19), control kidney specimens showed predominantly VEGF staining in tubules and to a lesser extent expression of VEGF-A in the glomeruli, probably expressed by podocytes (Figure 3A). Unexpectedly, the presence of VEGF in D+ HUS patients in both glomeruli and tubules was markedly reduced as compared to control sections (Figure 3B). To evaluate whether the reduced presence of VEGF was accompanied by a change in the expression of VEGF-A receptors, the expression of VEGF-receptors, Flt-1 and KDR were visualized. In control specimens both Flt-1 and KDR were observed in the tubular area (Figure 3 C+E) and almost no staining for Flt-1 or KDR was found within the glomerulus. Biopsy samples of D+ HUS patients showed a strong expression of both Flt-1 and KDR in glomeruli whereas limited expression was observed in tubular area (Figure 3D+F).

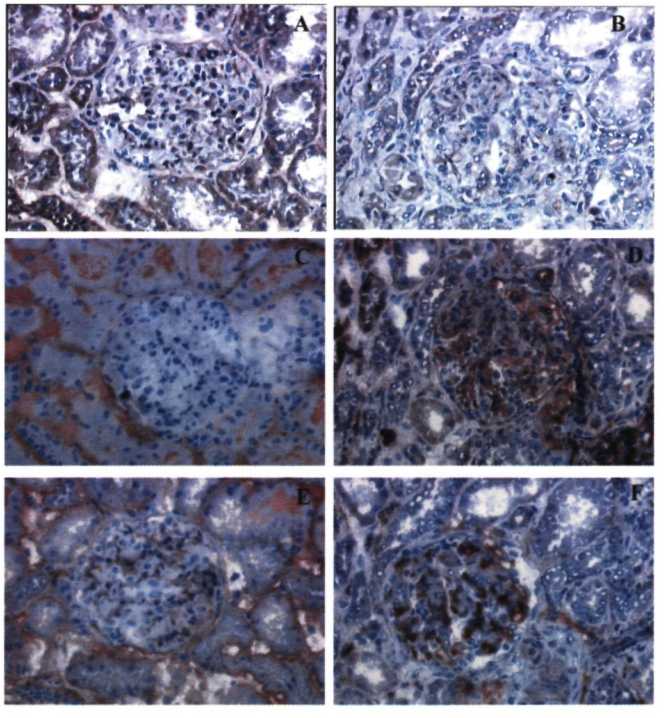



**Figure 3 Immunohistochemistry** of frozen sections of HUS-patients taken in the second or third week of the disease versus control (thin basement syndrome). **Figure A** VEGF expression in controls is predominantly seen in tubules and in lesser extent in the glomerulus. The cells that express VEGF in the glomerulus are most probably podocytes. The amount of VEGF present in glomeruli and tubuli in D+ HUS patients was strongly reduced **(Figure B)**. In serial sections, the receptors for VEGF-A, Flt-1 **(C, D)** and KDR **(E, F)**, of control and D+ HUS biopsy material were visualized. The expression of both receptors was enhanced in D+ HUS patients compared to controls. Magnification 400x.

## DISCUSSION

In the present study we demonstrate that in patients with D+ HUS levels of VEGF in serum and plasma were significantly elevated during the second and third week of the disease when compared to healthy controls and compared to patients with end-stage renal failure ($P<0.001$ and $P<0.05$ respectively). VEGF levels were not significantly elevated on the day of admission compared to controls. Furthermore, a significant relationship between VEGF levels and severity of the disease according the classification of Gianantonio (5) was found.

At the time D+ HUS patients were improving, VEGF levels in serum increased. This is suggestive for the conclusion that VEGF plays a role in the second phase of the disease, namely recovery. It has been described that VEGF can inhibit apoptosis of endothelial cells (21) and can mediate endothelial cell proliferation, neovascularization and eventually can induce the formation of fenestrae (239-242) Besides, increased levels of VEGF might induce an increase

in NO-production leading to vasodilatation and inhibition of leukocyte and platelet endothelium interactions. This may prevent the (further) formation of thrombi (239); all processes that can be important for repairing the damage of glomeruli and arterioles of the kidney as seen in HUS. The importance of VEGF in renal diseases in which thrombotic microangiopathy like in HUS plays a role was recently studied in an experimental model (243). The thrombotic microangiopathy was induced in rats by the administration of anti-glomerular endothelial cell IgG. Twenty-four hours later, half of the rats received VEGF. Renal function was evaluated and compared to control animals that received a placebo. Rats treated with VEGF had more glomeruli with intact endothelium and less glomerular ischemia compared to controls. Furthermore, the administration of VEGF was associated with an improved renal function indicating that VEGF might indeed have a role in repair and recovery in these animals. It is believed that endothelial damage of mainly the glomeruli hallmarks the pathogenesis of D+ HUS. In exactly these areas increased expression of KDR and Flt-1 was found. Other investigators found an increase in VEGF receptors and VEGF after vascular injury (244). Furthermore, an increased expression of VEGF receptors might be found after a period of hypoxia (245,246). The thrombotic microangiopathy in the kidney of D+ HUS patients will cause a period of hypoxia. The increased glomerular expression of KDR and Flt-1 is suggestive for the conclusion that more VEGF will be bound there and that for this reason more VEGF will be found in these regions than in control biopsies. However, to our surprise the amount of VEGF was reduced in both glomeruli and tubules of D+ HUS patients, despite the elevated levels of VEGF found in serum and plasma. It is important to realize that kidney biopsies were taken on day 9, 14 and 15. VEGF levels started to increase between day 14 and 21. For this reason, it may be possible that at a later time point of the disease, increased presence of VEGF can be found corresponding to the increased expression of the VEGF receptors Flt-1 and KDR. Unfortunately, no material of that time period was present.

In other glomerular diseases, like diffuse endocapillary proliferative glomerulonephritis, crescentic glomerulonephritis, and lupus erythematosus decreased expression of VEGF has also been found within the glomerulus (247). Furthermore, in patients with crescentic glomerulonephritis elevated serum levels of VEGF are found at the same time (248). Our data do not provide evidence that an increased VEGF production in the kidney occurs and contributes to the elevated VEGF levels. Furthermore, VEGF levels in patients with end stage renal failure (CAPD-patients) were not elevated compared to healthy controls indicating that renal function is not related to VEGF levels. A possible source for the increased VEGF levels found in D+ HUS patients may be platelets. Microthrombi are found in the kidney of D+ HUS patients and *in vitro* studies have shown that platelets can release VEGF during aggregation

(233,234). Multivariate analysis showed a relationship with platelet count however no direct relationship was found between VEGF levels in serum or plasma and number of circulating platelets or with platelet factor 4 suggesting that there is another source that plays a role. White blood cells may represent such a source. In inflammatory bowel disease, it has been described that peripheral blood mononuclear cells were one of the sources for increased levels of VEGF (249,250). Furthermore, *in vitro* studies have shown that activated neutrophils express VEGF and that they can release VEGF (230,251,252). Elevated levels of IL-8 and elastase are found in HUS patients indicating that neutrophils of HUS patients are indeed activated (79,80). However, these data should be interpreted with some precaution. Because in hemorrhagic colitis elevated number of white blood cells have been found, even in the absence of renal failure, it has to be considered that the increased number of white blood cells found in HUS is no more than a reflection of intestinal infection. It has been described that inflamed tissue in bowel disease can cause the release of VEGF (251). All patients suffering of HUS in this study went trough a prodromal phase of (bloody) diarrhea. Therefore it would be reasonable to suggest that the intestine might be the main origin of elevated VEGF levels in D+ HUS and that the relationship found between VEGF levels and number of white blood cells is just a reflection of the intestinal infection seen in these patients. However, no direct evidence for this was obtained during this study.

In conclusion, VEGF levels in serum and plasma are significantly increased in the second and third week of the disease in D+ HUS patients. In addition, D+HUS is associated with reduced presence of VEGF and increased VEGF receptor localization in the kidney. A good candidate for the increased VEGF production might be the inflamed intestine. Our data suggest that VEGF have a role in the recovery phase of the disease, probably by repairing endothelial damage.

# SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The hemolytic uremic syndrome (HUS) is the most common cause of acute renal failure in childhood in Europe. The pathogenesis of this syndrome is hallmarked by endothelial damage of predominantly the glomerular capillaries (2-4,9). An infection with a verocytotoxin producing Escherichia coli (VTEC) plays a prominent role in the etiology of HUS (10,118). It is generally thought that verocytotoxin (VT), after entering the circulation is transported through the circulation by an unknown mechanism. Subsequently, VT will bind to endothelial cells that express VT binding receptors, followed by endothelial cell damage and death. The endothelial damage is believed to be the primary events leading to a thrombotic microangiopathy and the development of acute renal failure (44). The reason why the damage preferentially is seen in the kidney is still not clear.

The purpose of this thesis was to identify the transport mechanism of VT through the systemic circulation and to gain more insight into the complex mechanism of the relationship between an infection with VTEC and the development of acute renal failure. Particularly the role of vasodilators and vasoconstrictors that normally regulate the perfusion of the kidney were investigated.

In **chapter 1,** an overview of the current knowledge about the hemolytic uremic syndrome is given. An infection with a VT producing *E. coli* plays a central role in the etiology of most cases of HUS and therefore the interaction of VT with his functional receptor and the mode of cytotoxic action are discussed in this chapter. Furthermore, the possible interactions of VT with different cells in the systemic circulation and with human endothelial and mesangial cells are considered.

The study presented in **chapter 2,** shows that *in vitro* VT is rapidly and completely bound to polymorphonuclear leukocytes (PMN) and not to other components of the blood. This binding occurs to a neutral glycolipid present on PMN with a Kd for VT-binding that is a 100-fold less than that of the classical VT-receptor globotriaosylceramide (Gb3). Furthermore, thin layer chromatography of extracted neutral glycolipids on PMN showed two small bands with an Rf value just below the Gb4. Further studies will focus on the exact nature of this receptor. In line with the difference in affinity for VT, PMN that had bound VT were able to transfer the toxin to cultured glomerular endothelial cells. Furthermore, after transfer of the toxin protein synthesis inhibition and finally cell death occurred. The data obtained in this study strongly suggests that PMN transport VT from the intestine to the endothelium in target organs. However, several other suggestions considering the transfer of VT from the intestine to the kidney have been made earlier. Bitzan and colleagues (54) showed that VT was able to bind to

erythrocytes *in vitro* depending on the P-blood group glycolipids present on erythrocytes The Pk-antigen, and to a lesser extent the $P_1$ antigen were able to bind VT, but not the $P_2$-antigen Based on their results, we investigated whether VT bound to isolated human erythrocytes of eight healthy donors with different P blood group phenotypes No significant binding was observed in these donors The fact that no binding of VT to human erythrocytes was observed when incubated in whole blood is strongly suggestive for the conclusion that erythrocytes do not play a pivotal role in transfer of the toxin Little binding of VT to human monocytes has also been described (76,77) This binding increased significantly after stimulation by lipopolysaccharide In our study in whole blood, however, no significant binding was observed For this reason, it is reasonable to suggest that human monocytes do not play a role in transport of VT from the intestine to the kidney

Cooling et al (74) reported the binding of VT (= SLT) to two different glycolipids on platelets obtained by apheresis, besides Gb3, a novel glycosphingolipid different from either Gb3 or the P1 phenotype was capable of binding of VT On the contrary, Yagi et al (75) indicated that isolated leukocyte-depleted human platelets have no receptor for VT They suggested that enhanced platelet aggregation observed after incubation with VT-1 (= SLT-1) is depended on cytokine production by the presence of other cells In our study, no binding to platelets in whole blood environment or when isolated was found, and therefore we conclude that the relative contribution of platelets in transporting VT through the systemic circulation appears to be limited as compared to PMN That PMN play a seminal role in the pathogenesis of HUS has been suggested several times PMN are elevated in the acute phase of the disease and the number of PMN present in the acute phase of the disease is predictive for the outcome of the disease (79,105) Furthermore, renal autopsy material of HUS patients showed an increased presence of PMN in the glomeruli (82) In addition, Forsyth and colleagues (81) showed that PMN isolated from D+ HUS patients in the acute phase of the disease can damage cultured endothelial cells, probably through the release of intracellular components like elastase, or by the formation of superoxide (80, 109,110) Interestingly, in our *in vitro* study, PMN previously loaded with VT showed to be cytotoxic to non-stimulated glomerular endothelial cells whereas PMN alone had no effect Although several ways of transport of VT have been suggested, VT itself has never been detected in the systemic circulation A study performed with rabbits showed that after injection the VT-1 was detectable in serum obtained from rabbits but also that there was a short serum half-life of VT-1 (2 minutes) (70) It was concluded that VT is probably rapidly cleared from the systemic circulation and for this reason not detectable in D+ HUS patients A more reasonable explanation for this short serum half-life would be the rapid binding of VT to PMN, as we observed *in vitro* In 85% of all cases with D+

HUS in Western Europe, infections with VT-2 producing *E. coli* are found (21), therefore, we investigated whether VT-2 bound to PMN could be detected in the systemic circulation of D+ HUS patients. In **chapter 3,** we demonstrate for the first time as far as we know the presence of VT-2 in the systemic circulation of patients during the acute phase of HUS. VT-2 was bound to PMN in the systemic circulation of nine out of ten patients in the acute phase of the disease. PMN isolated from 11 healthy volunteers and six patients with different infectious diseases were used as negative control samples. One patient with atypical HUS repeatedly exhibited no positive staining in the acute phase of the disease. Furthermore, we were able to study time course of VT-binding to PMN in five of the ten patients. The binding decreased in four patients within a week but the last patient studied showed no decrease at all and died in the second week of the disease. The finding of VT bound to PMN in the systemic circulation of D+ HUS patients forms the missing link between the intestinal infection and the development of renal failure as seen in HUS. Furthermore, this finding opens new approaches for treatment in the future. We believe that VT passes the intestinal-blood barrier during the acute phase of the disease and subsequently binds to PMN and in this way is transported to target organs. Acheson and colleagues showed that when PMN transmigrate across intestinal epithelial cells an associated movement of VT is seen in the opposite direction. Interfering in the VT-passage of the intestinal-blood barrier might prevent further transfer of VT by PMN and subsequently damage to target organs. Synsorb Pk might be such a substance that can interfere. *In vitro* studies have shown that Synsorb Pk, which is a synthetic analog of the Gb3 receptor, can bind VT and can neutralize VT when mixed *in vitro* with VT-positive stools from children with D+ HUS (116,117). Administration of Synsorb Pk, to patients with D+ HUS, but also to the siblings and parents of the patients might therefore be beneficial. Another possibility might be treatment with monoclonal antibodies against VT. By giving monoclonal antibodies, passing of the bound VT to the endothelium might be prevented. VT will stay on the PMN that are finally removed from the circulation. Experiments with antibodies against Stx-2 in piglets and rabbits seemed to be protective depending on the time and dose given (254-257). In addition, a study performed with cloned Stx-2 B-subunit in pigs seemed to prevent the binding of the toxin to target cells and in this way prevented the development of edema disease (258). In *vitro* experiments performed with a humanized monoclonal antibody against VT-2 developed by a Japanese group look hopeful. However, these antibodies are not available for clinical treatment yet. Therefore, other additional therapy might be required in severe cases of HUS. We propose, that on the basis of the finding that VT is bound to PMN in the systemic circulation in the acute phase of the disease in D+ HUS patients, leukopheresis could be considered to prevent further transfer of the toxin or protecting target organs for more damage in severe cases.

Because mostly young children develop HUS, we hypothesized that the easier entry of VT into the circulation in young children might be essential for the development of hemolytic uremic syndrome In **chapter 4**, it was evaluated whether VT was present in the systemic circulation of household members of D+ HUS patients to investigate whether this theory is correct Stool and serological analysis was performed to determine the strain of the VTEC involved To our surprise, more than 80% of the household members were positive for VT binding whereas only 10% was positive for VTEC infection in stool and/or serological analysis The number of PMN positive for VT-binding was similar to that found in D+ HUS patients However, because D+ HUS patients have an increased number of leukocytes and household members have not, relatively more VT will be present in the systemic circulation of D+ HUS patients Furthermore, it is also possible that daily measurements of VT bound to the surface of PMN will show a difference between young children and adults Improvement of the technique to quantify VT bound to PMN will be necessary to determine the exact amounts of VT entering and present in the systemic circulation This study shows that the total number of household members of D+ HUS patients being infected is underestimated based on the examination of stool and serological analysis alone Furthermore, this study suggests that the entry of the toxin into the circulation is an important but not the only factor that determines whether someone develops HUS or not One of these additional factors might be lipopolysaccharide (LPS) LPS, or bacterial endotoxin, is a component of the outer cell membrane of gram-negative bacteria (259) The host response to LPS depends on the amount of LPS-binding proteins, degree of cellular responsiveness to LPS and the presence of the active part of LPS (260) In patients who develop HUS, serum antibodies against LPS can be found whereas in household members this is rarely detected Therefore, it is possible that less LPS is entering the circulation of household members than in D+ HUS patients and that the amount of LPS entering the circulation determines whether someone develops HUS Although serum antibodies against LPS are detected in the circulation of D+ HUS patients, LPS itself has never been found in D+ HUS patients In **chapter 5**, we demonstrate for the first time the presence of LPS bound to PMN in the beginning of the acute phase of the disease in D+ HUS patients Furthermore, we demonstrated that the binding of LPS, and not of VT leads to the activation of PMN

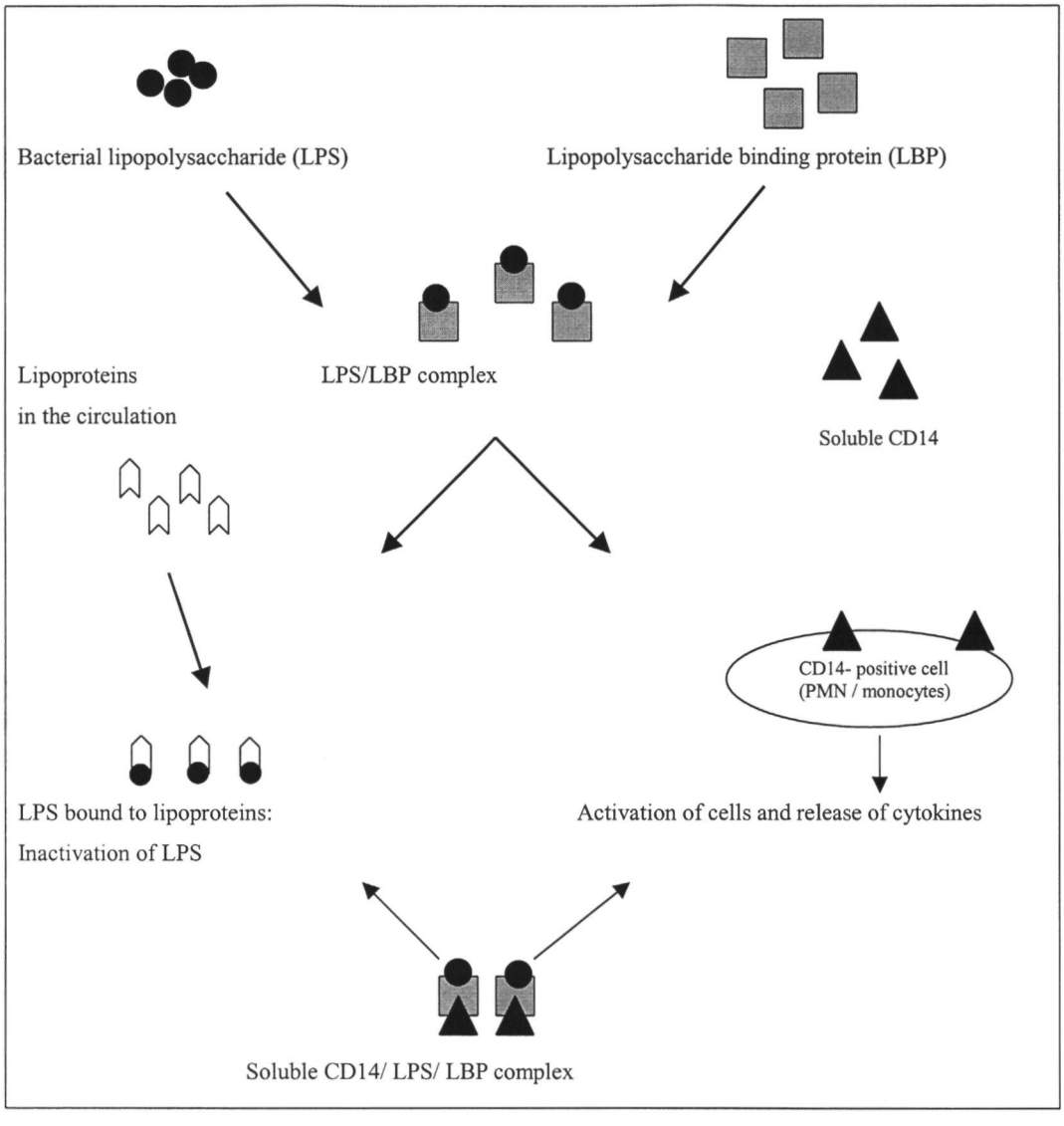



**Figure 1.** The interaction of lipopolysaccharide with different structures in the circulation. LPS can bind to the lipopolysaccharide binding protein (LBP) and can in this way be transported to lipoproteins, to CD14-bearing cells like monocytes and polymorphonuclear leukocytes (PMN) or to soluble CD14. The formation of Soluble CD14/LPS/LBP binding complex can subsequently lead to activation of cells that do not express CD14 themselves, like endothelial cells.

Activation of PMN by LPS led to up-regulation of the CD11/CD18 or anti-ICAM-1 complex and at the same time a reduced expression of L-selectin as described by other investigators (262) VT was able to induce *in vitro* ICAM-1 expression on endothelial cells The increased expression of the CD11/CD18 complex and the simultaneous up-regulation of ICAM-1 on endothelial cells might lead to increased adhesion of PMN to the endothelium Interestingly, it has been described that PMN isolated from HUS patients adhere more avidly to the endothelium than PMN isolated from healthy controls Furthermore, Zoja and colleagues reported that VT-1 could cause increased adhesion of PMN to the endothelium under flow conditions, by up-regulating adhesive proteins (78) Besides the up-regulation of CD11/CD18 complex, also an increased expression of CD66 and CD63 was observed indicating the release of the contents of azurophilic granulae (263) Incubations in whole blood performed with VT and LPS in different concentrations showed a release of elastase, corresponding with the increased expression of CD63 on PMN In line with these results, is the finding of elevated elastase levels in patients with D+ HUS (80) Future investigations in patients with HUS have to show whether the expression of adhesion-molecules on PMN is indeed increased and whether other activation markers will be present Apparently, LPS plays an important role in activation of PMN and probably activates other target cells such as monocytes and endothelial cells Elevated levels of lipopolysaccharide binding protein (LBP) in patients with D+ HUS have been found compared to healthy controls and to patients with only VTEC infection (141) LBP is an acute phase protein and hepatically synthesized Furthermore, LBP is the main LPS binding protein that is responsible for transporting LPS to lipoproteins, to CD14-bearing cells, which include monocytes and PMN but also to soluble CD14 (152) Transport of LPS to lipoproteins will lead to inactivation of LPS (Figure 1) It has even been suggested that high levels of lipoproteins, like high-density lipoprotein (HDL), might work protective against LPS challenges The formation of LPS-LBP-soluble CD14 complexes can lead subsequently to the activation of endothelial cells that do not express CD14 themselves In the study presented in chapter 5, we only investigated the presence of LPS bound to PMN and it cannot be excluded that LPS bound to lipoproteins and to soluble CD14 are also present in blood plasma during the acute phase of the disease Therefore, it would be of interest to study in the future whether LPS bound to soluble CD14 and/or lipoproteins can be detected in D+ HUS patients and patients that only have VTEC infection Maybe differences in distribution patterns of LPS or total amount of LPS presence in the circulation of HUS patients and those with only VTEC infection

will form a key role in the explanation why some develop HUS and others not. Apparently, VT itself is not cytotoxic to PMN, resembling the monocytes (76).

Under basal conditions, VT does not affect protein synthesis in endothelial cells. *In vitro* studies performed with human endothelial cells (56) showed that these cells have to be stimulated with inflammatory cytokines or LPS to increase susceptibility for VT. However, it might be possible that VT induces in basal conditions, factors that are involved in perfusion. In normal circumstances, equilibrium is seen between different factors such as endothelin-1 (ET-1) and NO. Nitric oxide is a potent vasodilatator and prevents adhesion and aggregation of platelets to the endothelium whereas ET-1 acts as a vasoconstricting factor (264,265). Injury or activation of the endothelium changes its regulatory functions leading to an imbalance between the relaxing and constricting factors and loss of anti-thrombotic properties. In **chapter 6**, the influence of VT on NO and ET-1 production of human glomerular endothelial cells was investigated. Furthermore, the effect of VT on NO production of human mesangial cells was studied *in vitro*. Human mesangial cells express in non-stimulated conditions the functional receptor of VT, Gb3, whereas glomerular endothelial cells have to be stimulated (86). For this reason, non-stimulated and stimulated cells were investigated in this study. VT-1 reduced basal NO production in *vitro* of human mesangial cells and to a lesser extent of GMVEC. The reduction of NO production by VT-1 found in human mesangial cells is in the micromolar range and that of GMVEC, in the nanomolar range. A concentration of 10 fmol/L was sufficient to cause maximal reduction of non-stimulated mesangial cells whereas a concentration of five nmol/L was necessary to cause a significant reduction of NO production by non-stimulated human GMVEC. This observation and the fact that endothelial cells make at least 1000–fold less NO, at least *in vitro*, makes it likely that the decrease of NO production of human mesangial cells, caused by VT-1, has more impact than the decrease observed in basal NO-formation of GMVEC. VT did not influence the production of ET-1 in non-stimulated GMVEC suggesting that this substance does not play a role in the initial phase of the disease. These data seem to be in contrast with observations of Bitzan and colleagues (166). They studied the effect of VT on NO and ET-1 production by bovine aortic endothelial cells. No significant changes in NO synthesis were observed but VT appeared to increase ET-1 production and preproET-1 mRNA transcript levels without affecting protein synthesis in quiescent bovine aortic endothelial cells. In our study, VT had no effect on ET-1 or on preproET-1 mRNA level in non-stimulated cells. However, it has to be considered that bovine aorta endothelial cells may react differently after exposure to VT as compared to GMVEC. The only change in ET-1 production found in this study was a decrease in ET-1 production when GMVEC were previously stimulated with TNFα.

It is of interest to notice that the kidney is the only organ in the human body that contains mesangial cells and therefore may be the only organ that will show vasoconstriction because of VT action. This may partly explain why the kidney is the main organ involved in the pathogenesis of HUS. It has to be considered that the data obtained in this study only represent what happens *in vitro,* in static conditions. Although it is anticipated that the data obtained in this study are in general representative for what happens *in vivo* in HUS patients, it cannot be excluded that the flowing blood system may influence such data. It might therefore be of interest for future studies to investigate the effect of VT under physiological flow conditions.

Inflammatory mediators are considered to play an important role in the pathogenesis of HUS. For example, *in vitro* studies have shown that VT is not cytotoxic to confluent glomerular endothelial and human umbilical vein endothelial cells and requires pre-exposure to inflammatory cytokines to induce cytotoxicity (55,56). In addition, elevated levels of TNFα and IL-6 have been found in urine and plasma of patients with D+ HUS as also MCP-1 and IL-8 (205). It has been suggested that monocytes may be an important source of these mediators (76,77). Furthermore, Harel and colleagues reported that VT (= SLT), injected into transgenic mice that bear a chloramphenicol acetyltransferase reporter (CAT) gene coupled to a TNFα promotor, was able to induce CAT activity in the kidney indicating an increased local TNFα synthesis (177). If inflammatory mediators indeed play an important role, then it might be possible that polymorphisms in one of these genes could influence the outcome of the disease.

Plasminogen activator inhibitor I (PAI-I) is also believed to play a role in the pathogenesis of HUS (188). PAI-I is a major regulatory protein of the fibrinolytic system and can rapidly inactivate tissue type and urokinase-type plasminogen activator (t-PA and u-PA)(49). Elevated PAI-I levels have been found in patients with D+ HUS and some studies suggest that there is an association with a worse outcome in D+ HUS (186-188). Polymorphism in the PAI-I gene could to lead to an increased or decreased PAI-I production and in this way influence the course of the disease in HUS (267). Therefore, polymorphisms of these two genes were studied in **chapter 7**. The role of the polymorphisms at position-308 (G/A) and –863 (C/A) in the tumor necrosis factor α gene and of the 4G/5G polymorphism in the PAI-I in relationship to the severity of the disease was investigated. In addition, levels of PAI-I and TNFα in plasma were measured in former HUS patients and compared to healthy controls. Levels of PAI-I and TNFα were in the normal range in both former HUS patients and in controls. Interestingly however, levels of PAI-I in HUS patients, who recovered several years ago, were significantly higher compared to age-matched healthy controls. It was evaluated whether the presence of 4G/5G polymorphism in the PAI-I gene could be an explanation for

this difference However, no significant difference in frequency of this polymorphism was found in patients versus controls The presence of other polymorphisms in this gene might form an explanation Another possibility might be the presence of a polymorphism in the angiontensin-converting enzyme (198) It has been described that elevated levels of angiontensin II can lead to increased PAI-I synthesis Therefore, a polymorphism in this gene resulting in elevated angiontensin II levels might contribute to the elevated PAI-I levels observed in former HUS patients No relationship was found between severity of the disease and the presence of the polymorphism according the classification of Gianantonio (5) It would be intriguing to study in the future the role of other polymorphisms and to evaluate whether these polymorphisms can help in understanding why some children develop a severe form of HUS and others only have diarrhea

As mentioned before, the primary site of damage in HUS is the endothelium of the kidney The endothelial damage is probably caused by two distinct mechanisms First, by the inhibition of protein synthesis in the cell and second by the induction of a process called programmed cell death or apoptosis (206) Several *in vitro* studies have pointed out that VT can induce apoptosis in endothelial cells, renal tubular epithelial cells, and other cell types (85,89,218,219) It seems that apoptosis induced by VT, is caspase-3 and -8 dependent after cross-linking the Gb3 receptor (268) There are only a few *in vivo* data present suggesting that apoptosis might indeed play a role in HUS Therefore, we studied in **chapter 8** the presence of apoptosis in renal biopsy material of seven D+ HUS patients and seven controls (minimal change disease or thin basement syndrome) and evaluated whether the presence of apoptotic cells was related to the severity of the disease To investigate the presence of apoptosis a stringent terminal deoxynucleotidyl nick-end labeling (TUNEL) technique was used Recently, it was shown that non-apoptotic cells that show signs of active gene transcription could be labeled nonspecifically by the TUNEL technique (216) In a true apoptotic cell, the nuclear DNA is cleaved into fragments and processes like DNA repair and simultaneously the RNA transcription/splicing activity disappears Therefore, the TUNEL technique was combined with a staining of RNA-synthesis and splicing factor, SC-35 True apoptotic cells are TUNEL positive but RNA splicing negative, whereas non-apoptotic TUNEL positive cells will be positive for the RNA splicing factor Using this combined method, an increased number of apoptic cells were observed in glomeruli and tubules of patients with D+ HUS compared to controls Most apoptotic cells were detected in tubules (80%) of the HUS patients and not in the glomeruli (20%) Furthermore, an increased staining for RNA splicing factor was observed in the tubular epithelial cells of D+ HUS patients compared to controls, indicating higher transcription activity of these cells Therefore, these cells are prone for non-specific labeling

with the TUNEL technique. Indeed non-specifical labeling of these cells were observed indicating that one should be careful interpreting results obtained by TUNEL technique alone. The finding of apoptosis in tubules and glomeruli of the D+ HUS patients is in line with the results reported by Karpman et al (84). She studied apoptosis in autopsy material of two children and in biopsy material of one child with D+ HUS. The amount of apoptotic cells observed in this study was much higher than in ours. However, apoptosis was studied using the TUNEL technique alone. Delay in fixation of the autopsy material, proteinase K pretreatment, the presence of calcium-containing vesicles and increased RNA-splicing activity, all could have caused non specific labeling in this study and therefore an overestimation of apoptotic cells might be expected (212,216). Alternatively, the occurrence of apoptosis in biopsy material of the seven D+ HUS patients in our study might be underestimated. Biopsies studied were taken in the second or third week of the disease and it is possible that patients were already recovering at that time. It is possible that the high RNA synthesis and splicing activity found in the tubular epithelial cells are related to a phase of repair and re-establishment. However, studying biopsy material of HUS patients in an earlier phase of the disease is not possible because the risk of hemorrhage due to thrombocytopenia is too high.

It is of interest to note that most patients with D+ HUS recover spontaneously and that only supportive therapy, consisting of peritoneal dialysis or hemodialysis, correction of electrolyte imbalance and treatment of hypertension when necessary, is given. The fact that patients recover spontaneously suggests that there are also factors playing a role in repairing the damaged tissues. One of these substances might be vascular endothelial growth factor (VEGF). VEGF is related to platelet-derived growth factor and is a member of a growing family of closely related growth factors (230). VEGF can promote angiogenesis and can stabilize endothelial cells. It also increases endothelial permeability (230). Elevated serum VEGF levels have been found in several diseases, in which glomerular endothelial damage has a role (248, 269). The role of VEGF in HUS was studied to determine whether VEGF has a function in HUS. For this reason VEGF levels in serum and plasma of patients with D+ HUS were evaluated and the results are presented in **chapter 9**. VEGF levels in serum and plasma were significantly increased during the second and third week of the disease. At that time, D+ HUS patients were already improving suggesting that VEGF plays a role in the second phase of the disease, namely repair and recovery. Interestingly, VEGF levels found in D+ HUS patients correlated with severity of the disease according to the classification of Gianantonio (5). To investigate whether the increased plasma VEGF level was associated with an increase in VEGF production in the kidney, the presence of VEGF in renal biopsy material of D+ HUS children and controls was investigated. Unexpectedly, the presence of VEGF in D+ HUS patients in

both glomeruli and tubules was markedly reduced as compared to control sections. In addition, no significantly elevated VEGF levels were observed in patients with chronic renal failure compared to controls indicating that decreased renal function is not related to VEGF levels. The biological function of VEGF is mainly mediated by interaction with two tyrosine kinase receptors: Flt-1 (fms-like tyrosine kinase) and KDR/Flk-1 (kinase domain-containing receptor/ fetal liver kinase) (230,270,271). For this reason we evaluated whether the reduced presence of VEGF was accompanied by a change in the expression of VEGF receptors. A strong expression of both Flt-1 and KDR in glomeruli was observed in D+ HUS patients whereas limited expression was observed in tubular area. On the contrary, VEGF staining in control sections was observed in the tubular area and almost no staining was found in the glomeruli. The reduced staining of VEGF in the kidney was surprising considering the elevated levels of serum VEGF levels. However, it have to be considered that biopsies were taken in the second or beginning of the third week. At that time, VEGF levels started to increase. Therefore, it may be possible that biopsies that are taken in a later time period will show increased presence of VEGF. Unfortunately, no kidney material of this period was available. Another possibility that has to be considered is, that VEGF may be localized intracellularly, beyond the reach of the antibody. It has been described that VEGF bound to his receptors can be internalized via a classical receptor-mediated endocytosis pathway and accumulates in the endosomal or nuclear compartment (271,272). Finally, we can not exclude that VEGF measured in D+ HUS patients may be bound partly to the soluble receptor of VEGF and for this reason can not bind to receptors expressed on the damaged endothelium.

In other glomerular diseases, such as diffuse endocapillary proliferative glomerulonephritis and lupus erythematosus decreased expression of VEGF has been found within the glomerulus (247). Furthermore, in patients with crescentic glomerulonephritis elevated serum levels of VEGF have been found. Recently, the role of VEGF in renal diseases in which thrombotic microangiopathy plays a role similar as in HUS, was studied in an experimental model (243). A thrombotic microangiopathy was induced by the administration of anti-glomerular endothelial cell IgG in rats. Rats, that were given VEGF had more glomeruli with intact endothelium and less glomerular ischemia compared to controls. The administration of VEGF was associated with an improved renal function. It has been described that VEGF can inhibit apoptosis of endothelial cells and can mediate endothelial cell proliferation, neovascularization and eventually can induce the formation of fenestrae (241,242). In addition, increased levels of VEGF might induce an increase in NO-production leading to vasodilatation and inhibition of leukocyte and platelet endothelium interactions (239). This may prevent the (further) formation of thrombi in the kidney. The elevated levels of VEGF found in HUS patients were related to

the number of white blood cells, which may suggest that these cells might be a source for the VEGF. However, the finding that VEGF levels increased at the time white blood cells were normalizing suggests that this is not probable. Future investigation might lead to the identification of the source releasing VEGF. The development of an animal model would give probably more insight in this aspect of the pathogenesis of HUS. Although several attempts have been made, no good animal model for the pathogenesis of HUS is available although VT susceptibility in different animals has been described. For example, infection with VT2e causes edema disease in young pigs showing symptoms of non-hemorrhagic diarrhea and systemic vascular damage (273). However, no renal damage is seen. In addition, mice injected with VT show tubular damage but no glomerular damage is seen (274,275). Recent data of Fernandez et al. show that the (Java) monkey is probably the only animal that might serve as model for the hemolytic uremic syndrome as seen in HUS. PMN of the monkey are capable of binding VT and transferring the toxin to GMVEC. Furthermore, staining with the B-subunit of VT demonstrated the presence of the VT receptor Gb3 in both glomeruli and tubules of this animal. Development of this monkey animal model will give more insight into the pathogenesis of HUS and probably gives opportunities to investigate possible treatment.

## CONCLUSION

The data presented in this thesis form the missing link between the intestinal infection and the development of renal failure seen in HUS. For the first time, as far as we know, the presence of VT and LPS in the systemic circulation of D+ HUS patients was demonstrated. Both, VT and LPS were bound to PMN. LPS bound to PMN, but not VT, led to up-regulation of adhesion-molecules and degranulation of PMN. The finding of VT bound to PMN in household members of D+ HUS patients indicates that not only the entry of VT into the circulation determines whether HUS will develop. Additional factors, like LPS and genetic differences, probably determine what the consequences of the VTEC infection will be. Probably, LPS is not bound only to PMN but also to human monocytes in HUS leading to the activation of these cells. A schematic representation of the possible mechanism leading to HUS and subsequently recovery is shown in Figure 2. VT was able to decrease NO production of mesangial cells and to a lesser extent of GMVEC *in vitro*. No effect of VT was observed considering ET-1 synthesis under basal conditions suggesting that ET-1 does not play a role in the initial phase of the disease. The reduced NO production observed *in vitro* may be an explanation for the acute renal failure and the thrombotic microangiopathy as seen *in vivo* in HUS. Stimulation of GMVEV and subsequently incubation with VT led to reduced ET-1 production in endothelial cells and increased NO production of mesangial cells.

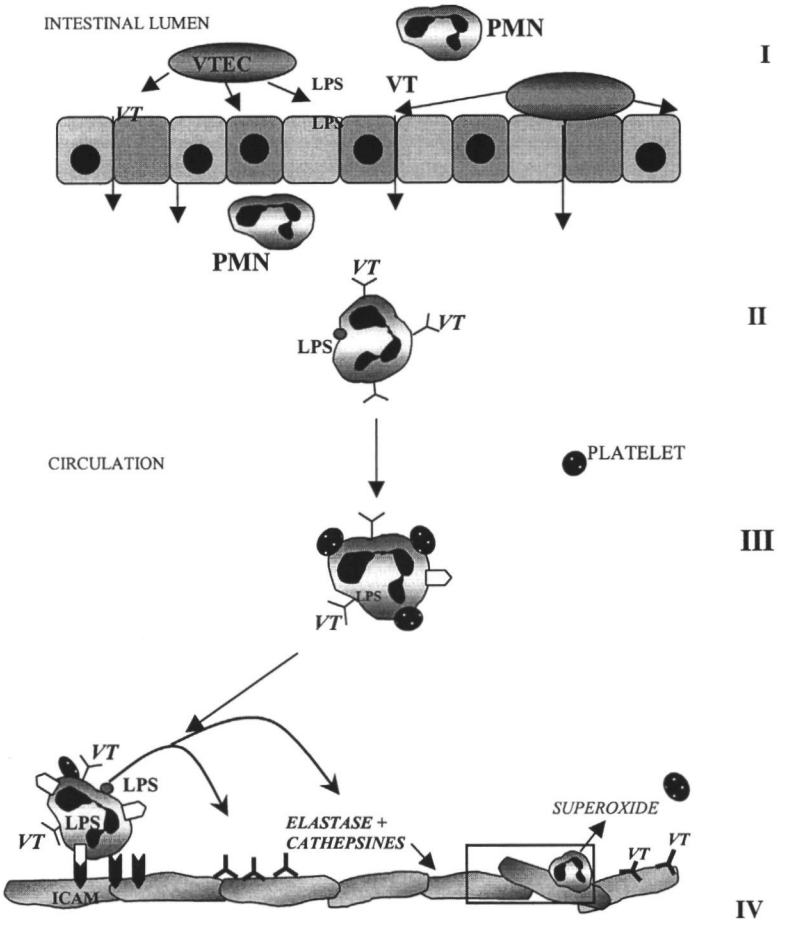
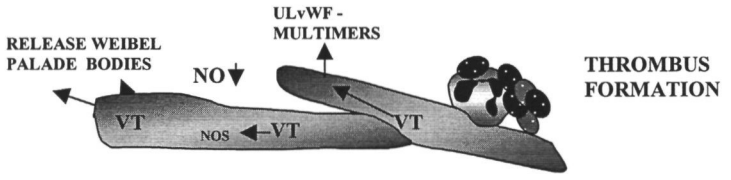
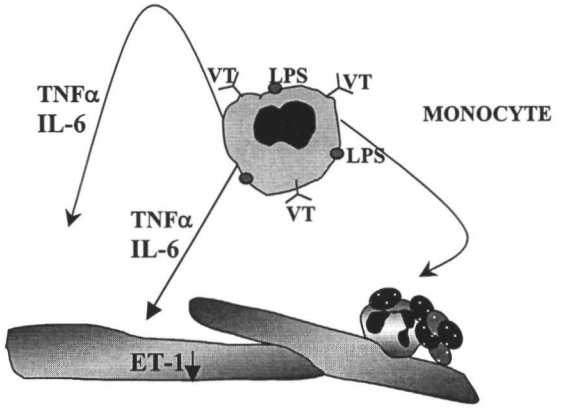



**Figure 2** Proposed mechanism of the pathogenesis of D+ HUS. Verocytotoxin producing *E. coli* (VTEC) binds to the epithelial cells of the intestine. After binding, VT and LPS enter the systemic circulation and will bind to PMN (**I +II**). Activation of PMN will occur as a consequence of LPS binding (**III**). LPS will induce the expression of adhesion molecules on PMN (CD11b/CD18 complex) but also on endothelial cells (P-selectin and ICAM-1). PMN will adhere firmly to the endothelium inducing the release of elastase, cathepsins and superoxide (**IV**).




V




VI

Furthermore, cytokines like TNFα will be released inducing the expression of globotriaosylceramide (Gb3). Transfer of VT will occur leading to reduced production of nitric oxide, inhibition protein synthesis and subsequently cell death (**V**). LPS stimulated monocytes will enter the damaged area, bind VT, and will produce large amounts of cytokines. This will lead to increased NO synthesis of mesangial cells and reduced endothelin-1 production in endothelial cells (**VI**).

It is of interest to notice, that the kidney is the only organ in the human body that contains mesangial cells. Therefore, reduction of NO production may occur only in this organ during the development of HUS because of the presence of VT. This would at least partly explain why the kidney is the major organ involved in HUS.

Whereas this thesis has provided new insights into the development of HUS, it is not completely clear why mainly young infants are affected and secondly why the kidney is the main target. It may be that the entry of LPS together with VT will form partially the explanation why especially younger children are affected. However, this would lead immediately to a new question; why is there a difference in the entry of LPS and VT between children and adults? This has to be investigated in the future.

The *in vitro* data obtained in this thesis showing that VT can reduce NO synthesis of mesangial cells may explain why the kidney is the main organ involved in HUS, however, more studies will be needed to prove this is indeed the case. To gain more insight into these questions, future research should be performed as indicated in the general discussion. Two aspects are especially of interest to focus on: First, the amount of LPS entering the circulation and its distribution pattern in patients with HUS and those with only VTEC infection and secondly, to study the effects of VT in a circulating system.

In addition, data presented in this thesis shows that both VT and LPS are transported by PMN in the systemic circulation of D+ HUS patients. The finding of this opens possibilities for treatment of these patients. Preventing the transfer of the toxin to target cells and inactivation of LPS still present in the circulation are especially of interest. Future research should also focus on this aspect. At the moment, prevention of VTEC infection is the best we can do. Treatment of HUS patients and household members with Synsorb-Pk might be beneficial. However, in a randomized double blind, placebo controlled trial, no significant reduction in the incidence of HUS was seen (28). On the other hand, it might be possible that Synsorb-Pk was given too late and for this reason no significant effect was observed. After admission to the hospital, symptomatic treatment can be offered to the HUS patients. Stabilizing electrolyte balance, treatment of the acute renal failure with dialysis are only supportive therapies. Maybe in the near future, based on the studies presented in this thesis, it will be possible to interfere between toxin binding to PMN and the transfer of the toxin and in this way preventing the development of the more severe form of HUS. Perhaps that also the inactivation of LPS present in the circulation of D+ HUS patients forms a therapeutic possibility. What can be done at the moment, is educating parents and in particular young children to prepare food well and taking hygienic precautions as much as possible to reduce the incidence of HUS.

***Hoofdstuk 1.*** Het hemolytisch uremisch syndroom (HUS), is een syndroom dat met name op de kinderleeftijd vaak wordt gezien. Het ziektebeeld kenmerkt zich door het optreden van bloedarmoede (hemolytische anemie), een tekort aan bloedplaatjes (thrombocytopenie) en het plotseling verminderen van de nierfunctie (acute nierinsufficiëntie). HUS is de meest voorkomende oorzaak van acute nierinsufficiëntie op de kinderleeftijd in Europa. De pathogenese van het ziektebeeld kenmerkt zich door beschadiging van endotheelcellen die de binnenkant van in de nier gelegen bolvormige vaatkluwentjes (glomeruli) bekleden. In ernstige gevallen wordt niet alleen endotheelschade gezien in de nier maar kunnen ook andere organen, zoals de alvleesklier en hersenen aangedaan zijn. Er zijn twee subtypes te onderscheiden, te weten de typische of epidemische vorm van HUS en een atypische vorm. In tegenstelling tot de atypische vorm, kenmerkt de typische of epidemische vorm van HUS zich door een prodromale fase van acute, vaak bloederige diarree en wordt daarom ook wel D+ (diarree geassocieerde) HUS genoemd. D+ HUS wordt in de meerderheid van de gevallen veroorzaakt door een infectie met een verocytotoxine producerende *Escherichia coli* (VTEC).

De belangrijkste bron van besmetting is veelal de consumptie van besmet rundvlees. Wanneer het vlees onvoldoende verhit wordt, kunnen de bacteriën overleven en zich vermenigvuldigen. De verocytotoxines (VT) zijn opgebouwd uit twee delen: Een gedeelte dat biologisch activiteit kan ontplooien (A-subunit) en een gedeelte dat ervoor zorgt dat het toxine kan binden aan doelwit cellen (B-subunits). Binding van VT aan doelwit cellen kan plaatsvinden als er een specifieke receptor voor VT aanwezig is. De meest beschreven receptor voor VT is globotriaosylceramide (Gb3). Deze receptor bevindt zich met name op endotheelcellen. Wanneer VT door middel van de B-subunits gebonden is aan de receptor op het celoppervlak van de doelwit cellen, kan internalisatie plaats vinden door middel van endocytosis. In de cel kan het toxine de eiwitsynthese op ribosomaal niveau remmen, hetgeen uiteindelijk tot celdood kan leiden. Behalve remming van de eiwitsynthese kan VT in bepaalde celtypes ook apoptosis (geprogrammeerde celdood) induceren.

Het complexe mechanisme dat uiteindelijk leidt tot acuut nierfalen in met name kinderen wordt nog steeds niet volledig begrepen. In het algemeen wordt aangenomen dat VT de bloedbaan binnen treedt, getransporteerd wordt door de systemische circulatie door een vooralsnog onbekend mechanisme, en vervolgens bindt aan endotheelcellen die de Gb3 receptor tot expressie brengen. Verschillende *in vitro* studies hebben uitgewezen dat gekweekte endotheelcellen afkomstig uit navelstrengen en uit de glomeruli van de nier, onder normale

omstandigheden niet gevoelig zijn voor de schadelijke effecten van VT. Door stimulatie van de cellen met ontstekingsmediatoren, zoals tumor necrosis factor alfa (TNFα), is het mogelijk om het aantal receptoren op deze cellen op te reguleren waarna een toxisch effect kan worden waargenomen.
Doelstelling van dit promotieonderzoek was het identificeren van het transport mechanisme van VT door de systemische circulatie en het verkrijgen van meer inzicht in factoren die een mogelijke rol spelen in het ontstaan van de acute nierinsufficiëntie in HUS. Factoren die ons inziens met name hierbij een rol zouden kunnen spelen zijn het vaatverwijdende (vasodilatator) stofje stikstofmono-oxide en het vaatvernauwende (vasoconstrictor) endotheline-1. Verandering in de productie van één of beide stofjes door het glomerulaire endotheel onder invloed van VT zou een verklaring kunnen zijn voor het ontstaan van het nierfalen in HUS.

***Hoofdstuk 2.*** Verschillende studies hebben aangetoond dat een VTEC infectie een belangrijke rol speelt bij het ontstaan van HUS, het is echter nog nooit iemand gelukt om de aanwezigheid van VT in de systemische circulatie van patiënten met D+ HUS aan te tonen. In deze studie hebben we daarom onderzocht welke fractie van het bloed verantwoordelijk was voor de binding en overdracht van VT aan doelwit cellen. Hieruit bleek dat VT in vol bloed, in een korte tijd volledig bond aan granulocyten. Er werd geen binding aan andere componenten van het bloed waargenomen. Door middel van Scatchard plot analyse werd duidelijk dat de binding van VT aan granulocyten teweeg werd gebracht door de aanwezigheid van één type receptor voor VT met een affiniteit die een factor 100 lager is dan die van de klassieke receptor voor VT, de Gb3 receptor. Met behulp van dunne laag chromatografie kon bovendien worden aangetoond dat het gaat om een neutraal glycolipid bestaande uit twee banden met een ander migratie patroon vergeleken met de Gb3 receptor op endotheel. Vanwege het verschil in affiniteit tussen de receptor aanwezig op granulocyten en die op endotheel, bleek het VT van de granulocyt overgedragen te kunnen worden aan het endotheel. Na overdracht van VT trad een biologisch effect op in de vorm van eiwitsynthese remming en celdood.

***Hoofdstuk 3.*** Zoals al eerder vermeld is VT zelf nog nooit gedetecteerd in de circulatie van HUS patiënten. In een studie waarbij konijnen geïnjecteerd werden met radioactief gelabeld VT, kon VT voor een zeer korte tijd in het serum gemeten worden. De halfwaardetijd van het toxine was ongeveer 2 minuten in het serum. Hieruit werd de conclusie getrokken dat VT snel geklaard wordt uit de systemische circulatie en daarom niet detecteerbaar is in HUS patiënten. De binding van VT aan granulocyten van D+ HUS patiënten zou misschien een betere verklaring kunnen zijn. Analyse van granulocyten geïsoleerd uit bloed van D+ HUS patiënten

in de acute fase van het ziektebeeld, liet zien dat VT inderdaad gebonden aan granulocyten aanwezig was. Er was bovendien een sterke associatie tussen de aanwezigheid van VT gebonden aan granulocyten en tegelijkertijd de aanwezigheid van diarree. Een afname van het totaal aantal granulocyten positief voor de aanwezigheid van VT correleerde met een verbetering van het klinische beeld. Eén patiënt in deze studie liet geen afname zien en overleed binnen een week na binnenkomst in het ziekenhuis. De aanwezigheid van VT gebonden aan granulocyten in de systemische circulatie van HUS patiënten bevestigd de hypothese dat granulocyten waarschijnlijk verantwoordelijk zijn voor transport en overdracht van VT vanuit de darm naar doelwit organen.

***Hoofdstuk 4.*** Het is nog steeds niet begrijpbaar waarom met name op de kinderleeftijd de typische vorm van HUS wordt gezien. Op basis van de bevinding van VT in de systemische circulatie van D+ HUS patiënten ontwierpen we daarom de hypothese dat de passage van de darmwand en vervolgens het bereiken van de circulatie een essentiële stap was voor het ontwikkelen van een HUS. Verassend genoeg bleek dit echter niet te kloppen. Meer dan 80% van de familieleden van HUS patiënten bleek positief te zijn voor de aanwezigheid van VT gebonden aan granulocyten tijdens de acute fase van het ziektebeeld. In slechts 23% van de gevallen kon de infectie ook in de feces of serologisch worden vastgesteld. De detectie van VT gebonden aan granulocyten maakt het mogelijk om op een snelle en gemakkelijke manier vast te stellen of er inderdaad een infectie is en geeft meer inzicht in het aantal personen daadwerkelijk geïnfecteerd met VTEC. De aanwezigheid van VT in de circulatie van familieleden geeft bovendien aan dat niet alleen de passage van de darmwand en vervolgens het transport van VT door de circulatie bepalend of iemand een hemolytisch uremisch syndroom ontwikkeld. Het is echter niet uitgesloten dat de hoeveelheid toxine die uiteindelijk de systemische circulatie bereikt en getransporteerd kan worden naar doelwit cellen wel een rol speelt.

***Hoofdstuk 5*** Lipopolysaccharide (LPS), ook wel endotoxine genoemd, is afkomstig van gram-negatieve bacteriën zoals ook van de *E. coli*. Door de interactie van het afweersysteem met LPS worden een aantal processen geactiveerd die allen leiden tot bescherming tegen gram-negatieve bacteriën. Zo wordt bijvoorbeeld het stollingssysteem geactiveerd maar ook monocyten en granulocyten. Als er echter teveel LPS in de circulatie terechtkomt kan dit nadelige gevolgen hebben. In hoge concentraties kan LPS leiden tot een lage bloeddruk (hypotensie), beschadiging van verschillende organen zoals nieren en longen en het ontstaan van bloedstolseltjes (thrombi). In HUS patiënten worden antilichamen tegen LPS, met name

serotype O157, gevonden. Het endotoxine zelf is echter nog nooit gedetecteerd. In deze studie hebben we zover we weten, voor het eerst de aanwezigheid van LPS gebonden aan granulocyten in de circulatie van HUS patiënten kunnen aantonen. Aangezien zowel LPS als VT gebonden is aan granulocyten in de acute fase van HUS, hebben we onderzocht welke van deze twee, of misschien wel beiden leiden tot de activatie van de granulocyten. Hierbij bleek dat LPS opregulatie van verschillende adhesiefactoren zoals het CD11/Cd18 complex (= anti-ICAM-1) en CD66 gaf maar VT zelf geen enkel effect daarop had. LPS, maar ook VT, bleek bovendien in staat te zijn om elastase release van granulocyten te induceren. Deze resultaten suggereren dat LPS in combinatie met VT een belangrijke rol spelen in het ontstaan van HUS.

***Hoofstuk 6*** Stikstofmono-oxide, afgekort NO (nitric oxide), is een belangrijke vasodilatator. Tegelijkertijd voorkomt NO de adhesie van plaatjes en leukocyten aan het endotheel. Endotheline-1 (ET-1) heeft juist een tegenovergestelde werking, het zorgt voor samenknijpen van de vaten (vasoconstrictie). Beide substanties kunnen gemaakt worden door endotheelcellen en oefenen hun werking uit op de daaronder liggende spiercellen. Onder de glomerulaire endotheelcellen in de nier liggen geen normale gladde spiercellen maar mesangiale cellen. Deze mesangiale cellen spelen een belangrijke rol bij het in stand houden van de glomerulaire hemodynamiek en kunnen ook NO produceren maar niet ET-1. VT bleek in staat te zijn om in basale omstandigheden de NO productie van zowel mesangiale cellen als van glomerulaire endotheelcellen te remmen. De afname van de NO synthese door mesangiale cellen was een factor 1000 hoger dan dat van de glomerulaire endotheelcellen. Dit suggereert dat veranderingen in de NO synthese door de mesangiale cellen meer impact zal hebben in de ontwikkeling van HUS dan dat door de glomerulaire endotheelcellen. Onder basale, niet gestimuleerde omstandigheden werd geen enkel effect van VT-1 op de ET-1 productie waargenomen. In het geval van gestimuleerde endotheelcellen bleek de ET-1 productie naar beneden te gaan. Gebaseerd op deze gegevens is het dan ook waarschijnlijk dat in de begin fase van het ziektebeeld met name veranderingen in de NO-productie een belangrijke rol zullen spelen.

***Hoofstuk 7*** Tijdens deze studie werden twee polymorphisms in het TNFα gen onderzocht namelijk op positie –308 (G/A) en op positie –863 (C/A) en het 4G/5G polymorphisme in het plasminogen activator inhibitor I gen (PAI-I) in voormalige HUS patiënten aangezien beide factoren een belangrijke rol spelen in de pathogenese van HUS. Er werd geen verschil gezien tussen frequentie van het voorkomen van deze polymorphisms in HUS patiënten, patiënten die alleen de VTEC infectie hadden en controles. Er werd bovendien geen relatie gevonden tussen

de ernst van het ziektebeeld volgens de classificatie van Gianantonio en de aanwezigheid van het polymorphisme hetgeen suggereert dat deze polymorphisms geen invloed hebben op het beloop van het ziektebeeld. Opvallend genoeg bleken HUS patiënten enkele jaren na het doormaken van het ziektebeeld verhoogde PAI-I spiegels te hebben vergeleken met gezonde controles. Geen relatie kon worden aangetoond met het 4G/5G polymorphisme hetgeen suggereert dat andere polymorphisms in dit gen of mogelijk andere genen een rol spelen. Toekomstig onderzoek zal dit moeten uitwijzen.

***Hoofdstuk 8*** Verschillende *in vitro* studies hebben aangetoond dat VT niet alleen de eiwitsynthese kan remmen maar ook apoptosis kan induceren in verschillende celtypen. In deze studie werd nierbiopsie materiaal van 7 HUS patiënten in de tweede of derde week van het ziektebeeld en 7 controles (patiënten met dunne basaal membraan of minimal change nefrotisch syndroom) onderzocht op de aanwezigheid van apoptosis door middel een verbeterde TUNEL techniek waarbij een extra kleuring voor RNA synthese activiteit werd uitgevoerd. Nierbiopsie materiaal van HUS patiënten lieten een sterk toegenomen RNA synthese activiteit van de tubuli zien. Met name in deze cellen werd ook aspecifieke aankleuring van de TUNEL techniek waargenomen hetgeen bevestigend dat men voorzichtig moet zijn met het interpreteren van resultaten verkregen met TUNEL techniek alleen. De TUNEL techniek in combinatie met de kleuring voor RNA synthese activiteit toonde aan dat zowel in glomeruli als tubuli van HUS patiënten verhoogde aantallen apoptotische cellen aanwezig waren vergeleken met controles. De hoge RNA synthese activiteit die wordt waargenomen zou mogelijk een reflectie kunnen zijn van cellen die bezig zijn met reparatie van de schade die tijdens het ziektebeeld is opgetreden.

***Hoofdstuk 9*** Het merendeel van de HUS patiënten herstelt zelf na het doormaken van een HUS. Vaak is alleen ondersteunende therapie in de vorm van dialyse en correctie van het elektrolyten evenwicht noodzakelijk. Dit impliceert dat er bepaalde factoren zijn die de herstelfase van het ziektebeeld mogelijk beïnvloeden of zelfs bevorderen. Vascular endothelial growth factor (VEGF) zou een van deze factoren kunnen zijn. Analyse van serum en plasma van HUS patiënten liet een toename van VEGF spiegels zien in de tweede en derde week van het ziektebeeld. Op dat moment was de klinische toestand van de patiënt al weer aan het verbeteren. Om te onderzoeken of de verhoogde VEGF waarden gevonden in plasma en serum afkomstig waren van een toegenomen VEGF productie in de nier werd nierbiopsie materiaal uit de tweede of begin derde week van het ziektebeeld van drie patiënten met HUS onderzocht. Hieruit bleek dat er geen verhoogde maar juist een verlaagde VEGF expressie in zowel

glomeruli als tubuli van de HUS patiënten was ten opzichte van controles. De expressie van de receptoren voor VEGF, Flt-1 en KDR, was tegelijkertijd toegenomen. Aangezien VEGF pas in het eind van de tweede week cq derde week stijgt is het zeer aannemelijk dat in een latere fase van het ziektebeeld wel verhoogde VEGF expressie kan worden waargenomen in de nier. Helaas was geen materiaal uit een latere periode van het ziektebeeld beschikbaar.

**Conclusie:**
De bevinding van VT gebonden aan granulocyten in de circulatie van HUS patiënten biedt perspectieven voor behandeling. Het verhinderen van de overdracht van VT aan doelwit cellen zou de ernst van het ziektebeeld ten goede kunnen beïnvloeden Als immers VT niet meer wordt overgedragen aan doelwit cellen kan er ook niet meer schade ontstaan. Een van de mogelijkheden om te interferen zou het toepassen van leukoferese kunnen zijn. Nadeel is echter dat patiënten deze methode erg ingrijpend is. Een beter alternatief zou een behandeling zijn met gehumaniseerde monoklonale antilichamen gericht tegen het toxine. Helaas is deze mogelijkheid momenteel nog in de experimentele fase en daardoor nog niet toepasbaar voor D+ HUS patiënten. De aanwezigheid van LPS in de circulatie van HUS patiënten zou hierin ook belangrijk kunnen zijn. Als immers LPS een belangrijke rol speelt in het ontstaan van het ziektebeeld dan zou inactivering van LPS bijvoorbeeld door antilichamen of door toediening van recombinant eiwit dat LPS inactiveert (BPI) beschermend kunnen werken. Toekomstig onderzoek zou zich hierop moeten richten. De bevinding dat VT in staat is om de productie van NO en ET-1 te beïnvloeden geeft meer inzicht in de thrombotische microangiopathy en het ontstaan van nierfalen in HUS. Echter bij toekomstig onderzoek moet ook de rol van andere factoren zoals thromboxaan en prostacycline in overweging worden genomen.

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## DANKWOORD

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Maroeska

## CURRICULUM VITAE

The author of this thesis was born on February 8th , 1973 in Bredevoort, the Netherlands. She attended high school in Aalten, Christelijke Scholengemeenschap Aalten (VWO), Gelderland, from which she graduated in 1991 Subsequently, she started to study medicine at the University Medical Center St Radboud of Nijmegen, the Netherlands

After passing her masters'and final examination (MD, cum laude) in 1995 and 1997, respectively, she started to work as a research fellow at the Department of Pediatrics at the University Medical Center St Radboud of Nijmegen, the Netherlands (Head of the Department Pediatrics: Prof Dr. R C.A. Sengers). During the following 3.5 years she worked on the research project described in this thesis. Studies were performed at the laboratory of the Department of Pediatrics and the Gaubius Laboratory TNO-PG, Leiden, the Netherlands She was awarded by the Dutch Society of Thrombosis and Hemostasis (January 2001) and the Dutch Society of Nephrology (March 2001) for her achievements as investigator. In June 2001, she returned to work in the clinic at the Department of Pediatric Oncology and Hematology (Head Prof Dr. P Hoogerbrugge) of the the University Medical Center St Radboud of Nijmegen, the Netherlands.

## List of publications


1. *te Loo DM*, Monnens LA, van der Velden TJ, Vermeer MA, Preyers F, Demacker PN, Heuvel LP, van Hinsbergh VW (2000) Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. *Blood 95 (11): 3396-3402*

2. *te Loo DM*, Hinsbergh VW, Heuvel LP, Monnens LA (2001) Detection of verocytotoxin bound to circulating polymorphonuclear leukocytes of patients with hemolytic uremic syndrome. *J Am Soc Nephrol 12(4): 800-806*

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4. *te Loo DM*, Monnens LA, van den Heuvel LP, Gubler MC, Kockx MM (2001) Detection of apoptosis in kidney biopsies of patients with D+ hemolytic uremic syndrome. *Pediatr Res 49(3): 413-416*

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9. Bitzan M, *te Loo DMWM*. Interaction of Shiga toxin with endothelial cells. In: Ebel F, Philpot D (eds) Shiga Toxin-producing Escherichia coli: Methods and Protocols. Humana Press INc., Totowa, NJ, USA *In press*

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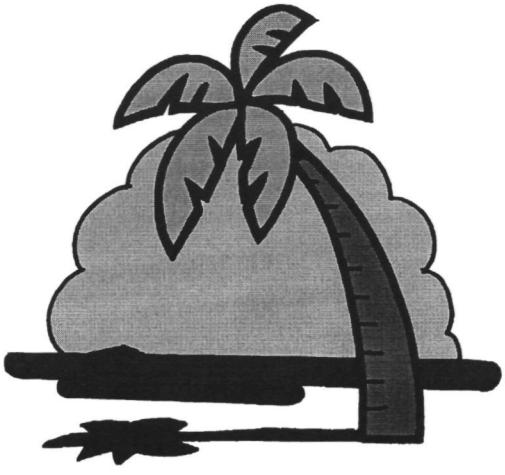
11. *te Loo DM*, Löwik MM, Monnens LA, van der Velden TJ, van Hinsbergh VW, van den Heuvel LP. Relevance of polymorphisms in the tumor necrosis factor α and plasminogen activator inhibitor-1 genes in the hemolytic uremic syndrome. *Submitted.*

12. *te Loo DM*, Bosma NJ, van Hinsbergh VW, Span PN, de Waal RM, Clarijs R, Sweep CG, Monnens LA, van den Heuvel LP. Elevated levels of vascular endothelial growth factor in serum of patients with D+ hemolytic uremic syndrome. *Submitted*



*I always keep my promise.*

# STELLINGEN

Behorend bij het proefschrift

*"De pathogenesis of the hemolytic uremic syndrome: the role of the granulocyte"*

- Met betrekking tot de pathogenese van het hemolytisch uremisch syndroom, is het vinden van verocytotoxine gebonden aan granulocyten te vergelijken met het binnenhalen van het paard van Troje ! *(dit proefschrift)*

- Het vinden van verocytotoxine gebonden aan granulocyten in de circulatie van HUS patiënten vormt de ontbrekende schakel tussen enerzijds de darminfectie en anderzijds het ontstaan van schade in onder andere de nier.*(dit proefschrift)*

- Diagnose en behandeling van cystinose dient te berusten op de meting van cystine in de granulocyte en niet in het totaal aantal leukocyten.

- Lipopolysaccharide speelt naast verocytotoxine een belangrijke rol in de pathogenese van het hemolytisch uremisch syndroom (*dit proefschrift*).

- De gevonden remming van de NO synthese van mesangiale cellen *in vitro*, veroorzaakt door incubatie met verocytotoxine, vormt mogelijk een belangrijk deel van de verklaring waarom met name in de nier vasoconstrictie en microthrombi ontstaan. De nier is immers het enige orgaan in het menselijke lichaam dat mesangiale cellen bevat. (*dit proefschrift*)

- Het feit dat er nog geen genetische oorzaak gevonden is voor het ontstaan van de typische vorm van HUS wil nog niet betekenen dat genetische factoren ook geen rol spelen in dit ziektebeeld. (*dit proefschrift*)

- Our research competitors are easily identified. They never criticise our work, neither in reports nor lectures, they simply fail to recognise its very existence. *(Lancet, augustus 2001)*

- Een positieve waardering dient vaker te worden uitgesproken !

- We moeten zelf de verandering zijn, die we in de wereld willen zien. *(Ghandi)*

- Het is opmerkelijk dat de therapie van een zo ernstig ziektebeeld als de Diamond Blackfan anemie zo eenvoudig kan zijn. Helaas betekent echter een eenvoudige therapie niet, dat ook de pathogenese wordt begrepen.

- De mogelijkheid om de minimal residual disease (MRD-) activiteit te kunnen meten bij patienten met acute leukemie zal ongetwijfeld meer inzicht geven in welke behandeling het beste en wanneer gegeven kan worden.

- De verklaring van de X-gebonden vorm van hypophosphatemische rachitis is vermoedelijk gelegen in de gestoorde afbraak van FGF-23. *(Bowe et al. Biochem. Biophys. Res. Comm. 2001).*

- Het is merkwaardig dat er wordt gedacht dat artsen door middel van financiële vergoeding gestimuleerd kunnen worden om zich in te zetten voor werven van weefseldonoren.

- Iemand die boven aan de ladder staat mag niet vergeten dat hij ooit de eerste stap heeft gezet beneden aan de ladder.

- Terrorism is a consequence of wider political and social change and thrives therefore especially in countries enduring state failure. (*Lancet, October 2001*).

- Today the real test of power is not the capacity to make war but capacity to prevent it. (*Anne O'Hara McCormick*)