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Novel *in vivo* therapeutic approaches  
to *Escherichia coli* O157:H7 infection

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DEPARTMENT OF PEDIATRICS | CLINICAL SCIENCES LUND | LUND UNIVERSITY





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# Novel *in vivo* therapeutic approaches to *Escherichia coli* O157:H7 infection

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<b>Title and subtitle:</b> Novel <i>in vivo</i> therapeutic approaches to <i>Escherichia coli</i> O157:H7 infection		
<p><b>Abstract</b> Shiga toxin (Stx), the unique virulence factor released by enterohemorrhagic <i>Escherichia coli</i> (EHEC), associated with gastrointestinal infection and in severe cases hemolytic uremic syndrome (HUS). Up until now, no effective therapies have been developed to control disease progression. In this thesis, four novel treatment strategies have been investigated targeting different aspects of EHEC pathogenesis. An established mouse model was used in which mice were infected with EHEC intragastrically. During EHEC infection, extracellular vesicles (EVs) are involved in the transport of Stx from the gut to the kidney. High levels of prothrombotic EVs that expose phosphatidylserine and tissue factor have been detected in patients with EHEC-associated HUS.</p> <p>In the first study, treatment with annexin A5 (anxA5) induced an increase in phagocytic uptake of EVs <i>in vitro</i>. Administration of anxA5 to EHEC-infected mice resulted in lower levels of circulating platelet-derived EVs and delayed disease development.</p> <p>In the second study, Stx triggered adenosine triphosphate (ATP) release from HeLa cells and similar results were obtained in a mouse model. ATP signals via purinergic receptors. Inhibition of purinergic P2X receptors with NF449 or suramin inhibited Stx-induced calcium influx and EV release. NF449 protected cells from Stx-induced toxicity and suramin decreased EV release even <i>in vivo</i> in mice.</p> <p>The third study targeted extracellular ATP by using apyrase in EHEC-infected mice. Apyrase cleaves extracellular ATP and adenosine diphosphate (ADP). Mice were injected with apyrase intraperitoneally. Treatment protected the mouse intestines from damage and delayed the onset of disease symptoms. Apyrase decreased bacterial release of Stx2 by reducing RecA involved in the SOS response and bacteriophage activation. In addition, apyrase lowered platelet aggregation when platelets were co-incubated with Stx2 and <i>E. coli</i> O157:H7 lipopolysaccharide in the presence of collagen. Thus, apyrase had a dual protective effect on both the bacterial release of toxin and host cell activation and injury.</p> <p>The fourth study focused on the effect of immunoglobulin G (IgG) on EHEC infection. Intraperitoneal administration of murine IgG to EHEC-infected mice had a clear beneficial effect on bacterial colonization, survival and intestinal and renal pathology. <i>In vitro</i> studies utilized both mouse and human IgG and showed that the Fc domain bound to the EHEC virulence factor, EspP, <i>E. coli</i> secreted serine protease. EspP is involved in bacterial adherence to the intestine, and intestinal injury during EHEC infection. It has potent enzymatic activity that was inhibited by the interaction with IgG. This indicates that the protective effects of IgG administration in EHEC-infected mice could be due to the interaction between IgG and EspP.</p> <p>In summary, this thesis investigated novel treatment strategies targeting different aspects of EHEC pathogenesis such as bacterial colonization, release of Stx from EHEC, phagocytic clearance of Stx-containing and prothrombotic EVs in the circulation and protection against the cytotoxic and prothrombotic effects of Stx using both <i>in vitro</i> and <i>in vivo</i> experimental set-ups. The results show that treatments such as anxA5, apyrase and IgG, when administered at an early stage of infection, may have therapeutic potential in EHEC infection.</p>		
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# Novel *in vivo* therapeutic approaches to *Escherichia coli* O157:H7 infection

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#### Cover photo

Immunofluorescence microscopy image of blood cell-derived extracellular vesicles in red, taken up by differentiated human monocytic cells (THP-1) in green, by Ashmita Tontanahal

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# List of papers

This thesis is based on the following papers:

- I. **Tontanahal A**, Arvidsson I, Karpman D. Annexin induces cellular uptake of extracellular vesicles and delays disease in *Escherichia coli* O157:H7 infection. *Microorganisms*. 2021; 9(6):1143.
- II. Johansson KE, Ståhl AL, Arvidsson I, Loos S, **Tontanahal A**, Rebetz J, Chromek M, Kristoffersson AC, Johannes L, Karpman D. Shiga toxin signals via ATP and its effect is blocked by purinergic receptor antagonism. *Sci Reports*. 2019; 9(1):14362.
- III. Arvidsson I, **Tontanahal A**, Johansson KE, Kristoffersson AC, Kellnerová S, Berger M, Dobrindt U, Karpman D. Apyrase exerts protective effects in *Escherichia coli* O157:H7 infection by decreasing bacterial Shiga toxin release and host cell injury: in vivo and in vitro studies. Manuscript.
- IV. **Tontanahal A**, Sperandio V, Kovbasnjuk O, Loos S, Kristoffersson AC, Karpman D, Arvidsson I. IgG binds *Escherichia coli* serine protease EspP and protects mice from *E. coli* O157:H7 infection. Submitted.

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Johansson KE, Willysson A, Kristoffersson AC, **Tontanahal A**, Gillet D, Ståhl AL, Karpman D. Shiga toxin-bearing microvesicles exert a cytotoxic effect on recipient cells only when the cells express toxin receptor. *Front Cell Infect Microbiol*. 2020;10:212.

Karpman D, **Tontanahal A**. Extracellular vesicles in renal inflammatory and infectious diseases. *Free Radic Biol Med*. 2021; 171:42-54.

# Abbreviations

A/E	Attaching and effacing lesion
ADP	Adenosine diphosphate
AnxA5	Annexin A5
ATP	Adenosine triphosphate
BiP	Binding immunoglobulin protein
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
Esp	<i>Escherichia coli</i> secreted protein
ER	Endoplasmic reticulum
EVs	Extracellular vesicles
Gb3	Globotriaosylceramide
Gb4	Globotetraosylceramide
GP	Glycoprotein
HUS	Hemolytic uremic syndrome
IgG	Immunoglobulin G
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
OMVs	Outer membrane vesicles
PS	Phosphatidylserine
Stx	Shiga toxin
TLR	Toll-like receptor
T3SS	Type three secretion system



# Abstract

Shiga toxin (Stx), the unique virulence factor released by enterohemorrhagic *Escherichia coli* (EHEC), associated with gastrointestinal infection and in severe cases hemolytic uremic syndrome (HUS). Up until now, no effective therapies have been developed to control disease progression. In this thesis, four novel treatment strategies have been investigated targeting different aspects of EHEC pathogenesis. An established mouse model was used in which mice were infected with EHEC intragastrically. During EHEC infection, extracellular vesicles (EVs) are involved in the transport of Stx from the gut to the kidney. High levels of prothrombotic EVs that expose phosphatidylserine and tissue factor have been detected in patients with EHEC-associated HUS.

In the first study, treatment with annexin A5 (anxA5) induced an increase in phagocytic uptake of EVs *in vitro*. Administration of anxA5 to EHEC-infected mice resulted in lower levels of circulating platelet-derived EVs and delayed disease development.

In the second study, Stx triggered adenosine triphosphate (ATP) release from HeLa cells and similar results were obtained in a mouse model. ATP signals via purinergic receptors. Inhibition of purinergic P2X receptors with NF449 or suramin inhibited Stx-induced calcium influx and EV release. NF449 protected cells from Stx-induced toxicity and suramin decreased EV release even *in vivo* in mice.

The third study targeted extracellular ATP by using apyrase in EHEC-infected mice. Apyrase cleaves extracellular ATP and adenosine diphosphate (ADP). Mice were injected with apyrase intraperitoneally. Treatment protected the mouse intestines from damage and delayed the onset of disease symptoms. Apyrase decreased bacterial release of Stx2 by reducing RecA involved in the SOS response and bacteriophage activation. In addition, apyrase lowered platelet aggregation when platelets were co-incubated with Stx2 and *E. coli* O157:H7 lipopolysaccharide in the presence of collagen. Thus, apyrase had a dual protective effect on both the bacterial release of toxin and host cell activation and injury.

The fourth study focused on the effect of immunoglobulin G (IgG) on EHEC infection. Intraperitoneal administration of murine IgG to EHEC-infected mice had a clear beneficial effect on bacterial colonization, survival and intestinal and renal pathology. *In vitro* studies utilized both mouse and human IgG and showed that the Fc domain bound to the EHEC virulence factor, EspP, *E. coli* secreted serine



protease. EspP is involved in bacterial adherence to the intestine, and intestinal injury during EHEC infection. It has potent enzymatic activity that was inhibited by the interaction with IgG. This indicates that the protective effects of IgG administration in EHEC-infected mice could be due to the interaction between IgG and EspP.

In summary, this thesis investigated novel treatment strategies targeting different aspects of EHEC pathogenesis such as bacterial colonization, release of Stx from EHEC, phagocytic clearance of Stx-containing and prothrombotic EVs in the circulation and protection against the cytotoxic and prothrombotic effects of Stx using both *in vitro* and *in vivo* experimental set-ups. The results show that treatments such as anxA5, apyrase and IgG, when administered at an early stage of infection, may have therapeutic potential in EHEC infection.

**Keywords:** EHEC, hemolytic uremic syndrome, Shiga toxin, extracellular vesicles, annexin A5, purinergic signaling, ATP, apyrase, IgG, EspP

# Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), is a highly potent human pathogen causing gastrointestinal infection and in severe cases leading to the development of hemolytic uremic syndrome (HUS).<sup>1</sup> HUS is defined by acute kidney injury, hemolytic anemia and thrombocytopenia, occurring simultaneously.<sup>2</sup> After ingestion of contaminated food or water, EHEC migrates through the gastrointestinal tract and colonizes the larger intestine.<sup>3</sup> EHEC is a non-invasive bacterium and elicits its effects by release of virulence factors, both in the intestine and systemically, leading to infection of the gastrointestinal tract and in severe cases bloody diarrhea due to hemorrhagic colitis.

Shiga toxin (Stx), a bacteriophage-encoded protein, is considered to be the main virulence factor released by EHEC upon bacterial cell lysis.<sup>4,5</sup> Stx is an AB<sub>5</sub> toxin with an enzymatic A-subunit and one pentameric binding B-subunit.<sup>6</sup> The toxin binds to the glycolipid globotriaosylceramide<sup>7</sup> or globotetraosylceramide receptors, termed Gb<sub>3</sub> and Gb<sub>4</sub>, respectively.<sup>8</sup> Following binding, Stx is taken up by the cell and undergoes retrograde transport first to the trans-Golgi network and then the endoplasmic reticulum (ER), before being released into the cytosol.<sup>9</sup> In the cytosol, Stx interacts with the ribosome where it cleaves an adenine off the 28S rRNA, thereby inhibiting protein synthesis and resulting in cell death.<sup>6</sup>

After adherence of EHEC to the intestinal epithelial cells, Stx is released in the intestine. Severe intestinal damage allows the toxin to gain access into the circulation and thereafter reach the main target organ, the kidney. Stx has been shown to cause intestinal damage in mice<sup>10</sup> and be associated with dysentery in a primate model of *Shigella dysenteriae*.<sup>11</sup> Studies have shown that minimal amounts of free Stx are detected in patient serum.<sup>12</sup> However, Stx was bound to platelets,<sup>13-15</sup> monocytes,<sup>14, 16</sup> neutrophils<sup>14, 17, 18</sup> and red blood cells in patient samples.<sup>19, 20</sup> Binding of Stx to these blood cells induces the release of Stx-containing extracellular vesicles (EVs).<sup>14,20,21</sup> EVs are plasma membrane-derived vesicles that are released during cellular activation and apoptosis. EVs mediate cell-to-cell communication via transfer of receptors, nucleic acids and proteins from the parent cell to neighboring and distant cells.<sup>22</sup> Stx-positive blood cell-derived EVs circulate in the plasma of EHEC-infected patients and in EHEC-infected mice.<sup>21</sup> Toxin within vesicles were detected in the kidney of one patient and in EHEC-infected mice.<sup>21</sup> This indicates that Stx is transported systemically within blood cell-derived EVs which are then taken up by kidney cells. Upon reaching the kidney cells, the EVs

are internalized, the toxin is released and inhibits protein synthesis leading to cell death.<sup>21</sup>

To-date no effective treatment strategies have been developed against EHEC infection to control disease progression. Management involves symptomatic treatment and treatment with antibiotics are not recommended. Antibiotic treatment has been shown to induce the bacterial SOS response, leading to an increase in Stx production and release.<sup>23</sup> Antibiotic treatment has also been shown to increase the risk of development of HUS during the diarrheal phase of infection,<sup>24</sup> suggesting that its use during EHEC infection could be contraindicative. This thesis addresses four novel treatment strategies that targets various aspects of EHEC pathogenesis.

# Enterohemorrhagic *Escherichia coli*

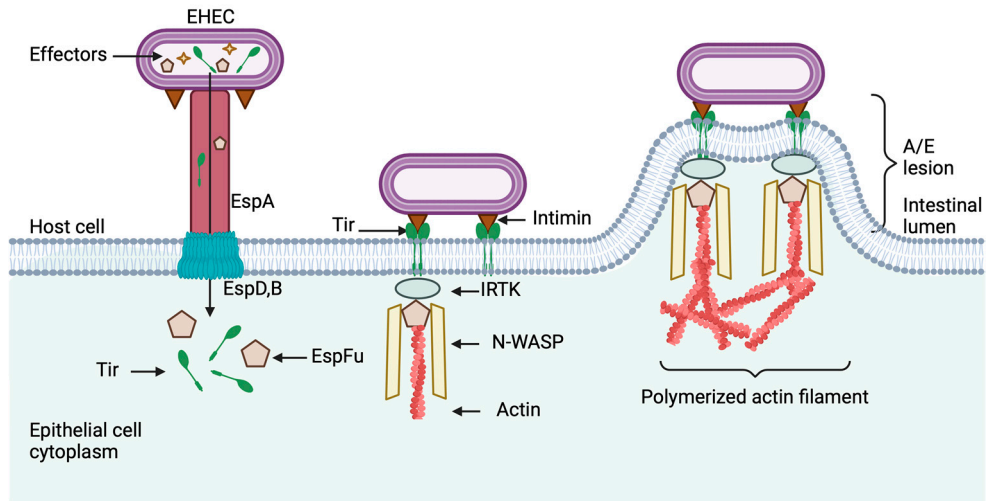
Enterohemorrhagic *Escherichia coli* (EHEC) is a pathogenic form of *E. coli*. EHEC has a zoonotic origin and cattle are recognized as its main reservoir.<sup>25</sup> Infection in humans mainly occurs by ingesting contaminated food or water. Upon ingestion, EHEC is transported through the gastrointestinal tract and colonizes the large intestine.<sup>26</sup> EHEC is non-invasive and does not cause bacteremia or septicemia<sup>3</sup> but it elicits its effects in the intestine, and systemically, by the release of virulence factors leading to gastrointestinal infection and bloody diarrhea, followed, in certain cases, by systemic spread of virulence factors.

Several pathogenic serotypes of EHEC have been identified and linked with human disease. They are subdivided in relation to the presence of lipopolysaccharide (LPS, O-antigen) and the flagella (H-antigen). *E. coli* O157:H7 was first isolated during an outbreak in Michigan and is considered to be the most common clinical isolate of EHEC associated with human disease.<sup>27</sup> Other EHEC serotypes that have been linked with human disease include O157:NM, O145:NM, O104:H21, O26:H11, O103:H2, O111:NM, O121:H9, O91:H21, O113:H21 and many others.<sup>28</sup>

## EHEC adhesion to cells in the gastrointestinal tract

Adherence of EHEC to the intestinal epithelium is regulated by several proteins encoded by genes located in the locus of enterocyte effacement (LEE). Some of these proteins are responsible for the formation of attaching and effacing lesions (A/E lesions). In order for the bacteria to attach intimately to the host intestinal epithelial cells, EHEC express several proteins that assemble to form the type III secretion system (T3SS) (Figure 1). The T3SS consists of a needle-like structure containing the *E. coli* secreted protein (Esp)A filament which forms a hollow channel through which effector proteins can be translocated from the bacterium into the host cell. The EspA filament attaches to a translocation pore composed of EspB and EspD<sup>29</sup> on the host cell membrane through which effector proteins are delivered to the host. Through this pore, EHEC transports translocated intimin receptor (Tir) as well as Esp F-like protein from prophage U (EspF<sub>U</sub>).<sup>30, 31</sup> Tir injected into the host cell adopts a hairpin loop confirmation and the extracellular domain of Tir binds to the bacterial outer membrane protein, intimin.<sup>32</sup> This results in clustering of Tir and initiation of a signaling cascade causing polymerization of actin and formation of a

pedestal, a characteristic of A/E lesion. EspF<sub>U</sub> along with Tir, recruit Wiskott-Aldrich syndrome protein (N-WASP) and contributes to actin polymerization.<sup>31, 33</sup> A complex formed by Tir, N-WASP and EspF<sub>U</sub> initiates the formation of actin assembly in the host cell. Insulin receptor tyrosine kinase (IRTK) has been identified as the link between Tir and EspF<sub>U</sub>. IRTK participates in the formation of actin pedestal-like structures (reviewed in<sup>34</sup>). Adherence of EHEC to intestinal epithelial cells followed by colonization of the bowel is regulated by the interplay between the commensal bacteria located in the intestine and EHEC, discussed in the following section.



**Figure 1: Schematic representation of an attaching and effacing lesion formed by EHEC.**

EHEC attaches to the intestinal epithelial cells via the type 3 secretion system and injects effector proteins and Tir (translocated intimin receptor) into the cell cytoplasm. Tir is inserted into the cell membrane, dimerizes and binds to intimin. EspF<sub>U</sub> (*E. coli* secreted F-like protein from prophage U) along with Tir triggers polymerization of actin filaments in the cell cytoskeleton and subsequent formation of a pedestal-like structure resulting in an attaching and effacing lesion (A/E lesion).

Successful EHEC colonization is achieved by overcoming the host barriers that protect from invading pathogens. These include biological, mechanical and chemical barriers.<sup>35</sup> The biological barrier constitutes the intestinal microflora and studies have shown that modifying the intestinal microflora aids in establishing a successful colonization.<sup>36</sup> The mechanical barrier is provided by the mucus layer lining the intestinal epithelial cells. Studies have shown that the thickness and composition of the mucus layer is essential for protection against adherence of pathogens. The third line of protection offered by the host is a chemical barrier. Intestinal epithelial cells produce several antimicrobial peptides and proteins that, in turn, protect the integrity of the mucus layer.<sup>37</sup> Taken together, EHEC needs to

overcome these barriers in order to establish intestinal colonization. The role of the biological barrier in EHEC colonization is further discussed below.

## Interplay between intestinal microflora and EHEC

The ability of EHEC to interact with the host microflora is genetically determined by quorum sensing, a mechanism employed by certain pathogenic bacteria to communicate with neighboring bacteria by the release of hormone-like compounds called autoinducers.<sup>38</sup> EHEC responds to specific signals that activate its virulence genes. These signals include a bacterial signal called autoinducer-3, produced by the intestinal commensal microflora, as well as adrenaline and noradrenaline, produced by the host. These signals trigger sensory kinases in the EHEC membrane which leads to activation of a regulatory cascade called quorum sensing.<sup>39</sup> This cascade activation in turn triggers activation of genes involved in motility, expression of LEE proteins and expression of EHEC virulence factors.

Two serine kinases have been identified in EHEC that are activated in response to adrenaline and noradrenaline.<sup>40</sup> One such sensory kinase is QseC. The role of QseC in EHEC-associated virulence has been studied using *qseC* mutants that attenuated the bacterial virulence in an animal model of EHEC infection.<sup>41</sup> *In vitro* and *in vivo* studies have shown that QseC is involved in pathogenesis of EHEC and inter-kingdom signalling.<sup>42</sup> QseEF is another sensory kinase that influences the formation of A/E lesions on intestinal epithelial cells by EHEC.<sup>43</sup> Both QseC and QseEF are involved in regulation of genes responsible for production of EHEC virulence factors.<sup>40</sup> Thus signaling via autoinducers produced by the microflora as well as QseC and QseEF plays a vital role in regulating expression of virulence genes in response to the intestinal microenvironment (reviewed in<sup>44</sup>).

## Key players of EHEC

The interaction between host microbiota and EHEC has been linked to activation of genes responsible for expression of Shiga toxin (Stx), the most potent virulence factor of EHEC.<sup>38,39</sup> Following adherence of EHEC to the host's intestinal epithelial cells after overcoming the barriers set up by the host, Stx is released in the intestine. Other factors that contribute to EHEC virulence include LPS, EHEC-hemolysin, subtilase and *E. coli* secreted serine protease, EspP.

## Shiga toxin

Kiyoshi Shiga was the first to describe *Shigella dysenteriae*, a Stx-producing bacterium that could cause bloody diarrhea.<sup>45</sup> Like *Shigella*, EHEC also produce Stx.<sup>46</sup> Stx in EHEC is encoded by a bacteriophage inserted into the bacterial chromosome and is released upon bacterial cell lysis.<sup>4,5</sup>

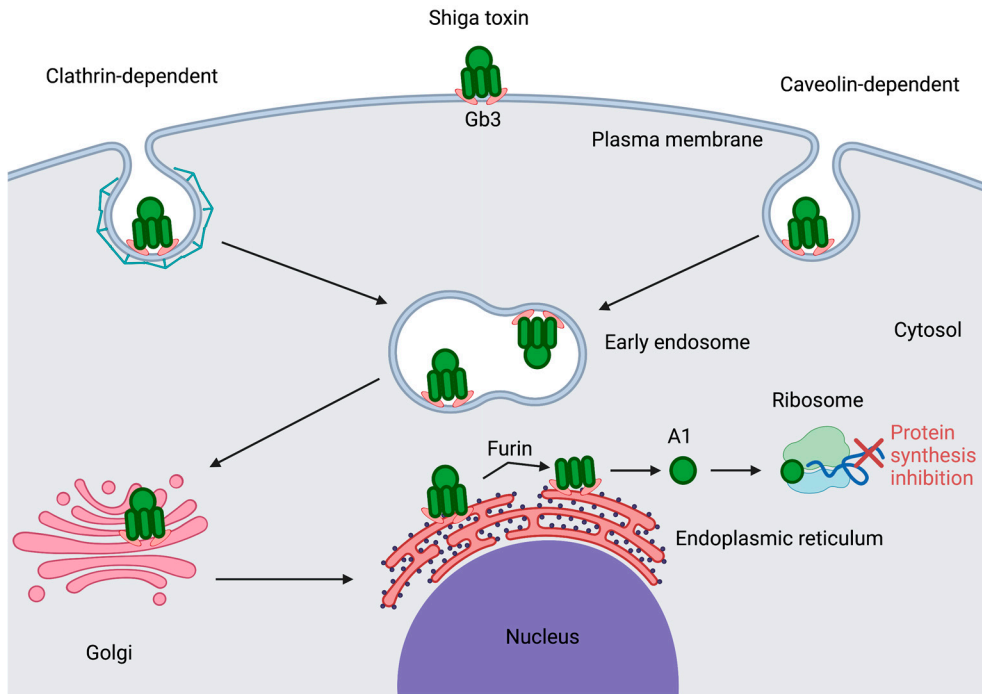
### *Shiga toxin and its receptors*

Stx can be sub-divided into two major groups, Stx1 and Stx2. Stx produced by *Shigella dysenteriae* differs by a single amino acid from Stx1 produced by *E. coli*,<sup>47</sup> while Stx1 and Stx2 share 56% amino acid homology.<sup>48</sup> Several variants of both Stx1 and Stx2 have been identified. The known variants of Stx1 include Stx1a, Stx1c and Stx1d<sup>49</sup> and for Stx2, the variants include Stx2a-h.<sup>50</sup> All variants of Stx, have the conserved structure consisting of the A and B-subunits described above (reviewed in<sup>51</sup>). Except for Stx2e,<sup>52</sup> all Stxs bind to the cellular glycosphingolipid receptor Gb3<sup>7</sup> or Gb4.<sup>8</sup> Binding is considered to be a necessary step for their cytotoxicity, therefore in line with this, mice and cells lacking Gb3 are insensitive to Stx.<sup>53,54</sup>

The Gb3 receptor is located in detergent-resistant membrane domains that are enriched with cholesterol and is required for the retrograde transport of Stx.<sup>55</sup> These specialized locations in the plasma membrane of the cell are called lipid rafts and are involved in transduction of cellular signals.<sup>56</sup> In HeLa cells, the interaction of Stx1 with the cells led to an increase in Gb3 expression in the lipid rafts and Stx is transported to the endoplasmic reticulum (ER).<sup>57</sup> An increase in Stx binding has also been achieved upon cholesterol extraction from adult renal tissue and led to an intracellular transport of the toxin to the lysosome.<sup>58</sup> These studies suggest that the interaction between Stx and its receptors could vary depending on the cell type and microenvironment of the membrane where the receptor is located.

### *Uptake and intracellular transport of Shiga toxin*

After binding to its cell surface receptors, Stx is taken up by the cell. This can occur by several endocytic pathways including clathrin-dependent pathways or caveolin-dependent pathways.<sup>59,60</sup> Receptor-bound toxin molecules cluster in the membrane and induce invaginations.<sup>61</sup> These invaginations containing Stx are stabilized with the help of endophilin and actin, while dynamin is responsible for the formation of intracellular vesicles by inducing scission of invaginations.<sup>62</sup> Thus the intracellular vesicles containing Stx fuse with the early endosome, and are then transported further to the trans-Golgi network and subsequently to the ER (Figure 2) (reviewed in<sup>63</sup>).



**Figure 2: Schematic representation of Shiga toxin retrograde transport in cells.**

Shiga toxin binds to Gb3 expressed on the cell surface. The toxin is taken up into the early endosome, after which it is transported to the Golgi apparatus and finally reaching the endoplasmic reticulum (ER). Before reaching the cytosol furin in the ER cleaves the A subunit of the toxin.

The intracellular route of toxin transport is dependent on the localization of Gb3 in the cell membrane. In macrophages and dendritic cells, Gb3 is not localized in lipid rafts and thereby the toxin is directed towards lysosomes, while in HeLa cells, the toxin is transported to the ER, owing to the localization of Gb3 in lipid rafts.<sup>64</sup> The retrograde transport of the toxin from the endosome to the trans-Golgi network is well regulated and is essential for its toxic effects.<sup>65</sup>

When Stx reaches the ER, the A-subunit is enzymatically cleaved by furin.<sup>66</sup> Furin is an enzyme that cleaves the disulfide bond linking the A1 and A2 moiety of the toxin. The A1 moiety is unfolded by binding immunoglobulin protein (BiP) which is an ER chaperone.<sup>67</sup> This chaperone is associated with a translocase and helps in refolding and translocation of the A1 moiety from the ER into the cytosol.<sup>67, 68</sup>



### *Intracellular function of Shiga toxin*

In the cytosol, Stx binds to ribosomes and exerts its cytotoxic effect by the depurination of an adenine from the 28s rRNA.<sup>69</sup> This leads to the catalytic inactivation of the ribosome and inhibits protein synthesis.<sup>6</sup> Stx has also been shown to induce apoptosis in several different cell types such as intestinal epithelial cells, renal proximal tubular epithelial cells and glomerular cells.<sup>70-72</sup> The mechanism by which Stx induces apoptosis has been studied in epithelial cells, showing that apoptosis is induced by activation of caspase 3, 8 and 9.<sup>73-75</sup> Studies have also shown that Stx enhances the expression of Bax,<sup>76, 77</sup> which in turn induces the release of cytochrome C, leading to promotion of apoptosis.<sup>78</sup> Bcl-xL is a potent inhibitor of Bax. Cells are protected from Stx-induced apoptosis by downregulating Bax and upregulating Bcl-xL, for example by ouabain.<sup>77</sup> While Stx-induced apoptosis can be mediated by caspase, apoptosis can also be mediated by a caspase-independent pathway.<sup>79</sup> Stx inhibited expression of an anti-apoptotic protein associated with early onset of apoptosis in endothelial cells.<sup>80</sup>

Stx has been shown to activate stress-associated signaling pathways called the ribotoxic stress response or ER stress response. Signaling via these pathways induces release of cytokines through activation of transcription factors<sup>81</sup> and in turn leads to apoptosis, thus contributing to Stx-associated disease (reviewed in<sup>82</sup>). Stx induces a ribotoxic stress response by activation of p38 and c-Jun N-terminal kinase mitogen activated protein kinase (MAPK).<sup>70</sup> This stress response is activated by the depurination reaction occurring in the ribosome. Toxoid, a modified form of Stx that lacks its toxic activity but is still antigenic, cannot induce activation of MAPK and hence does not induce ribotoxic stress.<sup>83</sup>

Stx can also induce ER stress signaling. As mentioned above, Stx upon localization to the ER, is recognized by BiP.<sup>67</sup> BiP is a protein regulating the ER stress response by surveying protein folding and inhibiting stress-inducing proteins. In the ER, when an incorrectly folded protein is identified by BiP, BiP associates with this protein. BiP then loses its inhibitory effect on the ER stress proteins which leads to ER stress signaling.<sup>84</sup>

In summary, Stx exerts multiple mechanisms of cytotoxicity including ribonuclease activity, induction of ER stress and apoptosis.

### *Release of Shiga toxin from the bacteria and its transport across epithelial cells*

Stx released in the intestine may migrate through or between cells in order to gain access to the circulation and thereafter be transported to the kidney. Stx has been shown to cause intestinal damage in animal models of *E. coli* O157:H7 infection<sup>10</sup> and hemorrhagic colitis in *Shigella dysenteriae* 1 infection.<sup>11</sup> The exact mechanism by which Stx is transported across the intestinal epithelial cells has not been completely elucidated while several mechanisms have been described.

In the intestine, Stx is released following bacterial cell lysis from EHEC<sup>85</sup> and/or via outer membrane vesicles (OMVs).<sup>86</sup> OMVs are vesicles secreted from the outer membrane of all Gram-negative bacteria.<sup>87</sup> Stx can be transported across the intestinal epithelial cells via transcytosis, as has been described for both Stx1 and Stx2.<sup>88</sup> Furthermore, the toxin has been shown to be transported via the paracellular route, by which the toxin passes the intestinal epithelium in the intercellular space between cells. This process is enhanced by the transmigration of neutrophils in the opposite direction, towards the intestinal lumen.<sup>89</sup>

Stx has also been shown to be taken up by intestinal cells via macropinocytosis. This is followed by the release of Stx to the basolateral environment by transcytosis. Inhibition of macropinocytosis by inhibiting proteins involved in actin remodeling during the formation of macropinocytic blebs, reduced the transport of Stx across the intestinal epithelial cells.<sup>90</sup>

Irrespective of the mechanism by which Stx is transported across the intestinal cells, it encounters a layer of connective tissue, the lamina propria. Stx has been demonstrated in the lamina propria in a study involving intestinal organ culture.<sup>91</sup> Studies have shown that EHEC infection in humans can cause severe intestinal inflammation and mucosal damage.<sup>10, 92</sup> Stx leaks through the damaged mucosal barrier, enters the circulation and interacts with blood cells. This aspect will be further discussed under the host response.

## **Lipopolysaccharide**

LPS is an abundant outer membrane protein found in gram-negative bacteria. LPS activates the innate immune response and when LPS is found in systemic circulation it is termed as endotoxemia. Studies have shown the presence of serum antibodies against O157LPS and elevated levels of LPS binding proteins in patients during the acute phase of *E. coli* O157:H7 infection.<sup>93, 94</sup> During bacterial proliferation or lysis, fragments of the bacterial membrane containing LPS may gain access to the circulation. In the circulation, LPS interacts with CD14, an anchored protein expressed on the cell surface. This interaction transfers LPS to toll-like receptor (TLR)4/MD2.<sup>95</sup> LPS can also interact with soluble CD14.<sup>96</sup> The interaction between LPS and CD14 can induce intracellular signaling via myeloid differentiation primary response 88 (MyD88).<sup>97</sup> Binding of LPS to TLR4 induces the release of pro-inflammatory cytokines and type 1 interferon, resulting in a strong immune response (reviewed in<sup>98</sup>).

The role of LPS in the pathogenesis of EHEC-associated disease has been studied in animal models. Coadministration of LPS and Stx1 in baboons, showed that LPS potentiated Stx-induced toxicity by upregulating renal Stx receptor.<sup>99</sup> Lethal effects of Stx2 were also enhanced upon LPS pretreatment of both rabbits and mice,<sup>100-102</sup> indicating that LPS and Stx could act synergistically. The synergistic effect of LPS

and Stx has also been shown in *in vitro* studies involving human umbilical vein endothelial cells showing that preincubation with LPS enhanced the cytotoxicity of Stx.<sup>103</sup>

LPS from *E. coli* O157 has been shown to bind and activate platelets through TLR4/CD62 receptor complex. In mice, O157LPS binds to TLR4 on platelets and leads to platelet activation.<sup>104</sup> These mice also had reduced platelet counts which could be due to a direct consequence of LPS binding to platelets.<sup>104</sup> Similarly, interaction between LPS and human platelets led to activation of platelets as measured by expression of glycoprotein (GP)IIb/IIIa receptor.<sup>104</sup> GPIIb/IIIa is a major protein involved in platelet adhesion and aggregation.<sup>105</sup> LPS has been found to be bound to platelets of children with EHEC-associated HUS and this binding was also detected even before the children developed HUS.<sup>104</sup> During HUS, many factors can contribute to platelet activation such as endothelial damage, release of chemokines from monocytes<sup>106</sup> and endothelial cells<sup>107</sup> and direct activation of platelets by Stx and LPS.<sup>14</sup> Stx has been shown to bind platelets and activate them.<sup>13, 15</sup> This data suggests that platelet activation occurring during HUS could be due to a combined stimulatory effect, thus contributing to microthrombi formation during HUS.

## **Outer membrane vesicles**

OMVs are membrane vesicles that arise due to blebbing of the bacterial outer membrane of gram-negative bacteria.<sup>87</sup> Release of OMVs is a ubiquitous process and has been observed for several non-pathogenic and pathogenic bacteria including *E. coli* O157:H7.<sup>86</sup> OMVs have been shown to be carriers of bacterial toxins and other virulence factors, protecting their contents from the external environment, resulting in long distance transportation.<sup>65, 108-110</sup> Bacteria tend to increase the release of OMVs when they encounter stress signals in their surrounding environment. EHEC has been shown to increase the secretion of OMVs loaded with outer membrane proteases. These protect the bacteria from the anti-microbial protein, cathelicidin. Anti-microbial proteins are released by the host epithelial cells as a defense mechanism against pathogens. Thus, outer membrane protease-loaded OMVs act as a bacterial mechanism against cathelicidin.<sup>111</sup> EHEC-associated OMVs can also contain Stx, cytolethal dissecting toxin V, EHEC-hemolysin and flagellin.<sup>65, 86, 109</sup> These OMVs can transport virulence factors and cause host cells to arrest in the G2 phase. EHEC OMVs can induce apoptosis in human intestinal epithelial and microvascular endothelial cells.<sup>65, 112</sup> Furthermore, EHEC-associated OMVs carrying Stx2a have been shown to cause renal damage in a mouse model suggesting the role of OMVs in EHEC pathogenesis.<sup>113</sup>

### ***E. coli* secreted serine protease, EspP**

The release of Esps has been shown to be associated with the formation of A/E lesions in the host cell.<sup>114</sup> *E. coli* strains secrete serine proteases belonging to the protein family of serine protease autotransporters of *Enterobacteriaceae* (SPATE) via the type-V secretion system.<sup>115</sup> An EHEC secreted serine protease, EspP has been shown to be involved in adherence of EHEC to intestine of calves and bovine intestinal epithelial cells.<sup>116</sup> It has been suggested to play a part in the development of watery diarrhea by contributing to ion transport in human colonoid cells.<sup>117</sup> EspP has been related to the virulence of EHEC strains.<sup>118</sup> EspP has been shown to cleave coagulation factor V<sup>119</sup> and complement factors C3, C3b and C5<sup>120</sup> although it is unclear if these enzymatic processes take place *in vivo*. *In vitro* it can also cleave pepsin.<sup>121</sup>



# EHEC-induced hemolytic uremic syndrome

HUS manifests with the simultaneous development of non-immune hemolytic anemia, thrombocytopenia and acute kidney injury.

Hemolytic anemia during HUS is associated with the fragmentation of red blood cells, resulting in generation of schistocytes.<sup>122</sup> It is assumed that red blood cells are damaged upon passage through narrow and partially occluded vessels. Additionally, oxidative damage of the red blood cells leads to alterations in membrane fluidity, which has also been described as a contributing factor to hemolysis observed during HUS.<sup>123</sup> EHEC-associated HUS patients were found to have complement deposition on red blood cells and similarly, Stx induces complement deposition on red blood cells *in vitro*.<sup>20</sup> These data suggests that hemolysis occurring during HUS could be partly due to complement activation on red blood cells induced by Stx.

Thrombocytopenia in HUS is secondary to platelet consumption associated with their activation and secondary to interaction with damaged endothelial cells.<sup>15, 34</sup> Platelets can be activated either by Stx and LPS directly<sup>14, 15</sup> or by cytokines released by activated cells.<sup>106, 107</sup> On damaged endothelial cells, platelets interact with fibrinogen, collagen and von Willebrand factor, leading to the formation of aggregates.<sup>124</sup> *In vitro* stimulation of platelets with Stx and LPS leads to platelet activation<sup>15, 104</sup> and release of platelet-derived EVs.<sup>14, 125</sup> Platelet-derived EVs have been found in HUS patients indicative of *in vivo* platelet activation.<sup>104</sup> During HUS, platelets are involved in inflammatory processes by interacting with leukocytes<sup>14</sup> and by release of proinflammatory cytokines.<sup>126</sup> Activation of platelets leads to their deposition on the endothelium and the formation of microthrombi during HUS.

Acute kidney injury during HUS is characterized by severe renal cortical injury.<sup>72</sup> This manifests with vessel wall thickening, detachment of endothelial cells, microthrombi formation and occlusion of glomerular capillaries as well as severe tubular damage.<sup>72</sup> Studies have shown that Stx does in fact reach the kidney after being released in the intestine.<sup>21, 127, 128</sup> The toxin affects glomerular and tubular cells by inducing apoptosis.<sup>72</sup> In addition to this, activation and influx of neutrophils<sup>129</sup> occurs as well as aggregation of platelets on damaged endothelial cells resulting in the formation of microthrombi.<sup>126</sup> Elevated cytokine levels have been associated with renal damage in HUS patients.<sup>130</sup> Complement activation could also contribute

to renal tissue injury as Stx and other EHEC-associated virulence factors can activate the complement system.<sup>20, 131, 132</sup> Complement activation could contribute to toxin-induced cellular injury and trigger prothrombotic reactions.

# Host response during EHEC infection

## EHEC interaction with the intestinal epithelium

EHEC colonization of the intestine leads to an inflammatory response.<sup>35, 36</sup> The innate immune system is activated by pathogen-associated molecular patterns (PAMPs) including LPS and flagellin.<sup>133, 134</sup> Recognition of PAMPs initiates a signaling cascade mediated by TLR4 and 5. The intestinal epithelial cells in response to infection include release of pro-inflammatory cytokines TNF $\alpha$  and IL-8.<sup>135, 136</sup> IL-8 is considered as a potent neutrophil chemoattractant and an increase in IL-8 levels has been associated with an increased risk of development of HUS during EHEC infection in children.<sup>137, 138</sup> Neutrophil translocation across the intestinal epithelial cells promotes systemic cytokine release.<sup>89</sup> Rabbits challenged with Stx resulted in an increase of chemokine mRNA levels in the kidney and urine protein levels.<sup>139</sup> Children with HUS also exhibited elevated levels of cytokines in serum and urine.<sup>130</sup>

## Shiga toxin interaction with blood cells

There are minimal amounts of free Stx in the circulation in patients with HUS.<sup>12</sup> Toxin binds to blood cells (neutrophils, monocytes, platelets and red blood cells) and can thus circulate systemically.<sup>13, 14, 16, 19</sup> Stx is not toxic to any of these blood cells.<sup>16, 35</sup>

Stx binds to platelets via Gb3 and a glycosphingolipid receptor termed 0.03.<sup>13, 140</sup> Stx circulates bound to platelets during HUS.<sup>14</sup> Binding of Stx to platelets results in internalization of the toxin, leading to activation and aggregation on endothelial cells.<sup>15</sup> This in turn leads to platelet consumption involved in the formation of microthrombi on damaged endothelium, resulting in thrombocytopenia.<sup>34</sup> Low platelet counts on account of platelet consumption in renal microthrombi are a characteristic of HUS.

Stx binds to neutrophils as demonstrated in patients with HUS.<sup>12, 14, 17</sup> Stx also binds to platelet and neutrophil aggregates.<sup>14</sup> There is a rise in neutrophils during HUS which is associated with prognosis.<sup>141</sup> This rise in neutrophils could be due to the effect of Stx2-induced stimulation of polymorphonuclear neutrophil progenitors as shown by *in vivo* studies.<sup>142</sup> During EHEC infection, neutrophils are activated thus



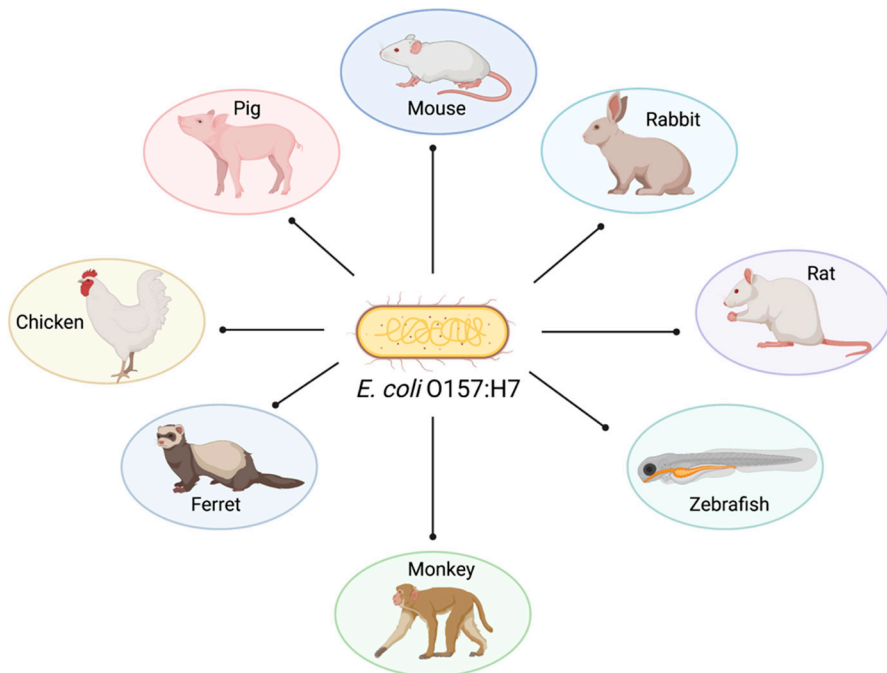
inducing release of proteases and reactive oxygen species.<sup>143</sup> These activated neutrophils adhere to the endothelium in the presence of Stx, leading to the expression of MCP-1 and IL-8 on the endothelial cell surface and leukocyte transmigration.<sup>81</sup> Thus Stx-induced activation of neutrophils, leading to degranulation and adherence to the endothelium could lead to increased endothelial damage.

Monocytes express Gb3 and can thereby bind Stx. Binding is enhanced in the presence of LPS. Stx does not inhibit protein synthesis in monocytes.<sup>16</sup> Binding of Stx induces the release of cytokines namely IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  as shown *in vitro*.<sup>16</sup> This in turn leads to activation of platelets<sup>106</sup> and monocytic cytokines can thereby participate in prothrombotic mechanisms associated with HUS. Stx2 and LPS induce tissue factor expression on monocytes and these activated monocytes can form complexes with platelets.<sup>14</sup> Additionally, Stx2 induces the release of monocyte-derived EVs bearing tissue factor which are found in the circulation in HUS patients.<sup>14</sup> Tissue factor-positive EVs can bind to platelets *in vitro* and participate in the prothrombotic processes occurring during HUS.<sup>14</sup>

Stx binds to the P<sup>k</sup> antigen (CD77) on red blood cells.<sup>19</sup> The P<sup>k</sup>, P and P<sub>1</sub> antigens give rise to five different red blood cell phenotypes namely P<sub>1</sub>, P<sub>2</sub>, P<sub>1</sub><sup>k</sup>, P<sub>2</sub><sup>k</sup> and p. All but the p red blood cell phenotype express the P<sup>k</sup> antigen and can bind Stx to varying extents.<sup>144</sup> One study suggested that strong expression of P<sub>1</sub> antigen on red blood cells could protect from toxin-mediated injury as the toxin would bind to red blood cells and thus protect the more sensitive cells from toxin-mediated injury.<sup>145</sup> *In vitro*, Stx2 stimulation of human red blood cells from donors with the P<sub>1</sub>, P<sub>2</sub>, P<sub>1</sub><sup>k</sup>, and P<sub>2</sub><sup>k</sup> phenotypes induced release of complement-coated red blood cell-derived EVs, indicating that EV release and complement activation could be related to expression of toxin receptor.<sup>20</sup> This *in vitro* study also showed that Stx2 induced release of hemoglobin and lactate dehydrogenase, indicative of Stx2-induced hemolysis. Hemolysis has also been demonstrated in a primate model injected with Stx<sup>146</sup> and mice infected with *E. coli* O157:H7 exhibited fragmented red blood cells.<sup>147</sup>

# Animal models of *E. coli* O157:H7 infection

Animal models of EHEC infection have been developed in order to reproduce aspects of the human disease and understand how HUS develops. To date, no small animal model has been developed that fully mimics human EHEC infection. The available animal models can help researchers understand aspects of EHEC pathogenesis and HUS. The most widely used animal model is the mouse while others such as rats, rabbits, pigs, zebrafish, ferrets, monkey and chickens have also been described (Figure 3). Here I will first describe animal models of EHEC infection followed by those with Stx injection to mimic human disease.



**Figure 3: Animal models used to study *E. coli* O157:H7 infection.**

Several animal models have been inoculated with *E. coli* O157:H7 in an attempt to mimic the human infection.

Mice infected with EHEC via the oral/intragastric route exhibit some but not all the attributes of human infection. EHEC infected mice exhibit intestinal colonization, weight loss, as well as systemic and neurological signs of disease.<sup>10, 133, 147-149</sup> EHEC infection in mice cannot reproduce certain aspects of human disease as mice infected with EHEC do not develop diarrhea. Infected mice exhibit intestinal pathology including inflammatory infiltrates, thickening of the submucosa, damaged crypts and edema<sup>133, 147</sup> and renal pathology including mesangial cell proliferation and deposition of fibrin in the glomeruli<sup>133</sup> along with desquamation of tubular epithelial cells and presence of red blood cells in the tubular lumen.<sup>133, 147</sup> EHEC-infected mice also exhibit low platelet and red blood cell counts and elevated plasma creatinine and blood urea nitrogen (BUN) levels.<sup>133, 150</sup> The streptomycin-treated mouse model was developed in order improve colonization of streptomycin-resistant *E. coli* O157:H7 to the gastrointestinal tract.<sup>149</sup> In this model, mice were successfully colonized when treated with streptomycin in drinking water to reduce the normal intestinal microflora prior to overnight fasting of only food.<sup>149</sup> This led to development of cortical tubular necrosis while no glomerular damage was observed in infected mice. Glomerular lesions in *E. coli* O157:H7 infection were observed in germ-free mice pretreated with TNF $\alpha$  prior to inoculation with *E. coli* O157:H7.<sup>151</sup> The germ-free *E. coli* O157:H7 model has been used in several studies that investigated bacterial internalization by the epithelium<sup>152</sup> and assessed the virulence of mutant *E. coli* O157:H7 strains.<sup>153</sup> However, the germ-free mice in comparison to the streptomycin-treated mice lack mucosal adherence of *E. coli* O157:H7 in the colon.<sup>154</sup>

Mouse models in which the normal microflora was not altered have also been utilized. C3H/HeN and C3H/HeJ mice inoculated intragastrical with high doses of *E. coli* O157:H7, developed gastrointestinal, neurological, and renal manifestations resulting in mortality and morbidity.<sup>147</sup>

Unlike human intestinal epithelial cells that have been reported to lack Gb3,<sup>91, 155</sup> murine intestinal epithelial cells in the distal colon express Gb3.<sup>156</sup> Therefore the difference in renal pathological findings between mice and humans could be attributed to the localization of Gb3 in the tissue. Gb3 is expressed in human glomerular endothelial cells<sup>157</sup> and tubular epithelial cells<sup>158</sup> while in mice, it is expressed only in the tubular epithelium but not in glomerular endothelium.<sup>54, 159</sup>

Rats were treated intraperitoneally with culture supernatant from recombinant *E. coli* expressing Stx2.<sup>160</sup> This model of rats exhibited watery diarrhea, hemolytic anemia due to vascular damage, thrombocytopenia and kidney injury associated with tubular and glomerular necrosis, similar to those described in humans.<sup>160</sup>

Rabbits infected orally/intragastrically with *E. coli* O157:H7 infection developed diarrhea, weight loss as well as renal vascular and glomerular lesions.<sup>161-163</sup> However, EHEC infected rabbits do not develop thrombocytopenia.<sup>164</sup>

Germ-free gnotobiotic piglets orally infected with Stx2-producing *E. coli* O157:H7 or *E. coli* O26:H11 developed intestinal manifestations such as damage to the intestinal mucosa and thrombotic microangiopathy in the kidney resembling HUS. Anemia, thrombocytopenia and kidney failure could not be reproduced in this model.<sup>165</sup>

Zebrafish have been used to study the colonization and pathogen-microbiota interactions during foodborne EHEC infection. This model allows the study of molecular aspects of EHEC colonization as well as pathogenesis at a single cell level.<sup>166</sup>

Ferrets treated with streptomycin and colonized with *E. coli* O157:H7 upon oral infection exhibited weight loss, glomerular lesions, hematuria and some even developed thrombocytopenia.<sup>167</sup>

Monkeys intragastrically infected with *E. coli* O157:H7 developed diarrhea. Histological analysis showed the formation of intestinal A/E lesions, epithelial cell damage, neutrophil infiltration and in the kidney, moderate tubular damage. Elevated levels of leukocytes and neutrophils was also observed. This model closely resembles the early stages of disease caused by *E. coli* O157:H7 infection.<sup>168</sup>

Chickens infected with *E. coli* O157:H7 were predominantly colonized in the cecum and exhibited formation of A/E lesions. No damage to other organs or other parts of the gastrointestinal tract was observed.<sup>169</sup>

In addition to animal models of EHEC infection, models have also been developed using purified Stx. Mice injected with Stx alone or in combination with LPS developed clinical signs of disease including thrombocytopenia, hemolysis, renal failure and weight loss.<sup>133, 170</sup> These mice exhibited platelet deposition in the glomeruli, glomerular damage due to red blood cell infiltration<sup>132, 170, 171</sup> and microthrombi in glomerular capillaries.<sup>170</sup> Tubular damage was also observed. An increase in BUN levels was demonstrated, indicative of kidney injury.<sup>102, 172</sup>

Rabbits injected with Stx exhibited gastrointestinal and neurological pathology. In the intestine, hemorrhages, edema and a damaged mucosal layer was observed. Similarly, hemorrhage, edema and microvascular thrombosis were observed in the spinal cord.<sup>173</sup>

Baboons infused intravenously with Stx1 developed renal failure and mucosal damage in the gastrointestinal tract. Glomerular lesions with swelling of podocytes and desquamation of capillary endothelium was observed along with glomerular microthrombi.<sup>146</sup>

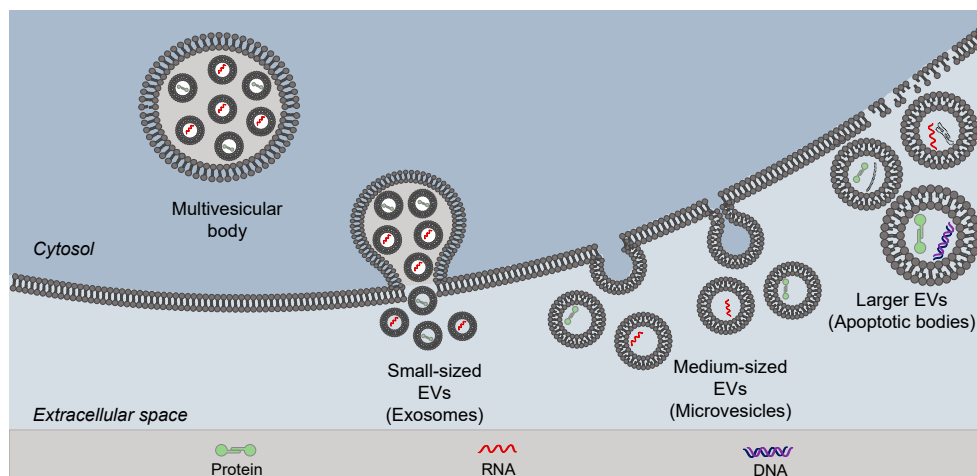
Greyhounds dogs injected with Stx1 or Stx2 developed severe bloody diarrhea, low platelet counts, anemia and thrombotic microangiopathy.<sup>174</sup> Of note, there is a natural disease in dogs termed Alabama Rot that resembles human HUS.<sup>175</sup>



# Extracellular vesicles

Studies have shown that patients with EHEC-associated HUS have elevated levels of EVs in the circulation derived from blood cells<sup>14, 176, 177</sup> and that EVs take part in all aspects of HUS, including transport of Stx from the gut to the kidney, hemolysis and thrombosis (reviewed in<sup>178</sup>). Therefore, the study of EVs is relevant in order to understand the pathogenesis and to develop effective treatment strategies. In the following section EVs and their role in EHEC-associated HUS is discussed.

Most cells shed EVs during normal physiology or upon activation. These vesicles express contents of the cell of origin such as RNAs, receptors, proteins and phospholipids on their surface.<sup>179</sup> EVs are divided or subclassified into three subtypes which are differentiated based on their release mechanisms, size and cellular markers located on the outer leaflet. According to the earlier classification, EVs included exosomes, microvesicles and apoptotic bodies. The new guidelines of the International Society for Extracellular Vesicles, encourages authors to classify EVs based on either physical characteristics such as size, density or biochemical composition or based on the cell of origin.<sup>180</sup> EVs are accordingly referred to as small EVs (< 200 nm in diameter), medium and/or large EVs (> 200 nm in diameter)<sup>180</sup> (Figure 4).



**Figure 4: Schematic representation of extracellular vesicle subtypes.**

Extracellular vesicles (EVs) being shed from a cell are classified based on size into small-sized EVs (exosomes), medium-sized EVs (microvesicles) and larger EVs (apoptotic bodies).

One of the main functions of vesicles is to mediate cell-to-cell communication between neighboring and distant cells.<sup>22</sup> EVs can also be a means by which the cell rids themselves of unwanted materials in order to maintain cellular integrity.<sup>181</sup>

## Types of EVs

Small-sized EVs, with a diameter < 200 nm are a type EVs that are formed by inward budding of early endosomal membranes into intraluminal vesicles that are packed into multivesicular bodies.<sup>182</sup> Fusion of these multivesicular bodies with the plasma membrane of the cells leads to release of the intraluminal vesicles that when extruded from the cell are small-sized EVs (i.e. exosomes).<sup>182</sup> These are secreted by all cell types and have been detected in many body fluids.<sup>183</sup> Small-sized EVs have been shown to carry various forms of cargo such as proteins, lipids and RNAs.<sup>184, 185</sup>

Medium-sized EVs, also termed microvesicles, with a diameter in the range of 200 – 1000 nm, are formed by direct outward budding of the cell plasma membrane. These can be released constitutively<sup>186</sup>, while an increase in their release has been associated with cellular activation.<sup>187</sup> Medium-sized EVs are mainly composed of cytosolic and plasma membrane-associated proteins of the parent cell. The role of medium-sized EVs in various cellular process have been extensively studied, including transport of RNAs, microRNAs, receptors, proteins and lipids.<sup>188</sup> EVs can also transfer foreign substances such as bacterial and parasitic virulence factors as these factors are protected from the host response when caged inside an EV.<sup>21, 189</sup> The physiological and pathological functions of EVs could include immune modulation, angiogenesis, thrombosis and tissue regeneration (reviewed in<sup>190</sup>).

Large-sized EVs, apoptotic bodies, sized > 1000 nm in diameter are released by cells undergoing apoptosis. They contain fragments of the parent cell including organelles, proteins and genomic DNA.<sup>191</sup> These EVs can be taken up by phagocytosis by immune cells thus lowering the inflammatory and apoptotic response.<sup>192</sup>

The protein content of larger-sized EVs is quite similar to that of the cell lysate however, the protein content of small- and medium-sized EVs can be enriched.<sup>193</sup> Thus EVs possess the unique property of packaging several types of cargo, which are then transported to other cells and result in alteration of the functions of the recipient cells.<sup>194</sup>

## Mechanisms of EV release

Release of EVs has been closely linked to intracellular calcium influx, cortical actin cleavage and loss of lipid membrane asymmetry.<sup>195, 196</sup> The plasma membrane of the cell is made up of asymmetrically distributed lipids. During homeostasis, PS and phosphatidylethanolamine (PE) are mainly located in the inner leaflet of the plasma membrane and this is maintained by calcium-dependent transmembrane enzymes namely flippases, floppases and scramblases. In a resting cell and under normal intracellular calcium levels, flippases are active, resulting in inward movement of PS and PE from the outer leaflet to the inner leaflet of the cell. Floppases are inactive during this state. Upon cellular activation, intracellular calcium rises, flippases are inactivated while floppases and scramblases are activated, resulting in externalization of PS and PE to the other leaflet.<sup>197</sup> The role of these transmembrane enzymes in juggling lipids within the cell membrane has been shown to be involved in the release of EVs.

Another factor controlling the release of EVs is the actin cytoskeleton, which stabilizes the plasma membrane. For EVs to be released, especially in the case of medium-sized EVs, which directly bud off from the plasma membrane, the actin cytoskeleton needs to be disrupted. Cells contain calpain which cleaves and disrupts the actin cytoskeleton in a calcium-dependent manner contributing to EV release.<sup>195</sup>

In order for medium-sized EVs to be successfully released into the extracellular space, they need to undergo a scission step. This is mainly controlled by the endosomal sorting complexes required for transport machinery.<sup>198</sup>

## Mechanisms of EV uptake

EVs are taken up by the recipient cells by phagocytosis, macropinocytosis, clathrin-dependent endocytosis, caveolin-dependent endocytosis and membrane fusion. Phagocytosis is a receptor-mediated uptake mechanism involving the formation of membrane invaginations whereby the cell extends its membrane and engulfs other cells or large particles. A specialized group of cells can accomplish phagocytosis such as macrophages, neutrophils, monocytes and dendritic cells. The receptors located on the surface of phagocytes can be non-opsonic or opsonic. Non-opsonic receptors directly detect molecular patterns on the particle that needs to be phagocytosed while opsonic receptors detect host-derived proteins (opsonins) associated with the particles. Following the recognition, the phagocytic receptors initiate a signaling process that leads to remodeling of the actin cytoskeleton, membrane extension and engulfment of the particle (reviewed in<sup>199</sup>). Phagocytes can internalize EVs.<sup>200</sup> Exposure of PS on the surface of EVs is an important signal for phagocytosis.<sup>200</sup>



Macropinocytosis is an endocytic pathway involving formation of membrane invaginations leading to uptake. This mechanism of uptake is similar to phagocytosis. However a direct interaction with EVs is not essential. Macropinocytosis is a rac1-, actin- and cholesterol-dependent mechanism and requires ion-exchanger activity.<sup>201</sup> Rac1 is a GTPase and its inhibition abrogates the uptake of EVs by microglia.<sup>202</sup>

Clathrin-dependent endocytosis involves internalization of EVs by inward budding of the plasma membrane resulting in clathrin forming a coat around the EVs. This clathrin-coated intracellular structure fuses with the early endosomes and mediates intracellular trafficking.<sup>203</sup>

Caveolin-dependent endocytosis like clathrin-dependent endocytosis, occurs by the formation of small membrane invaginations referred to as caveolae around the EVs leading to their internalization. Caveolae are sub-domains of lipid rafts of the plasma membrane that are rich in cholesterol, sphingolipids and caveolins.<sup>204</sup>

Membrane fusion is an alternative mechanism by which EVs can be internalized by recipient cells. This process occurs when two distinct lipid layers come in close proximity. The outer leaflets of the two membranes come in direct contact and form a hemi-fusion stalk along with the two membranes. The stalk then expands and forms a hemi-fusion diaphragm leading to the formation of a fusion pore. This results in mixing of the two hydrophobic cores and formation of one structure.<sup>205</sup> Proteins like SNAREs and Rab proteins are involved in this process.<sup>206</sup>

## Importance of EVs

EVs are shed both during physiological and pathological conditions into the extracellular environment and this occurs during the resting state or upon activation due to physical or chemical stress such as hypoxia, shear or oxidative stress.<sup>188</sup> The main role of EVs is to mediate cellular signaling in the target cells as well as to transfer cargo. EVs can also be utilized as biomarkers of disease as specific EVs are released during certain pathological processes.<sup>207</sup> EVs are found in a variety of biological fluids such as blood, urine, cerebrospinal fluid and saliva and their presence can be used as liquid biopsies.<sup>208</sup>

EVs are capable of horizontal transfer of mRNAs and microRNAs to the target cell and in turn regulate its protein expression.<sup>209</sup> EVs also contribute to thrombosis and coagulation. EVs derived from platelets and monocytes express tissue factor on their surface.<sup>14, 210</sup> Tissue factor forms a complex with coagulation factors FVII resulting in the activation of the extrinsic pathway of coagulation. The complex formed with tissue factor/FVII cleaves and activates FX.

Exposure of PS on EVs provides a platform for the assembly of the prothrombinase complex formed by FXa and FVa which participates in the conversion of prothrombin to thrombin.<sup>211</sup> PS-positive EVs are involved in coagulation by interacting with coagulation factor FV and FVII.<sup>212</sup> Platelet-derived EVs possess more procoagulant activity compared to their parent cell<sup>193</sup> and deposition of complement factors on EVs derived from platelets provide a membrane surface that allows assembly of the prothrombinase complex.<sup>213</sup> Furthermore, endothelial cell-derived EVs carrying von Willebrand factor have been shown to induce platelet aggregation suggesting the role of EVs in thrombosis.<sup>214</sup>



# Treatment strategies for EHEC infection

## Current treatment strategies

To date there are no available therapeutics against EHEC infection. The current management regimens for EHEC infection and HUS are supportive care and symptomatic treatments such as rehydration therapy and dialysis.<sup>215, 216</sup>

## Proposed experimental treatment strategies

Over the years, new therapeutic approaches have been developed for EHEC infection. These include antibodies against Stx, use of Gb3 mimics and inhibitors, vaccines, molecules blocking Stx-induced apoptosis and other alternatives targeting the pathogen. None of these are used in clinical practice but they will be shortly discussed in this section.

Antibodies targeting Stx, the primary agent causing organ damage in humans has been a prime target for therapeutic development. These antibodies aim to neutralize the toxin systemically during the earlier stages of infection. Studies using Stx-specific antibodies were able to neutralize the cytotoxic effect of the toxin both *in vitro* as well as in mice.<sup>217</sup> Rabbits immunized with Stx2 toxoid developed antibodies against Stx2 that protected *E. coli* O157:H7-infected mice by lowering the bacterial burden, and decreasing weight loss and death.<sup>218</sup> The most crucial aspects of immunization with Stx antibodies is the stage of infection and the dosage of the antibody, as the use of Stx antibodies has only been shown to protect EHEC infected patients from the development of HUS when administered shortly after the onset of diarrhea.<sup>219</sup> Protective effects of Stx-specific antibodies and their efficacy in piglet and mouse models cannot be applied to humans as both pigs and mice do not develop bloody diarrhea or HUS. Two monoclonal antibodies (Shigamabs®) against Stx1 and Stx2 were evaluated in phase II clinical trials as a treatment against Stx-producing *E. coli* infection (<https://www.pharmaceuticalonline.com/doc/thallion-and-lfb-terminate-shigamabs-collaboration-0001>). Shigamabs® act as a

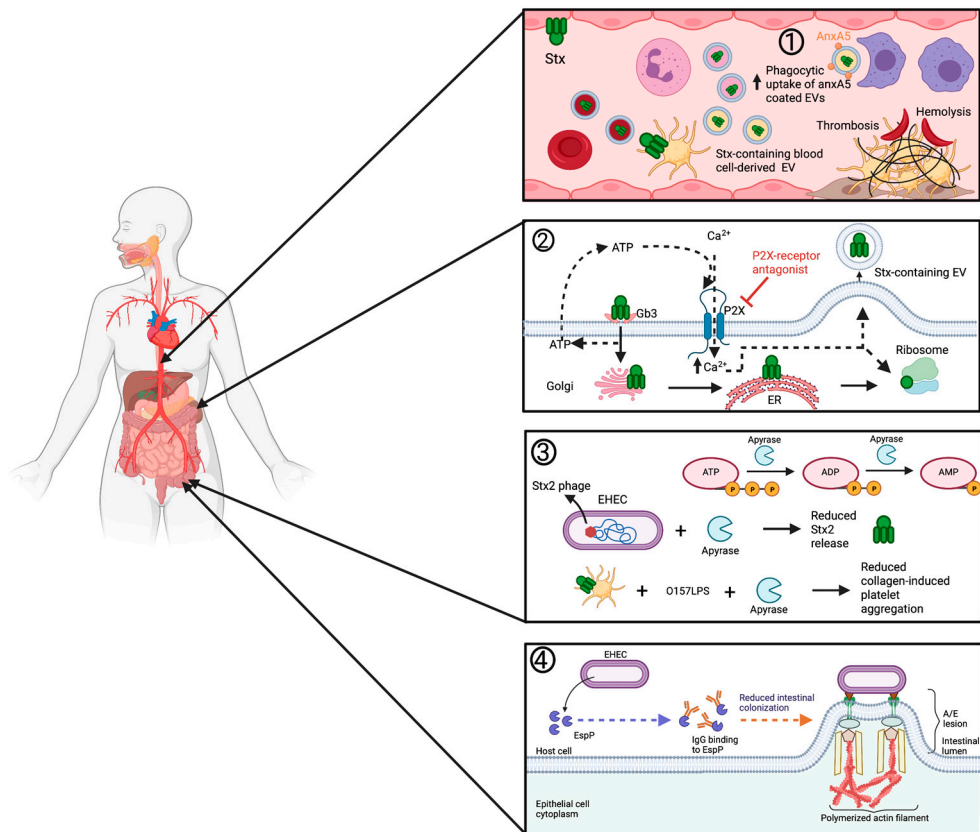
neutralizing agent by forming a complex with the toxins which are then eliminated from the body.

Gb3 mimics or analogues have been investigated as a treatment strategy that aimed at interfering with the interaction between Stx and its receptor. SYNSORB Pk was the first identified Gb3 analogue to be tested in children diagnosed with EHEC-associated HUS. However this treatment did not prove to be beneficial in this study as it was administered at a later time point during the course of the disease.<sup>220</sup> The lack of protective effects of this treatment could also be due to the impaired gastrointestinal motility among patients with diarrhea-associated HUS which may have limited the drug delivery to the distal intestine, where the bulk of the toxin is found during EHEC infection. Another factor that could explain the lack of protection in patients could be due to the interaction between EHEC and gastrointestinal epithelium which could hinder the capacity of the Gb3 analogue to bind to Stx *in vivo*.<sup>220</sup> However, carbohydrate based Gb3 analogs have been shown to be protective against damage caused by purified Stx<sup>221</sup> as well as EHEC infection in mice.<sup>222</sup> This treatment strategy like the antibody based treatment has a limitation with respect to the time point of administration which is very crucial in disease management.

Gb3 inhibitors aim at interfering with the synthesis of Gb3. This is achieved by inhibiting glucosylceramide synthase which is an enzyme involved in the rate-limiting step during the biosynthesis of Gb3. Oral administration of a Gb3 inhibitor in rats improved survival and protected the intestine and kidney from Stx-induced damage.<sup>223</sup> A more recent study showed a similar protective effect against Stx2-induced cytotoxicity in human renal tubular epithelial cells.<sup>224</sup>

# Potential treatment strategies investigated in this thesis

In this thesis, four novel treatment strategies have been investigated which potentially target different aspects of EHEC-associated HUS, including bacterial colonization and toxin release, intracellular signaling triggered by Stx and its systemic transport in EVs and effects of toxin on platelet activation. These strategies will be described here (Figure 5).



**Figure 5: Schematic representation of targets of novel treatment strategies included in this thesis.** 1) The effect of annexin A5 (anxA5) on uptake of blood cell-derived extracellular vesicles by phagocytes. 2) Inhibition of purinergic receptor signaling and its consequence on Shiga toxin (Stx)-induced effects on cells. 3) The effect of apyrase on Stx released from EHEC and on platelet activation. 4) The effect of immunoglobulin G (IgG) on EHEC colonization and its interaction with EspP.

## Annexin A5

Annexins are a highly conserved protein family that are characterized by their ability to interact with biological membranes in a calcium-dependent manner.<sup>225</sup> They are expressed in many organisms, and are termed based on their origin, so that annexin A is found in humans, B found in invertebrates, C in fungi, D in plants and E in *Protista*.<sup>225</sup> Human annexins vary in expression levels from universal expression in many organs and cells (for example A1, A2, A4, A5, A6, A7 and A11), to selective expression (A3 in neutrophils, A8 in placenta and skin, A9 in tongue, A10 in stomach and A13 in small intestine).<sup>226</sup> Several intracellular and extracellular functions of annexins have been identified such as membrane trafficking<sup>227</sup> and repair,<sup>228</sup> intracellular signaling, exocytosis,<sup>229</sup> endocytosis,<sup>230</sup> vesicle aggregation,<sup>231</sup> interaction with the cytoskeletal membrane,<sup>232</sup> ion channel regulators, anticoagulant<sup>233</sup> and anti-inflammatory properties.<sup>234</sup>

Among the annexins, Annexin A5 (AnxA5) is the most abundant protein and is expressed in most cells and tissues.<sup>235</sup> The most widely known function of anxA5 is its ability to bind specifically to PS, which is a commonly used detection tool for apoptotic cells and EVs.<sup>236</sup> AnxA5 stabilizes membranes and contributes to membrane repair. A ruptured membrane creates a microenvironment with high local calcium levels, which triggers anxA5 to bind to exposed PS and assemble to form a 2D-lattice for membrane repair.<sup>237</sup> AnxA5 is also known to have several other roles such as inhibiting prothrombinase complex formation leading to an anticoagulant effect<sup>233</sup> and inhibiting proinflammatory responses *in vivo*.<sup>234, 238</sup>

Studies have investigated the interaction between EVs and cells in the presence of anxA5. AnxA5 inhibits uptake of hypoxia-induced stem cell-derived EVs by human umbilical cord endothelial cells.<sup>239</sup> In contrast, a similar effect could not be detected when alveolar macrophage-derived EVs were incubated with alveolar epithelial cells in the presence of anxA5.<sup>240</sup> The mechanism by which the cell membrane is modified due to interactions between PS and anxA5 resulting in uptake of EVs is still quite unclear and suggests that the effect of anxA5 on uptake of vesicles is highly dependent on the experimental conditions. In this thesis, the effect of anxA5 on the interaction between EVs and phagocytic cells was investigated *in vitro* and the consequence of anxA5 treatment was further studied using an *in vivo* mouse model of *E. coli* O157:H7 infection.

## Purinergic receptors, ATP signaling and blockade

The purinergic receptor family is a group of receptors that are activated by nucleotides. They are divided into P1, P2Y and P2X receptors.<sup>241</sup> P1 receptors activated by adenosine.<sup>242</sup> There are eight P2Y receptors. These are G-protein coupled receptors activated by ADP, ATP, UDP and UTP.<sup>243</sup> There are seven P2X receptors which are activated by ATP.<sup>244</sup> Among the P2X receptors, P2X1 is the

only one present on platelets and is a calcium ion channel.<sup>245</sup> Purinergic signaling is known to be involved in immune responses, inflammation, pain, platelet aggregation, cell proliferation, differentiation, wound healing, ageing and regeneration among others.<sup>246, 247</sup>

Among the P2X receptors, P2X7 is highly expressed on inflammatory cells and is activated by a high concentration of ATP.<sup>248</sup> Stimulation of this purinergic receptor induces the influx of calcium and increased shedding of plasma membrane-derived EVs.<sup>249</sup> The release of EVs on account of P2X7 activation by ATP has been studied in microglia and dendritic cells.<sup>250, 251</sup> The release of EVs can be inhibited by the removal of extracellular calcium or by using P2X7 antagonists.<sup>250, 252</sup> The importance of purinergic signaling in Stx-induced complement-mediated hemolysis and EV release from red blood cells and their inhibition of EV release in the presence of purinergic receptor antagonists has been studied.<sup>20</sup> Complement deposition on red blood cells and red blood cell-derived EVs were observed in EHEC-associated HUS patients.<sup>20</sup> Detection of C3 and not C5b-9 on red blood cells from HUS patients could suggest that red blood cell lysis occurred when C5b-9 deposited on cells thus preventing its detection. While, red blood cell-derived EVs from patients were coated with C5b-9 suggesting that either the cells or the EVs were exposed to complement activation. Purinergic receptor antagonists inhibited Stx-induced hemolysis and EV release from red blood cells. These findings are in line with the previous studies that showed that purinergic receptors contribute to hemolysis.<sup>253, 254</sup>

ATP, an activator of purinergic signaling, is involved in platelet aggregation.<sup>255</sup> ATP is released from platelets by collagen and thrombin. Modulation of purinergic signaling by ATP degradation or the use of purinergic receptor antagonists led to reduced platelet aggregation.<sup>256, 257</sup> In line with this, platelets from P2X1-deficient mice showed reduced thrombus formation upon collagen stimulation *ex vivo*.<sup>258</sup>

In this thesis blockade of P2X and specifically P2X1 in the context of Stx-mediated cytotoxicity and EV release, both *in vitro* and *in vivo* has been investigated.

## **Apyrase**

Bacteria release extracellular ATP during their growth.<sup>259</sup> Extracellular ATP could be used as a source of nutrients or signaling molecule to communicate with other bacteria in the bacterial communities. Levels of ATP changes depending on the bacterial density suggesting that ATP could also be a signal for quorum sensing.<sup>259</sup> ATP released by commensal bacterium can activate dendritic cells in the lamina propria and induce release of inflammatory cytokines. This in turn led to the differentiation of Th17 cells (proinflammatory cells that play a crucial role during infection) and exacerbates colitis in mice.<sup>260</sup> ATP released by intestinal bacteria can also dampen the intestinal IgA response against enteropathogens.<sup>261</sup>



Extracellular ATP in the host cells can serve as a signaling molecule that triggers several pathological processes occurring during thrombosis and inflammation including chemotaxis,<sup>262</sup> activation of the inflammasome<sup>263</sup> and platelet activation.<sup>264</sup> Upon *in vitro* stimulation of cells with Stx, an increase in intracellular calcium levels is observed, triggering release of ATP.<sup>265</sup> Released ATP interacts with P2X receptors. ATP molecules can be degraded by enzymes called ecto-nucleotidases. Humans have two types of ecto-nucleotidases, namely CD39 and CD73. These are membrane bound enzymes and are generally expressed on endothelial cells as well as lymphocytes.<sup>266</sup> CD39 cleaves phosphate groups from ATP and ADP while CD73 cleaves the final phosphate group from AMP, resulting in adenosine. Ecto-nucleotidases play a crucial role in regulating extracellular ATP levels and purinergic receptor sensitivity to stimuli.

Apyrase is an ecto-ATPase capable of cleaving ATP, ADP and AMP.<sup>267</sup> It is a soluble enzyme usually extracted from potatoes. In this thesis, the effect of apyrase both in the host and on the bacteria was investigated. The effect of apyrase administration on survival, goblet cell depletion, intestinal apoptosis and levels of ATP and Stx2 in feces was investigated in our murine model of *E. coli* O157:H7 infection. Effect of apyrase on platelet aggregation and its effect on release of Stx2 from the bacteria in culture was investigated *in vitro*.

## **Immunoglobulin**

Antibodies against Stx<sup>268</sup> have been developed as a treatment strategy against EHEC infection. However, they have not been found to be clinically tested or effective. A specific immunoglobulin Y preparation isolated from egg yolks inhibited the growth of *E. coli* O157:H7.<sup>269</sup> Bovine colostrum concentrates (rich in IgG) given to pediatric patients with EHEC-associated diarrhea resulted in reduced frequency of loose stools.<sup>219</sup> Similarly, oral administration of IgG-enriched bovine colostrum protected mice from *E. coli* O157:H7 infection.<sup>270</sup> In this thesis, the effect of IgG administration and its specific interaction with EHEC proteins has been further investigated *in vivo* for its effects on colonization, intestinal cell injury, renal failure and death and *in vitro* for its effects on impairing the enzymatic activity of a bacteria protein.

# The present investigation

The overall aim of this study was to investigate novel pathogenic mechanisms and protective measures during *E. coli* O157:H7 infection using *in vivo* and *in vitro* models, and ultimately develop novel therapies.

## Specific aims

1. To investigate the interaction of anxA5 with EVs and phagocytes *in vitro* and to further assess its effect on the course of disease during *E. coli* O157:H7 infection *in vivo* with special focus on EVs.
2. To investigate the role of ATP and purinergic signaling in Stx-induced cellular activation and the role of purinergic receptor blockade on Stx-mediated calcium influx, cytotoxicity and EV release.
3. To evaluate the significance of ATP signaling during *E. coli* O157:H7 infection *in vivo* by treating infected mice with apyrase and determine the dual protective effect of apyrase on the bacteria and the host.
4. To analyze the effect of IgG in murine *E. coli* O157:H7 infection and its interaction with EspP.

# Methods

A short description of the methods used in the papers within this thesis is given below. For a detailed description of the methods, please refer to each individual paper.

## *Ethical considerations*

Samples were obtained from normal human subjects in papers **I**, **II** and **III**. Informed written consent was obtained from these healthy adult volunteers. The studies were conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Medical Faculty, Lund University.

An animal model was used in papers **I**, **II**, **III** and **IV**. All animal experiments were approved by the animal ethics committee of Lund University in accordance to the guidelines of the Swedish National Board of Agriculture and the European Union directive for the protection of animals used for scientific research.

## *Flow cytometry*

In papers **I** and **II**, flow cytometry was used to analyze EVs. Flow cytometry is a well-established research tool to characterize particles based on physical and chemical composition. Particles flow in a streamline motion where a single particle passes through a laser beam. Each particle is analyzed based on its light scattering ability. The light scattered is measured in two directions, forward scatter (FSC) that is indicative of its size and side scatter (SSC) that is indicative of the granularity of the particle. This method can be coupled to fluorescent proteins to characterize the particles even further. In this thesis, flow cytometry was used to identify membrane bound receptors and the cellular origin of EVs using fluorescently labelled antibodies. Intravesicular components, such as Stx within EVs were analyzed using detergent to permeabilize the EV membrane.

## *Cell culture*

Cervical epithelial (HeLa) cells were used in papers **I**, **II** and **III**, human monocyte (THP1) and murine monocyte (RAW264.7) cells were used in paper **I**, primary human umbilical vein endothelial (HUVEC) and primary glomerular endothelial (pGEC) cells were used in paper **III**.

## *Viability assay studying cell metabolism*

The viability of cells was quantified as a measure of metabolic activity using alamar blue in papers **I**, **II** and **III**. A shift in color of alamar blue occurs when it is reduced by NADH and NADPH. Measurements were carried out on cells treated in the

presence or absence of: anxA5 in paper **I**, NF449 (P2X1 inhibitor) in paper **II**, apyrase in paper **III** and exposed to free Stx or without Stx.

#### *Protein synthesis assay*

In paper **II**, protein synthesis was assayed in cells treated with or without NF449 and certain cells were exposed to Stx1. Protein synthesis was quantified by the amount of radioactive labelled methionine that is incorporated. The incorporation of methionine was measured using a scintillator, the generated signal was in proportion to the amount of protein synthesized.

#### *Caspase activity assay*

Caspase activity was assayed in paper **II**. The activity of terminal caspase 3/7 is quantified as a measure of cleaved substrate indicating that caspase 3/7 are activated which can in turn cleave a membrane permeable substrate that the cells have been treated with. This cleaved substrate generates green fluorescence upon excitation by blue laser. Cells undergoing apoptosis appear green in color. HeLa cells treated with or without NF449 in the presence of Stx were used in this assay.

#### *ATP determination*

Luciferase activity was used for ATP measurements in papers **II** and **III**. Luciferase is an enzyme obtained from fireflies, which is used as a catalyst to cleave ATP. This catalytic reaction led to light emission, in direct proportion to the ATP generated. ATP was assayed in HeLa cells supernatants in paper **II** and in mouse feces from EHEC-infected mice in paper **III**.

#### *Calcium imaging*

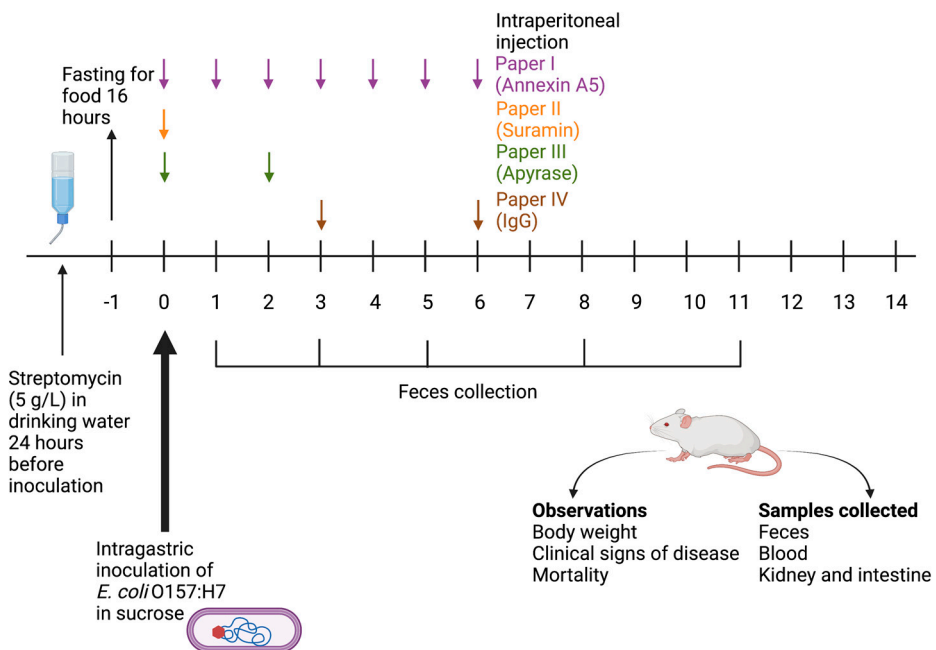
HeLa cells and human platelets were stained with a calcium sensitive fluorescent dye, Fluo-4 in paper **II**. This dye is cell-permeable and is cleaved upon entering the cell, thus losing its permeability property. Fluo-4 is brighter in the presence of a high calcium concentration. Calcium influx was quantified as a measure of fluorescence in Fluo-4 stained HeLa cells stimulated with Stx and in platelets stimulated with LPS and Stx.

#### *In vitro platelet aggregation*

Platelet aggregation was studied using optical light transmission aggregometry in paper **III**. Platelet-rich plasma from healthy volunteers were incubated with buffer alone, apyrase alone, LPS and Stx2 and LPS and Stx2 and apyrase for 5 mins prior to addition of collagen to induce platelet aggregation. Platelet poor-plasma is used to set the maximum amount of light being transmitted. The amount of light transmitted through the platelet rich-plasma was used as a measure of platelet aggregation.

## Mouse models

A mouse model was used in papers **I**, **II**, **III** and **IV**. Mice were treated with streptomycin in drinking water, in order to reduce the normal gut microflora and improve colonization of streptomycin-resistant *E. coli* O157:H7 (Stx-producing strain, 86-24). Mice were fasted for food but not water for 16 hours before bacterial inoculation to further improve colonization. Mice were inoculated with the streptomycin-resistant *E. coli* O157:H7 strain ( $10^9$  colony forming units/mL). Weight changes, bacterial colonization and development of clinical signs of disease were monitored throughout the experiment. Upon development of clinical signs of disease, >20% weight loss or at a predetermined end-point, the mice were sacrificed and blood was drawn, kidney and intestine were harvested (Figure 6).



**Figure 6: Schematic representation of an in vivo model of *E. coli* O157:H7 infection.**

In paper **II**, mice were also intraperitoneally injected with pure Stx2 (285, 142.5 and 71,25 ng/kg). The mice were monitored as above and samples were collected upon sacrifice.

## Blood urea nitrogen

Kidney function impairment was investigated by measurement of BUN levels in mouse plasma. This analysis was conducted in papers **I**, **III** and **IV**.

### *Light microscopy*

Murine kidney and intestine tissue were stained with hematoxylin and eosin or periodic acid-Schiff to visualize different tissue components using light microscopy in paper **III** and **IV**.

### *Immunofluorescence*

Immunofluorescent staining was performed in murine renal sections. In paper **I**, murine renal tissue was stained with antibodies against fibrinogen.

### *TUNEL assay*

In paper **III**, colons from mice, treated with or without apyrase were stained for apoptotic cells. Apoptotic cells incorporated alkyne tagged deoxyuridine triphosphate into the fragments of DNA and these alkyne groups were in turn tagged to a fluorescent probe. The renal tissues were analyzed using fluorescence microscopy.

### *Liquid chromatography-Mass spectrometry*

Liquid chromatography is a technique widely used to separate compounds based on their interaction with the stationary and mobile phase. Mass spectrometry is an analytical technique used to quantify or detect the presence of specific proteins in a sample based on the mass-to-charge ratio. First the samples are subjected to chromatographic separation, followed by which the samples are eluted and ionized in an ionization source and introduced into the mass analyzer. Here, the ions are either in an electric or magnetic field, where they are deflect based on their mass-to-charge ration and are detected by the ion detector. Mass spectrometers are connected to computer-based software platforms, which measures ion oscillation frequencies and are used to acquire mass spectra using image current detection. This acquired data is then fed to a data analysis program that detects ions and organizes them based on their individual mass-to-charge ratios and relative abundance in the samples. Based on its mass-to-charge ratios, ions can be identified using a database to predict the identity of the molecule. In paper **I**, mass spectrometry was used for detection of Stx2 in *E. coli* O157:H7-infected murine renal tissue. In paper **IV**, proteins in *E. coli* O157:H7 culture filtrates that interacted with either murine or human IgG were thereby identified.

# Results

## Paper I

AnxA5 treatment of blood- or HeLa cell-derived EVs induced an increase in cellular uptake by differentiated human monocytic cells (THP1) and murine macrophage cells (RAW264.7), respectively. AnxA5 had no effect on EV uptake when the recipient phagocytic cells were treated with anxA5, only EVs treated with anxA5 induced phagocytic uptake. HeLa cell-derived EVs induced an increase in PS exposure on RAW264.7 cells compared to the untreated cells which could explain the mechanism of interaction with anxA5-coated EVs.

The effect of intraperitoneal administration of anxA5 in an *in vivo* murine model of *E. coli* O157:H7 infection was investigated. Elevated levels of PS-positive EVs were detected in mice after *E. coli* O157:H7 inoculation before development of clinical signs of disease suggesting that murine EVs can bind anxA5.

*E. coli* O157:H7-infected mice developed clinical signs of disease from day 4 onwards. Mice treated with anxA5 (100 µg/kg) developed clinical signs of disease on days 6-7 and when treated with a higher concentration of anxA5 (500 µg/kg) developed clinical signs of disease on days 6-9. Thus, the effect of anxA5 on delaying developed of disease symptoms was dose-dependent.

No difference in bacterial colonization was observed between anxA5-treated and untreated mice. AnxA5 did not protect from kidney injury detected by elevated BUN as these measurements were either taken before the development of fulminant disease or after, when mice already had clinical disease.

In order to confirm that anxA5 treatment reached the circulation, plasma anxA5 levels were quantified. Mice treated with anxA5 had significantly higher levels of plasma anxA5 when compared to vehicle-treated controls and *E. coli* O157:H7-infected mice.

Plasma samples were also analyzed for levels of platelet-derived EVs, showing significantly lower levels of platelet-derived EVs in anxA5-treated *E. coli* O157:H7-infected mice when compared to vehicle-treated infected mice. This could explain the delay in development of symptoms, as EVs transport Stx to the kidney and fewer EVs would result in lower burden in the target organ.

## Paper II

Stx1 (1 µg/mL) induced the release of ATP from HeLa cells after 5 min. A significant increase in the levels of extracellular ATP was measured in Stx1-stimulated cells when compared to PBS-treated cells. Stimulation of HeLa cells with Stx1 (1 µg/mL) for 40 min induced an increase in the levels of free extracellular phosphate when compared to PBS-treated cells. This indicates that Stx1 induces release of ATP that is degraded extracellularly.

In a murine model, intraperitoneal injection of Stx2 (285 and 142.5 ng/kg) induced the release of ATP. Plasma ATP levels were significantly higher in mice injected with Stx2 (142.5 ng/kg). ATP levels in mice injected with a lower dose of Stx2 (71.25 ng/kg) were similar to untreated mice. Stx1 induced steady calcium influx into HeLa cells over time and lasting up to 270 seconds. Calcium influx in HeLa cells was inhibited in the presence of both, a specific P2X1 receptor antagonist, NF449 (60 µM) and a non-selective P2X antagonist, suramin (200 µM). Similarly, NF449 (60 µM) inhibited calcium influx induced by O157LPS (1 µg/mL) together with Stx1 or Stx2 (1 µg/mL) in human platelets.

The P2X1 antagonist NF449 (60 µM) did not affect the retrograde transport of Stx1B (1 µg/mL) to the ER in HeLa cells. Preincubation of HeLa cells with NF449 (60 µM) one hour prior to stimulation with Stx1 or Stx2 (7 ng/mL) for 24 hours protected cells from Stx-induced cytotoxicity as detected by metabolism and uptake of alamar blue. Likewise, the HeLa cells were protected from the effects of Stx1 on inhibition of protein synthesis when preincubated with NF449 for 30 min following incubation with Stx1 (7 ng/mL) for 4 hours. NF449 also had a protective effect on toxin-induced caspase activation in HeLa cells. Targeted silencing of the P2X1 receptor in HeLa cells confirmed that purinergic signaling contributed to caspase activation by Stx1.

The role of purinergic signaling in release of EVs was investigated in HeLa cells and platelets. Stx1B (130 ng/mL) induced the release of EVs from HeLa cells *in vitro*. In the presence of NF449 EV release was reduced, but statistical significance was not found. HeLa cell-derived EVs that were Stx1B-positive were significantly decreased in the presence of NF449. In platelets, both Stx1 or Stx2 (200 ng/mL) induced release of toxin-positive EVs. EV release induced by Stx2 was significantly reduced in the presence of NF449. Taken together the results indicate that ATP functions as a secondary messenger. ATP induces calcium influx by way of purinergic signaling. Purinergic signaling blockade protected against Stx-induced cytotoxicity and inhibited EV release both from HeLa cells and platelets particularly toxin-positive EVs.

Intraperitoneal injection of Stx2 (285 ng/kg) induced an increase in the release of platelet-derived and Stx2-positive EVs which were significantly reduced by treatment with suramin (60 mg/kg) administered intraperitoneally 16 hours before Stx2 injection. Additionally, EHEC inoculated mice had significantly higher



platelet-derived and Stx2-positive EVs also inhibited by suramin (20 mg/kg) pre-treatment administered intraperitoneally 1 hour before inoculation.

The collective results show that Stx mediates crucial effects such as calcium influx, decreased viability, induced apoptosis and EV release via ATP release and secondary purinergic signaling.

### **Paper III**

Experiments were designed to assess the effects of apyrase in *E. coli* O157:H7 infection in mice. *E. coli* O157:H7-infected and PBS-treated mice developed symptoms starting from day 3, post-inoculation. Mice infected with *E. coli* O157:H7 and treated with apyrase (15 U/mouse) intraperitoneally on days 0 and 2 after inoculation developed symptoms from day 5, post-inoculation. This resulted in a significant difference in survival between the apyrase-treated and PBS-treated *E. coli* O157:H7-infected mice.

Apyrase-treated *E. coli* O157:H7-infected mice were protected from weight loss in comparison to PBS-treated infected mice. Separately, mice were sacrificed on day 2 to analyze histopathology, goblet cell depletion and apoptosis in the intestine. Apyrase-treated *E. coli* O157:H7-infected mice were protected from goblet cell depletion and apoptosis in the intestine, as detected by the TUNEL assay, compared to the PBS-treated infected mice.

ATP levels in fecal samples were measured on day 1 and 3 post-inoculation. ATP levels on day 1 were significantly lower in the apyrase-treated and infected mice when compared with PBS-treated *E. coli* O157:H7-infected mice. Stx2 was detected in feces on days 1 and 3 post-inoculation in the *E. coli* O157:H7-infected mice, showing a tendency to higher Stx2 levels in feces from PBS-treated *E. coli* O157:H7-infected mice in comparison to the apyrase-treated and infected mice.

Apyrase lowered Stx2 release from *E. coli* O157:H7 in culture. A similar effect was noted using exogenously administered ATP. Apyrase (10 U/mL) and exogenous ATP (3 mM) also had an effect on lowering RecA levels in *E. coli* O157:H7 bacterial cell lysate indicating an effect on the SOS response.

The effect of apyrase on host cells exposed to Stx2 was evaluated. Apyrase (1 U/mL) preincubated with LPS (1 µg/mL) and Stx2 (200 ng/mL) significantly lowered collagen type-1-induced platelet aggregation. However, no protective effect of apyrase was detected on cell viability both in the case of HeLa and endothelial cells.

Taken together, apyrase had a protective effect on *E. coli* O157:H7-infected mice, lowering ATP release in the gut and protecting intestinal cells from damage most probably because it lowered Stx2 release from the bacteria. Additionally, apyrase decreased platelet activation and thereby had a dual effect both on the bacteria and on the host.

## Paper IV

This study investigated the effect of IgG treatment of mice on *E. coli* O157:H7 infection and attempted to determine the mechanism by which IgG affected the course of infection. Intraperitoneal administration of murine IgG (1 mg/mouse) on day 3 and 6 post-inoculation significantly improved survival in mice when infected with *E. coli* O157:H7 compared to untreated mice. Similar results were found in mice treated only on day 3.

*E. coli* O157:H7-infected mice treated with IgG on day 3 showed significantly lower bacterial colonization on day 8. All mice infected with *E. coli* O157:H7 exhibited weight loss starting from day 5, while the infected and IgG treated on day 3 and 6 remained stable.

Mice were evaluated for intestinal pathology including inflammatory infiltrates and goblet cell depletion and renal pathology consisting of tubular epithelial desquamation. Mice infected and treated with IgG exhibited less goblet cell depletion and less tubular epithelial desquamation when compared to untreated mice.

Renal dysfunction was evaluated by the measurement of BUN. Infected and untreated mice exhibited elevated levels of BUN which was significantly lower in infected and IgG treated mice.

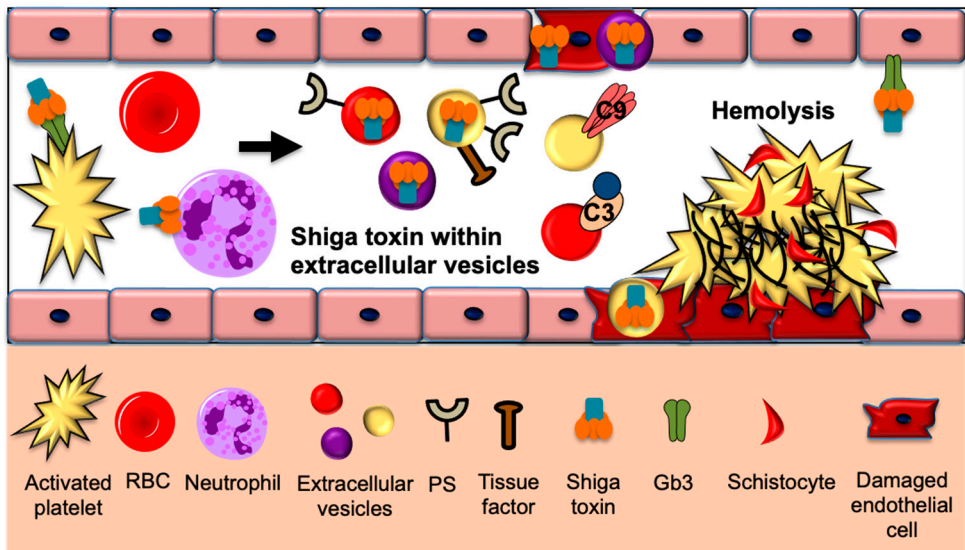
Both murine and human IgG bound to proteins present in *E. coli* O157:H7 culture filtrates. To identify the proteins that interacted with IgG, *E. coli* O157:H7 culture filtrates were separated on an immunoblot. Murine IgG detected six protein bands while human IgG detected seven proteins bands. Proteins corresponding to the detected bands were extracted in individual segments and these were then examined by mass spectrometry. EspP and translational elongation factor Tu were detected in five of the seven segments. EspP has been shown to contribute to bacterial virulence<sup>119</sup> and was further investigated. Binding of murine and human IgG to purified EspP was detected and mediated by the Fc fragment. This interaction between IgG and EspP was further confirmed by using a  $\Delta espP$  *E. coli* O157:H7 mutant lacking EspP.

The effect of IgG on enzymatic activity of EspP was investigated by pepsin cleavage using culture filtrates from both the wild-type and  $\Delta espP$  *E. coli* O157:H7 strains. Pepsin cleavage was achieved using the wild-type *E. coli* O157:H7 strain, as expected, and this cleavage was inhibited by both murine (5  $\mu$ g) and human IgG (100  $\mu$ g) after 16 hours of incubation.

Taken together this study shows that IgG can prevent disease in *E. coli* O157:H7-infected mice, and that murine and human IgG, the latter commercial preparation, bind to EspP from *E. coli* O157:H7 blocking its enzymatic activity and may thereby affect its potential in bacterial virulence.

## Discussion

There are no effective means to prevent or treat EHEC-associated HUS once it has occurred. Antibiotic treatment during the prodromal diarrheal phase of EHEC infection may increase the risk of developing HUS by inducing bacteriolysis and the release of Stx. This thesis focused on four novel strategies to reduce bacterial colonization, neutralize bacterial virulence, and protect host cells. The first step in the establishment of severe EHEC infection is bacterial colonization of the gut. This was reduced by treatment with IgG, as shown in paper **IV**. Note, that the treatment was given after inoculation which would mimic the situation during human infection. EspP is a known EHEC virulence factor associated with bacterial adherence to the intestinal cell lining and IgG binding to EspP interfered with the enzymatic effect of EspP. After colonization, EHEC releases Stx. Reducing the release of bacterial toxin, would decrease host cell injury, both in the intestine and systemically. This was shown by administration of apyrase in paper **III**. Not only did apyrase affect Stx release from EHEC by decreasing RecA, it also had an anti-thrombotic effect as it reduced collagen-mediated platelet aggregation when combined with Stx2 and O157LPS. Within the circulation, Stx can bind to blood cells and be released within blood cell-derived EVs that have prothrombotic and proinflammatory potential (Figure 7). These EVs can contain the toxin and transport it to the kidney. The third approach used in this thesis was to inhibit the release of EVs by blocking the purinergic P2X1 receptor involved in calcium influx and EV release, as shown in paper **II**. Importantly, this treatment also inhibited the cytotoxic effects of Stx. Once EVs are released and circulate with toxin, a treatment that could remove these harmful EVs could also have a protective potential. This was shown in paper **I**, in which, anxA5 coating of EVs led to their phagocytic removal, and protected mice during EHEC infection. Taken together, the four separate non-antibiotic approaches studied, using *in vivo* and *in vitro* methods, could inhibit essential aspects of EHEC and Stx virulence.



**Figure 7: Extracellular vesicles in EHEC-associated HUS.**

Shiga toxin bound to Gb3 on blood cells, activates them and induces release of extracellular vesicles (EVs). These blood cell-derived EVs express phosphatidylserine (PS) and tissue factor. Some platelet and red blood cell-derived EVs express complement factors (C3 and C9). This figure is modified from<sup>207</sup> (<http://creativecommons.org/licenses/by/4.0/>).

Volume expansion has been shown to be protective during EHEC infection. A combination of volume expansion with a treatment that delays the onset of Stx-mediated injury could prevent the development of severe HUS. Both anxA5 and apyrase resulted in a delay of disease onset. AnxA5 reduced systemic levels of prothrombotic EVs containing toxin while apyrase could reduce the real thrombotic effects on platelets. AnxA5 most probably delayed disease in mice by reducing the level of circulating toxin-positive EVs. Probable additional beneficial effects of anxA5 were not addressed here. AnxA5 was shown to have anti-inflammatory properties in an apoE<sup>(-/-)</sup> murine model of vascular inflammation by lowering leukocyte chemotaxis<sup>271</sup> and in a mouse model of endotoxemia by reducing cytokine release in response to LPS<sup>238</sup> and also inhibiting binding of LPS to TLR4.<sup>234</sup> Apyrase presumably delayed disease by reducing the SOS response and toxin release followed by a protective systemic effect on platelet activation.

The substances studied in this thesis have the potential to being used in humans. Trials with anxA5 are being conducted in healthy volunteers (<https://clinicaltrials.gov/ct2/show/NCT04850339>) as well as a treatment for COVID infection (<https://clinicaltrials.gov/ct2/show/NCT04748757>). Suramin is used as a treatment for parasite infection in humans.<sup>272</sup> Apyrase has been suggested as a treatment in humans for systemic inflammation, infections and wound healing during burns.<sup>273-275</sup> In line with this, a patent application for the route of

administration of apyrase in humans has been submitted (<https://patents.justia.com/patent/20170333532>). Herein apyrase was administered via the intraperitoneal route however this approach would not be appropriate during EHEC infection in humans as the gastrointestinal tract is inflamed, and thus other routes of administration should be investigated before a clinical trial can be planned. Intravenous and subcutaneous administration of IgG is already being used in the clinic for multiple indications. It could be a potential candidate for trials in patients with EHEC infection. Oral administration of IgG to patients with EHEC infection lead to reduced diarrhea<sup>219</sup> and could probably be protective during the early stages of infection. IgG has also been shown to have an effect on intestinal attachment of EHEC and colonization in mice.<sup>270</sup> These data indicate that IgG could be considered as a potential therapeutic and should be investigated further to identify the optimal route of administration and its effect on human EHEC infection should be assessed.

All animal models have their limitations. A critical assessment of the results presented here, that all contain the EHEC-infected mouse model, is addressed. This is especially important when comparing to the human disease. In humans, EHEC-induced gastrointestinal infection develops approximately 4 days after ingestion of the bacteria and, after an additional 3-7 days, HUS may develop in up to 15% of cases. Patients present with low platelet counts due to consumptive thrombocytopenia and platelet activation, which is due to toxin-induced activation of platelets and the endothelium,<sup>15, 34</sup> thus patients, at this point (about 10-14 days after ingestion of EHEC), cannot undergo intestinal or renal biopsy due to the serious risk of bleeding. Severe cases exhibit extensive intestinal damage showing hemorrhagic colitis, severe necrosis and erosions, while these indication of damage are hard to evaluate in milder cases.<sup>10</sup> In the most severe cases, the renal pathological lesion typically shows thrombotic microangiopathy and severe tubular injury.<sup>126, 276</sup>

The scientific humane approach to experiments involving animal will not allow research groups to follow severely ill animals for days and will therefore limit the duration of experimental procedure. In fact, our ethical permit requires that the mice should be sacrificed as soon as any symptoms develop. This aspect is applicable to understanding the differences between EHEC infections in human and animal, as we do not know at which point after development of initial symptoms (in humans) the pathological lesion develops. The pathological lesions are caused by systemic spread of Stx to target organs (as demonstrated in infected mice<sup>72, 147</sup>) as well as other virulence factors affecting the intestines.<sup>10</sup> The effect of toxin may be cumulative indicating that several days would be required in order to develop into fulminant thrombotic microangiopathy. The only animal models, in which this lesion has been demonstrated, either spontaneously or in a scientific setting, are baboons and dogs,<sup>146, 277</sup> which are harder to conduct intervention studies on.

Mice usually do not develop a spontaneous disease resembling HUS in humans after EHEC ingestion. For this reason, our group and others have established a variety of experimental settings that allow induction of disease leading to symptoms and

pathology of EHEC infection that strongly resemble the infection in humans. These include either a period of fasting prior to inoculation, administration of antibiotics to remove some of the intestinal microflora, administration of mitomycin (to release Stx) or the use of germ-free mice.<sup>278</sup> Certain models have been developed by treating mice with purified Stx with or without LPS. In this thesis, I used the first two settings as established in our lab previously, that is fasting and antibiotic (streptomycin) treatment before inoculation with *E. coli* O157:H7. Our group has demonstrated that mice infected with *E. coli* O157:H7 develop gastrointestinal, neurological, and renal symptoms, all resembling human disease.<sup>133, 147</sup> The infected mice have low red blood cell counts and fragmented red blood cells (as in humans), thrombocytopenia and renal failure, also characteristic features of HUS in humans.<sup>133, 147</sup> Furthermore, these mice also develop intestinal and renal lesions resembling human pathology<sup>21, 72</sup> although, as mentioned above, lesions in mice may not fully correspond to the severity of lesions as observed in humans as the infection persists for a much longer duration at the time when biopsies or autopsies are obtained.

However, mice do not develop thrombotic microangiopathy. Mice develop glomerular injury indicated by endothelial cell swelling and deposition of platelets<sup>132, 170, 171</sup> and fibrin deposition in glomeruli indicating thrombus formation<sup>133</sup> as well as glomerular cell apoptosis identical to that seen in humans after ingestion of EHEC.<sup>72, 77</sup> Both humans and mice develop identical tubular damage in the renal cortex<sup>72, 276</sup> along with severe tubular apoptosis. Lesions in the brain of sick mice corresponds to the damage seen in humans.<sup>279</sup> Taken together, the mouse model seems highly appropriate for the study of EHEC infection, with similar symptoms as well as renal and neurological lesions, and has been used in many studies addressing pathogen-mediated injury and the host response.

The importance of *E. coli* O157:H7 adhesins for colonization and pathogenesis has been demonstrated in mice.<sup>10, 278</sup> Several studies in mice have shown the importance of Stx for the virulence of EHEC strains.<sup>147, 280</sup> Our mouse model demonstrates the mechanism of Stx transfer from the intestine into the kidney within blood cell-derived EVs that are endocytosed by target organ cells (glomerular endothelial and tubular, both endothelial and epithelial cells) thus leading to cell death, as shown both, in patients and in mice.<sup>21</sup> Studies in mice have shown the importance of host response pathways such as the CXCR4/CXCR7/SDF-1 pathway<sup>171</sup> and LPS signaling,<sup>170</sup> diet<sup>281</sup> and anti-microbial peptides.<sup>36</sup> Mouse models have also been used to study therapeutic interventions such as targeted trafficking of Stx by administration of manganese,<sup>282</sup> usage of synthetic Retro compounds<sup>283, 284</sup> or blocking the toxin with antibodies.<sup>218</sup> The mouse model is thus highly suitable for the study of pathogen virulence and the host response, mimicking human disease.

Additionally, several *in vitro* assays involving endothelial and epithelial cells have been investigated in order to imitate different aspects of human *E. coli* O157:H7 infection. The expression levels of Gb3 on cells contributes to their susceptibility to Stx. In this thesis, HeLa cells have been used to investigate Stx-induced cell

activation and damage. Keeping in mind, that HeLa cells are cervical epithelial cells and not cells specific to the target organ, their usage should be assessed. HeLa cells have been shown to express the toxin receptor, Gb3 and that Stx binds to Gb3 via the B-subunit.<sup>285</sup> These cells have been shown to be highly sensitive to Stx and contribute to Stx-induced apoptosis.<sup>75</sup> Studies in HeLa cells suggest that Gb3 is not only required for Stx binding,<sup>57</sup> but is also essential for determining its route of intracellular transport. This indicates that, the association of Gb3 in lipid rafts could contribute to directing Stx towards retrograde transport.<sup>64</sup> These data suggest that HeLa cells that are sensitive to Stx could be used as a model to study Stx-mediated disease *in vitro*. In line with assessing the usage of HeLa cells in this thesis, in paper **I**, HeLa cells have been used as a source of EVs to investigate phagocytosis by murine phagocytes in the presence of anxA5. The effect of anxA5 on inducing phagocytosis was a proof of concept to show that this effect of anxA5 is most likely not dependent on the parent cell or the phagocyte and it is probably based on the interaction between the EV and the phagocyte. In the disease setting, elevated levels of EVs have been found in samples taken both from EHEC infected patients and mice infected with EHEC.<sup>21</sup> Thus usage of an *in vitro* set-up that was tailored to mimic the *in vivo* scenario occurring in a blood vessel would be relevant with regard to *E. coli* O157:H7 infection in humans. Thus, differentiated THP1 cells were combined with blood cell-derived EVs in the presence of anxA5 to mimic the *in vivo* scenario. Platelet activation has also been associated with EHEC-mediated HUS, making platelets a highly relevant cell type to study *in vitro*. Stx can activate and bind platelets<sup>126</sup> and in turn release of platelet-derived EVs induced by Stx was observed in paper **II**.<sup>21</sup> During EHEC infection, platelets, Stx and LPS can interact together. Stx and LPS have been found on platelets in patient samples and could induce platelet activation *in vitro*.<sup>15</sup> Stx induced calcium influx and EV release from platelets in paper **II** and a combination of Stx and O157LPS induced platelet aggregation in paper **III**. In summary, the *in vitro* models utilized in this thesis could be relevant in understanding the intracellular functioning of Stx and further its role in EHEC-associated HUS.

# Conclusions

- AnxA5 induces EV uptake by phagocytes *in vitro*. AnxA5-mediated clearance reduced circulating EVs, which during EHEC infection carry Stx, suggesting that a lower toxin burden will reach the kidney. AnxA5 delayed disease development in mice.
- Stx binding to cells induced release of ATP and subsequently leading to purinergic receptor activation. Stx-mediated calcium influx, cytotoxicity and EV release utilized ATP-mediated purinergic signaling via P2X receptors.
- Apyrase delayed disease development in EHEC-infected mice. It exerted a protective effect on the host with less goblet cell depletion and apoptosis of intestinal cells as well as less platelet activation in the presence of Stx2 and O157LPS. Apyrase also had an effect on *E. coli* O157:H7 which lead to a decrease in the release of Stx by a decrease in the SOS response. Apyrase was shown to have a protective effect both on reducing virulence of the bacterium and on the host cells. The latter most probably by reducing extracellular ATP.
- Administration of murine IgG to *E. coli* O157:H7-infected mice reduced colonization, improved survival and protected mice from renal and intestinal pathology. Both murine and human IgG bound to *E. coli* O157:H7 secreted protein, EspP *in vitro* via the Fc domain and inhibited its enzymatic activity. Thus IgG binds to EspP and may thereby exert a protective effect during *E. coli* O157:H7 infection.





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