Mechanisms of Shiga toxin-mediated signaling and toxicity

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2020

Document Version:
Publisher’s PDF, also known as Version of record

Link to publication

Citation for published version (APA):

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Mechanisms of Shiga toxin-mediated signaling and toxicity

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Mechanisms of Shiga toxin-mediated signaling and toxicity

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DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended in Belfrangesalen, Biomedicinskt Centrum, D15.
Friday the 17th of January 2020, at 9.00

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Dept of Pediatric Nephrology, Radboud University Medical Center, Nijmegen, The Netherlands
Abstract

Shiga toxin (Stx) is the main virulence factor of enterohemorrhagic *Escherichia coli* (EHEC). EHEC strains cause gastrointestinal infection and release Stx that can gain access to the circulation. Patients may develop hemolytic uremic syndrome with extensive kidney damage.

In the first paper we investigated if blood cell-derived microvesicles released during EHEC infection contain Stx. Toxin-containing microvesicles were found in plasma from EHEC-infected patients and mice, and in kidney cells. Toxin transferred within microvesicles retained its toxic potential. This study demonstrated a novel mechanism of Stx delivery to the kidney.

In the second paper the role of the Stx receptor, globotriaosylceramide (Gb3) in toxicity and intracellular transport of Stx delivered via microvesicles was investigated. Stx-positive microvesicles were taken up by both Gb3-positive and Gb3–negative cells but the toxin only exhibited toxicity when the cells expressed endogenous Gb3.

Stx induces calcium influx, cytotoxicity and microvesicle release in cells. In the third paper we found that ATP is released from HeLa cells after Stx stimulation and that ATP functions as a secondary messenger to promote all the above-mentioned toxin effects. Blocking purinergic P2X signaling with NF449 inhibited Stx-induced calcium influx, microvesicle release and protected cells from the cytotoxic effects.

In the fourth paper the role of extracellular ATP was investigated in a murine model of EHEC infection. Infected mice were injected with apyrase to cleave extracellular ATP. Apyrase protected the mice form EHEC-induced intestinal damage and delayed symptoms.

The fifth paper is a review that summarizes the role of extracellular vesicles in normal physiology and kidney diseases.

In summary this thesis defined a novel mechanism of Stx transfer to the kidneys, an ATP-mediated effect of Stx-signaling and the role of Gb3 on the cellular effect of Stx-positive microvesicles.

Key words: EHEC, hemolytic uremic syndrome, Shiga toxin, purinergic signaling, microvesicles

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Mechanisms of Shiga toxin-mediated signaling and toxicity

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Faculty of Medicine
Department of Pediatrics
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Lund University
Sweden
2020
Till minnet av min pappa
List of papers

This thesis is based on the following papers:


II. **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 the toxin receptor. Manuscript.


Permission to reprint the previously published articles has been granted by the respective publisher.
The following papers have been published but are not included in the thesis:


## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A/E</td>
<td>Attaching and effacing</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohemorrhagic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>Gb3</td>
<td>Globotriaosylceramide</td>
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<tr>
<td>Gb4</td>
<td>Globotetraosylceramide</td>
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<tr>
<td>HUS</td>
<td>Hemolytic uremic syndrome</td>
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<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>OMV</td>
<td>Outer membrane vesicles</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<tr>
<td>Stx</td>
<td>Shiga toxin</td>
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<tr>
<td>T3SS</td>
<td>Type three secretion system</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are gram-negative bacteria that may lead to disease in humans and in severe cases causes hemolytic uremic syndrome (HUS), characterized by thrombocytopenia, hemolytic anemia and acute kidney injury.\(^1,2\) EHEC enters the body via ingestion of contaminated food or water and passes through the gastrointestinal tract to the large intestine. EHEC is non-invasive and colonizes the large intestine.\(^3\) Within the intestine EHEC releases several virulence factors, including Shiga toxin (Stx), which is the main virulence factor. Stx must enter the circulation to reach and damage the target organs, including the kidney.\(^4-7\)

Free Stx is scarcely detectable in plasma from hemolytic uremic syndrome (HUS) patients.\(^8\) However, Stx may circulate bound to blood cells. Stx may bind to platelets,\(^9-11\) neutrophils\(^11-13\), monocytes\(^11, 14\) and red blood cells\(^15\) and thus be transported in the circulation, but this does not fully explain how it is transferred to target organ cells such as in the kidney. Stx-binding induces release of microvesicles from platelets, neutrophils, monocytes and red blood cells.\(^11, 15\) Microvesicles are small (100 – 1000 μm) vesicles that are shed directly from the plasma membrane.

The first paper of this thesis investigated the systemic transport of Stx via blood cell-derived microvesicles in the circulation and the delivery of the toxin-bearing vesicles to relevant kidney cells. The potential of toxin within microvesicles to exert a cytotoxic effect was further addressed.

Stx is endocytosed by cells following binding to its receptor, globotriaosylceramide (Gb3).\(^16\) After uptake, Stx is transported via the retrograde pathway to the endoplasmic reticulum (ER).\(^17\) From the ER the enzymatic A subunit of Stx is released into the cell cytosol where it exhibits RNA N-glycosidase activity cleaving an adenine base of the 28S rRNA, causing inhibition of protein synthesis and thereby leads to cell death.\(^18, 19\)

Stx has been detected within cells that lack the Gb3 receptor\(^20\) and may also indirectly affect cells that lack the toxin receptor.\(^21, 22\) This was also demonstrated in murine glomerular endothelial cells in the first paper of this thesis.\(^23\), which may indicate a receptor-independent mechanism of toxin uptake. In the second paper included in this thesis we investigated the importance of endogenous Gb3 in the recipient cell for the toxicity of microvesicle-delivered Stx in Gb3-positive and Gb3-negative cells.
Stx induces calcium influx, protein synthesis inhibition, apoptosis and microvesicle release in stimulated cells.\textsuperscript{5, 11, 15, 24-27} In the third paper of this thesis a secondary messenger contributing to these cellular effects of the toxin was identified.

Stx induced release of ATP \textit{in vitro} and \textit{in vivo} and the released ATP activated purinergic receptors on host cells. We showed that pharmacological blockade of purinergic P2X signaling inhibited Stx-mediated calcium influx, microvesicle-release and cytotoxicity \textit{in vitro}.

The fourth paper in the thesis investigated the role of extracellular ATP in an \textit{in vivo} mouse model of EHEC infection using the ATP-hydrolyzing enzyme apyrase. Apyrase-treatment delayed symptoms in mice. Furthermore, goblet cell depletion and intestinal apoptosis were reduced.

The fifth paper focused on describing the role of extracellular vesicles in normal physiology as well as biomarkers of kidney disease. Specifically the review mentions the role of microvesicles in HUS and in inflammatory renal diseases.

In summary, this thesis addresses the transport of Stx within the circulation to the kidneys via microvesicles, the importance of the Gb3 receptor in microvesicle-delivered Stx toxicity, the mechanism by which Stx induces cell signaling and the effect of extracellular ATP during EHEC infections. This thesis thereby provides novel insight in the pathogenesis of EHEC infection and may contribute to identifying possible targets for future therapeutic strategies.
Enterohemorrhagic *Escherichia coli*

EHEC are human pathogens. The bacteria colonize the intestine of graminivores asymptptomatically. Human infection occurs via the fecal-oral route by ingestion of contaminated food or water and has a global prevalence. EHEC may infect individuals in all age groups but is especially harmful to infants and the elderly and can in severe cases cause hemolytic uremic syndrome (HUS) leading to acute kidney injury and death. Infectious doses as low as 100 colony-forming units can cause disease symptoms. Ingested EHEC is transported in the gastrointestinal tract to the large intestine, which it colonizes. EHEC has developed efficient acid resistance, which explains its capability to survive the acidic environment of the stomach. EHEC is a non-invasive bacteria and once it has reached the large intestine it colonizes the intestinal epithelium but does not cause bacteremia or septicemia.

There are several pathogenic EHEC serotypes. These strains are named based on the terminal portion of the lipopolysaccharide (O-antigen) and the type of flagella (H-antigen) that the bacteria express. Serotypes associated with human disease include O157:H7, O26:H11, O103:H2, O111:NM, O121:H19 and O145:NM.

**EHEC adhesion**

The initial EHEC adhesion to the intestinal mucosa is mediated by several factors, such as fimbriae and the type three secretion system (T3SS) (Figure 1). This first adhesion is followed by a more intimate adhesion leading to attaching and effacing (A/E) lesions. A/E lesions are anchoring points that require transfer of bacterial proteins into the host epithelial cell. EHEC encodes the protein complex T3SS that resembles a syringe-like structure. T3SS attaches to host cells, linking the bacteria to the epithelial cell and allows for bacterial protein transfer into the recipient cell membrane. In order to transfer proteins to the host cell EHEC forms a pore in the host membrane composed of EspB and EspD. EHEC protein-transfer includes translocated intimin receptor (Tir) and *E. coli* secreted protein F-like protein from prophage U (EspFu). Tir proteins form transmembrane structures in the epithelial cell that enable binding to intimin that is present on EHEC. EspFu recruits the actin-modulating proteins Wilskott-Aldrich syndrome protein (N-WASP) and Insulin receptor substrate p53 (IRSp53) that connect the Tir
complexes to the actin network of the host cell and also form the pedestal-like actin structures that are typical for A/E lesions.\textsuperscript{42}

![Figure 1. Schematic figure depicting the formation of an attaching/effacing lesion.](image)

EHEC attaches to epithelial cells via the type 3 secretion system (T3SS) and transports Tir and EspFu into the host cell. Tir is inserted into the membrane of the recipient cell and forms dimers that function as anchoring points for the intimin receptor. EspFu promotes remodelling of the host cell’s actin cytoskeleton and subsequently forms pedestal-like structures. This figure was made using PowerPoint software v.14.7.7.

Certain determinants of the T3SS have been associated with bacterial virulence. For example, EspB, has been shown to induce diarrhea in healthy volunteers who ingested a wild-type strain and its isogenic $\Delta$EspB mutant.\textsuperscript{43} Intimin was associated with a higher mortality in a mouse model.\textsuperscript{44} The presence of intimin and T3SS effectors were associated with a higher symptom score in murine EHEC infection.\textsuperscript{45}

**EHEC virulence factors**

During EHEC infection the bacteria release several virulence factors. The main virulence factor is Shiga toxin (Stx). Other virulence factors include lipopolysaccharide (LPS), EHEC-hemolysin and subtilase.
Shiga toxin

Stx is the major virulence factor of EHEC. The toxin is named after the Japanese physician Kiyoshi Shiga who discovered *Shigella dysenteriae*, a Stx-producing bacteria that may cause dysentery.\(^46\) Shiga toxin is an AB5 toxin, consisting of an enzymatically active A-subunit and a binding, pentameric B-subunit.\(^47\)

**Release and transport of Shiga toxin**

Stx is released from EHEC via outer membrane vesicles (OMVs) or by bacterial lysis. It is released in the intestine and is essential for intestinal damage and hemorrhagic colitis as shown in animal models.\(^45\), \(^48\) For the toxin to exert its systemic effect it needs to be transported from the intestine into the circulation. It is not completely known how Stx is transported across the intestinal epithelium; however, several mechanisms have been suggested.

The tight proximity of intestinal cells was shown to be disrupted in response to EHEC infection *in vivo* and *in vitro*.\(^49\), \(^50\) Furthermore, during EHEC infection, Stx was shown to diffuse between monolayer cultures of the intestinal epithelial cell line T84 cells, a cell line lacking the Stx receptor.\(^51\) The translocation of Stx across intestinal epithelium was enhanced during inflammation with neutrophil extravasation into the intestinal lumen.\(^52\)

Stx may be taken up by intestinal cells by macropinocytosis, followed by release on the basal side via transcytosis.\(^20\) Free toxin has also been shown to cross monolayer cultures of T84 cells without disrupting the barrier function of the culture. The translocation of the toxin was shown to be energy-dependent, indicating a cell-mediated transport.\(^53\)

Uptake of Stx by intestinal cells may occur via receptor-mediated endocytosis, followed by subsequent release through cell death induced by the toxin. Human intestinal epithelial cells normally only express trace amounts of the Stx receptor.\(^54\) The fatty acid butyrate have been shown to increase expression of the toxin receptor in intestinal epithelial cells *in vitro*, providing a possible mechanism for Stx uptake and translocation.\(^55\)

Regardless of the mechanism through which Stx is translocated over the intestinal epithelial cell layer the toxin also needs to cross the lamina propria (a layer of connective tissue) to reach blood vessels. Stx has been found in the lamina propria in a study using *in vitro* intestine organ cultures.\(^56\) Access to the bloodstream may be a result of the massive inflammation and damage of the intestine. Once Stx has gained access to blood vessels it can interact with and damage intestinal endothelial cells.\(^57\)
The transport of Stx in the circulation was a focus of this thesis. This aspect is of interest because only trace amounts of free Stx have been found in patient blood.\(^8\) Stx can bind to platelets,\(^11\) monocytes,\(^11\) red blood cells,\(^15\) and neutrophils.\(^11, 12\) Binding may lead to cellular uptake, as has been shown in platelets and monocytes.\(^10, 58\) Cells that bind Stx shed microvesicles, which will be described below.

From the circulation Stx targets various organs, such as the kidney\(^{59, 60}\) and the central nervous system.\(^{61}\) Target cells in the kidney include glomerular endothelial cells, tubular cells, mesangial cells and podocytes.\(^4-7, 62-64\)

### Shiga toxin 1 and 2

Stx is a collective name for Stx1 and Stx2. While Stx1 has an almost identical amino acid sequence to the Stx produced by *Shigella dysenteriae*, save for one amino acid, Stx1 and Stx2 share only 56% amino acid homology.\(^{65, 66}\) Both Stx1 and Stx2 are released by EHEC but Stx2 has been associated with more severe disease during HUS.\(^{67}\) The two toxins share the same receptors but with different affinities, as will be described below. There are currently three known variants of Stx1: Stx1a, Stx1c and Stx1d and seven variants of Stx2: Stx2a-g.\(^{68, 69}\)

### The Shiga toxin receptor

Stx binds to the neutral glycosphingolipids globotriaosylceramide (Gb3) or globotetraosylceramide (Gb4) (Figure 2).\(^{70}\) There is also evidence for binding of Stx2e to globopentaosylceramide.\(^{71}\) Neutral glycosphingolipids consist of a ceramide base with carbohydrates attached to the hydrophilic part. Ceramide in turn consists of sphingosine with an attached fatty acid that varies in length. The first step in Gb3 biogenesis is attachment of glucose (Glu) to ceramide via glucosylceramide synthase, producing glucosylceramide. This is followed by addition of two galactose (Gal) molecules to produce Gb3. An additional addition of N-acetylgalactosamine results in Gb4. The enzyme responsible for the final step in Gb3 synthesis is lactosylceramide 4-α-galactosyltransferase and the presence of this enzyme is a critical determinant in cellular Stx-sensitivity.\(^{72}\)

Glycosphingolipids are present in the membrane of most mammalian cells and are enriched in membrane domains known as lipid rafts.\(^{73, 74}\) Lipid rafts, or detergent-resistant membrane domains, are dynamic structures of the membrane with increased concentrations of long-tailed lipids, cholesterol and trans-membrane proteins. These structures promote endocytic events and are involved in cell signal transduction.\(^{74}\)

Stx1 and Stx2 both have affinity for Gb3 and Gb4.\(^{70}\) However, Stx1-binding requires the saccharide part of the glycosphingolipid, as the soluble glycan part of
Gb3 (Glu-Gal-Gal) is sufficient for Stx1-binding. In contrast Stx2, also utilizes the lipid residues for binding.\textsuperscript{70} Stx1 binds well to purified Gb4 but Stx2 require additional factors for Gb4-binding, such as cholesterol and phosphatidylcholine.\textsuperscript{70} The side-chain of the ceramide residue also affects Stx1- and Stx2-binding. Stx1 has increased affinity for side-chains with 20 or 22 carbons while Stx2 has optimal affinity for 18-carbon side-chains.\textsuperscript{75}

Each part of the pentameric B-subunit of Stx has three binding sites, resulting in 15 possible binding sites for the receptor per Stx molecule.\textsuperscript{76, 77} This induces clustering of Gb3 upon Stx binding and decreases the probability of dissociation.

![Figure 2. Schematic drawing of the Shiga toxin receptor globotriaosylceramide](image)

The Shiga toxin receptor globotriaosylceramide (Gb3) consists of a sphingosine backbone (blue), a side chain that varies in the number of carbons (green), a glucose attached to the sphingosine (purple) and two galactoses (red). Sphingosine together with a side-chain forms ceramide.

**Endocytosis and intracellular transport of Shiga toxin**

After binding of Stx to Gb3 and Gb4 in the outer plasma membrane leaflet, the toxin is internalized by the cell. Stx uptake has been shown to occur through several pathways, involving both clathrin-dependent and –independent mechanisms.\textsuperscript{78, 79} Upon binding, several Stx molecules cluster together and induce mechanical membrane invaginations in the plasma membrane.\textsuperscript{80} These invaginations are recognized and stabilized by the BAR-domain protein endophilin-A2 that together with actin and dynamin induce scission, producing
intracellular vesicles. The early vesicular structures fuse with early endosomes and are sorted and further transported to the trans-golgi network and the endoplasmic reticulum (ER) (Figure 3).

The transport route of the Stx is dependent on if the Gb3 that is encountered at the plasma membrane is associated with a detergent-resistant membrane environment. Interestingly, in human macrophages and dendritic cells Gb3 is separated from detergent-resistant membranes, leading to lysosomal transport of the toxin, while in HeLa cells, where Gb3 is associated with lipid rafts, Stx is transported to the ER. Furthermore, in HeLa cells Stx seems to be associated with detergent-resistant membranes throughout the retrograde transport.

During the transport route the A-subunit of the toxin is enzymatically cleaved, mainly by the protease furin, into Stx-A1 and -A2 moieties that are held together by disulfide bonds. In the ER the disulfide bonds are reduced and the A1-moiety is unfolded by the ER-chaperone Binding immunoglobulin Protein (BiP). BiP associates with an ER-translocase and refolds and translocates the A1-moiety over the ER membrane and into the cell cytosol.

Figure 3. Retrograde transport of Shiga toxin.

The B-subunit of Shiga toxin (Stx-B) binds to Gb3 and the toxin is taken up by the cell. The toxin is transported to the early endosome, followed by the Golgi apparatus and the endoplasmic reticulum (ER). In the ER the A-subunit of Stx (Stx-A) is cleaved by furin and a part of the A-subunit (A1) is released into the cytosol.
Protein synthesis inhibition and apoptosis

Stx induces inhibition of protein synthesis in several mammalian cell types. Once the A₁-moiety of Shiga toxin is transferred into the cytosol it depurinates an adenine base of the 28S rRNA in the 60S subunit of the ribosome and thereby inhibits mRNA translation.²⁶⁻⁸⁵

Stx causes apoptosis in a range of different cell types, such as intestinal mucosal cells, renal cortical cells and epithelial cells.⁵⁻²⁷⁻⁸⁶ Stx-induced apoptosis involves activation of the initiator caspases, caspase-8 and -9.²⁷⁻⁸⁷ These are in turn induced by Calpain and Cytochrome C. Cytochrome C is released from the mitochondria via pores consisting of Bak/Bax and Bak has been shown to be upregulated by Stx₂.⁸⁸⁻⁸⁹ Caspase-8 and -9 both activate the executioner caspases, caspase-3 and -7, which eventually executes the apoptotic program.⁸⁹

The mechanism for how Stx induces apoptosis is not completely understood, however multiple contributing pathways have been suggested. Upon Gb3-binding, Stx induces cellular activation, characterized by calcium influx from extracellular sources.²⁵⁻⁹⁰ This activation seems to be dependent on cross-linking of Gb3, as an IgM antibody, targeting several Gb3s, was shown to give rise to a similar response, while the corresponding IgG, targeting one Gb3, did not.²⁵ The B-subunit of Stx₁ alone has been shown to induce apoptosis in Gb3-positive cell lines.⁹¹

Stx induces ER stress signaling in target cells. When Stx reaches the ER it is recognized by the chaperone BiP as an incorrectly folded protein.⁸⁴ BiP acts as a surveyor of protein folding in the ER. Under normal conditions BiP inhibits proteins that control ER cell stress responses. When incorrectly folded proteins are present in the ER, BiP associates with these and thereby releases its inhibiton of ER stress proteins leading to ER stress signaling.⁹²⁻⁹³

Stx-mediated inhibition of protein synthesis induces the ribotoxic stress response and may cause apoptosis via activation of p38 mitogen activated protein kinase.⁹⁴ Interestingly, it is not the halted protein synthesis that gives rise to this response, but rather the site where the depurination occurs, as indicated by the lack of stress response caused by cycloheximide, a mechanistically distinct protein synthesis inhibitor.⁹³

Taken together, Stx-induced apoptosis may depend on several cell stress pathways.

Shiga toxin-induced signaling

Stx-binding to its receptor initiates signaling in the cell. The signaling is quite unique given that the glycosphingolipid receptor, to which Stx binds, lack means for signal transduction into the cell on its own. The mechanism for how Stx
initiates signaling through binding of membrane lipids is unknown. One theory is that the clustering of Gb3 that occurs through the multiple binding sites of the Stx B-subunit and subsequent clustering of Stx mechanically forces proximity and activation of adjacent receptors.

Stx1 binding leads to calcium influx in a range of cell types, including endothelial and epithelial cells.\textsuperscript{24, 25} The exact mechanism has not been elucidated but several factors have been shown to be involved in the signaling. The response seems to be linked to the phospholipase C (PLC) pathway, as Stx1-induced calcium influx in human umbilical vein endothelial cells was almost completely abrogated when treated with a PLC inhibitor.\textsuperscript{24}

The origin of the increased cytosolic calcium in response to Stx1-binding seems to come mainly from extracellular sources. Stx1-binding to HeLa cells in calcium-free solutions resulted in minor cytosolic calcium increases.\textsuperscript{25}

Not much is known about Stx2-induced signaling. Stx2 binding induces calcium influx in murine neurons\textsuperscript{95} but not in human endothelial cells.\textsuperscript{24} Stx2 does, however, activate protein kinase A (PKA) in endothelial cells, preceding release of von Willebrand factor.\textsuperscript{24} The activation occurs via an unconventional mechanism, as it is not preceded by elevation in cyclic AMP, which is the canonical pathway for PKA activation.\textsuperscript{24}

Lipopolysaccharide

LPS is an endotoxin found in almost all gram-negative bacteria.\textsuperscript{96} As the name suggests it consists of a lipid anchor, connecting it to the bacterial membrane, and a saccharide part that extends out from the membrane. The saccharide component of LPS is divided into a core region, closest to the lipid anchor, and the O-antigen, which constitutes the most distal part of LPS.\textsuperscript{97} Soluble LPS that has been released from the bacterial membrane is highly immunogenic. The core region of LPS binds to LPS-binding protein that in turn binds to and forms a complex with CD14. This complex facilitates activation of TLR4, starting a signaling via the adaptor protein myeloid differentiation primary response 88 (MyD88), that results in cytokine release and activation of the innate immune system.\textsuperscript{98}

Patients with EHEC-induced HUS have LPS bound to circulating blood cells and blood cell-aggregates.\textsuperscript{11, 99} LPS induce release of tissue factor from whole blood.\textsuperscript{11} MyD88 knock-out mice exhibit more severe symptoms and have a higher bacteria burden compared to wild-type mice in response to EHEC infection, suggesting a role for LPS.\textsuperscript{100}
EHEC-hemolysin

EHEC-hemolysin is a pore-forming toxin that is mainly released associated with OMVs.\textsuperscript{101} The toxin is delivered into target cells and translocates to the mitochondria. The toxin is inserted into the mitochondrial membrane followed by subsequent oligomerization.\textsuperscript{101} Clustered EHEC-hemolysin subunits cause pore-formation and lead to unregulated flow of ions and other small molecules across the mitochondrial membrane, ultimately leading to apoptosis. The importance of this phenomenon \textit{in vivo} is unknown. EHEC-hemolysin may also be released in soluble form from the bacteria and has been shown \textit{in vitro} to bind to and lyse red blood cells.\textsuperscript{102}

Subtilase

Subtilase is an AB\textsubscript{5} toxin that was identified following an outbreak of HUS that was caused by \textit{E. coli} O113:H21.\textsuperscript{103} It was later identified in other EHEC strains.\textsuperscript{104} The B-subunit of the toxin binds to glycans with terminal N-glycolylneuraminic acid, which is not naturally produced in human cells, but may be incorporated following ingestion of red meat and dairy products.\textsuperscript{105} Subtilase causes vascular injury, thrombosis and mortality when injected into mice.\textsuperscript{103} The cellular damage is mediated through cleavage of the ER protein BiP by the A-subunit of subtilase,\textsuperscript{106} which induces ER stress and subsequent cell death.\textsuperscript{107}

Outer membrane vesicles

OMVs are membrane-derived vesicles released from Gram-negative bacteria.\textsuperscript{108} Their diameter differs between different types of bacteria and ranges between 20 and 100 nm for vesicles released by \textit{E. coli} O157:H7.\textsuperscript{109} OMVs are released in response to various physiological stimuli, such as temperature change, which leads to loss of cross-linking between the proteoglycan layer and the outer membrane and disruption of the outer membrane fluidity.\textsuperscript{110-112} OMVs released by EHEC carries Stx, LPS and hemolysin. OMV-delivered Stx and hemolysin were shown to be taken up by both intestinal epithelial cells and renal microvascular endothelial cells, leading to cell death in these cells.\textsuperscript{101} In a mouse model, intraperitoneal injection of purified OMVs, derived from \textit{E. coli} O157:H7, containing both Stx2 and LPS were shown to induce HUS-like symptoms.\textsuperscript{113}
Host response during EHEC infection

Interaction of EHEC with intestinal epithelium

EHEC colonization causes an inflammatory response in the intestine. The initial response is mediated by the innate immune system through recognition of pathogen associated molecular patterns (PAMPs) such as LPS and flagellin that are present on the bacteria. PAMP recognition initiates signaling cascades, mainly induced by toll-like receptors (TLR)-4 and -5. The early inflammatory response in the intestine is enhanced by release of the pro-inflammatory cytokines IL-8 and TNF-α. IL-8 is a chemoattractant that recruits neutrophils. Neutrophils infiltrate the colonic mucosa layer during EHEC infections, possibly by IL-8-mediated chemotaxis. Furthermore, high levels of IL-8 in the intestine are associated with a worse outcome during EHEC infection.

In the intestine, the mucosal layer represents a major barrier during infection. The mucosal layer consists of epithelial cells and a layer of mucus, mainly produced by goblet cells. In addition there are antimicrobial peptides, such as cathelicidin, play a role in the preservation of the mucosal layer. Cathelicidin knock-out mice have a thinner mucosal layer and are more susceptible to EHEC infection compared to wild-type mice. During EHEC infection in humans there is massive damage and inflammation in the intestinal mucosal layer. Goblet cell depletion has been reported in EHEC infected mice. EHEC secretes a mucinase termed StcE that cleaves components of the intestinal mucus, thinning the mucus layer and increases bacterial adhesion.

Blood cell response to Shiga toxin

Stx may lead to both cell death and cellular activation, depending on the cell type to which it binds. Stx is non-toxic to monocytes and neutrophils but induces activation in these cells.

During EHEC-induced HUS, Stx is bound to platelets in the circulation. In vitro the toxin binds to Gb3 and a glycolipid called 0.03. However, Stx binding to platelets have been shown to occur only when the platelets are activated and this
seems to be dependent on how exposed the Gb3 on the platelet is. Following binding, Stx is taken up by the platelet. Following binding, Stx is taken up by the platelet.

Stx1-binding to isolated human monocytes causes activation and release of several cytokines, such as IL-1β, IL-8 and TNFα. In addition, both Stx1 and Stx2 induce release of IL-1β, IL-8 and TNFα from a monocytic cell line in vitro, with Stx2 being a greater inducer. Stx2-stimulation of monocytes cells leads to release of cytokines that in turn activates platelets. Importantly, both TNFα and IL-1β have been linked to increased Gb3 expression in endothelial cells and may play an important role in the EHEC pathogenesis. Stx2 activates the inflammasome complex in monocytic cells. Activation of the inflammasome complex requires a dual response in which ATP signaling constitutes one of the activating events. The inflammasome is important for IL-1β and caspase-1 maturation and pyroptosis, a caspase-1-dependent apoptotic-like process that has been implicated in Stx2 toxicity.

In neutrophils Stx2 has been shown to reduce cell death via activation of intracellular signaling pathways. Stx2 also induces release of reactive oxygen species in neutrophils, which may exacerbate EHEC infection.

Stx1 and Stx2 induce the release of von Willebrand factor from endothelial cells. von Willebrand factor is important in platelet adhesion to sites of endothelial injury and promotes platelet aggregation. von Willebrand may drive platelet consumption and thrombi formation during EHEC infection.
Clinical manifestations of EHEC infection

After oral EHEC ingestion clinical symptoms appear between 3 and 8 days and usually manifest as abdominal cramps and watery diarrhea and in some cases bloody diarrhea. EHEC infection may resolve spontaneously but about 15 % of the patients develop HUS, typically 2 – 12 days after the start of diarrhea. HUS manifests as the simultaneous occurrence of thrombocytopenia, hemolytic anemia and acute kidney injury. Patients may exhibit high blood pressure, jaundice, pallor and symptoms of renal failure.

Thrombocytopenia likely occurs as a consequence of damaged endothelium. Stx activates platelets in the presence of LPS or certain cytokines. Damaged endothelium leads to exposure of fibrinogen, collagen and von Willebrand factor, resulting in pro-thrombotic activation of platelets. The activation drives consumption of platelets and results in the formation of microthrombi. Interestingly, no major consumption of coagulation factors is seen in HUS.

Hemolytic anemia in HUS is non-immune, indicating that it is not induced by antibodies. The main hypothesis regarding the mechanism is that red blood cells are damaged as a result of microthrombi in the blood vessels, narrowing their path and mechanically rupturing them. This is supported by the presence of schistocytes (fragmented red blood cells) in blood smears from HUS patients. In addition, evidence for decreased membrane fluidity in the membrane of red blood cells, as a consequence of oxidative stress, has been described. This may cause cell rigidity and consequently higher risk of rupture.

During EHEC-induced HUS the kidney undergoes extensive injury to the renal cortex. This is mediated by several mechanisms, including formation of microthrombi that occlude the glomerular capillaries, detachment of glomerular endothelial cells, thickening of the vessel walls and severe tubular damage.
Purinergic signalling

Purinergic signalling comprises a family of receptors that bind and respond to extracellular nucleotides, such as adenosine triphosphate (ATP) or adenosine diphosphate (ADP), and adenosine. Purinergic receptors are expressed in most, if not all, cell types in the body. ATP is the main energy source for mammalian cells. Various stimuli or mechanical injury may cause release of ATP from cells. Extracellular ATP is an important damage-associated molecular pattern ligand and its signaling via purinergic receptors is involved in many different processes, of which inflammation is the most prominent.139

Receptor types

Purinergic receptors are divided into three different receptor classes: the metabotropic P1 and P2Y receptors and the ionotropic P2X receptors (Figure 4).140

P1 receptors

There are four different types of P1 receptors, all of which utilize adenosine as their main endogenous ligand. These receptors are expressed in the central nervous system, heart, kidney and adipose tissue.141 P1 receptors mainly, but not exclusively, have inhibitory effects on the cells on which they are expressed.142

P2Y receptors

P2Y receptors are present in almost all cells in the body and eight different types of P2Y receptors have been identified to date. P2Y receptors mainly bind ADP as endogenous ligands but can also use other adenine or uracil nucleotides.143

P2X receptors

Just as P2Y receptors, P2X receptors are expressed in most cell types in the body. There are seven different P2X receptor subtypes (P2X1–7) that form homo- or heterotrimeric complexes, utilizing ATP as the sole endogenous ligand.144 P2X
receptors are ionotropic and in the open state conformation they allow passage of specific ions, depending on the P2X receptor, in a concentration-dependent manner. The P2X$_1$ receptor is the only known P2X receptor present on platelets and has a high specificity for calcium ions.$^{145}$

**Release of ATP**

There are three main mechanisms of ATP release: passive release following mechanical insult to the plasma membrane, exocytosis of endosomally stored ATP and release of cytosolic ATP via transmembrane channels.$^{146,147}$

The most studied pathway of ATP-release is via transmembrane channels, such as the pannexin family. The pannexin 1 channel has been shown to open and release ATP in response to activation of the ER-associated calcium channel inositol triphosphate (IP3) receptor.$^{148,149}$ This is believed to occur through PLC-mediated cleavage of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and IP3 and subsequent binding of IP3 to IP3R (Figure 4).$^{150}$ In addition, depletion of ER-stored calcium activates the ER-associated calcium sensor protein STIM1 that in turn opens several membrane channels, including calcium channels and possibly also ATP-conducting channels.$^{151}$ This mechanism is believed to be important for re-stocking of calcium in the ER. $^{152}$ Several studies have demonstrated a direct association between PLC-activation and ATP release and this pathway likely represents the current most well studied pathway of ATP-release.$^{148,153-155}$

ATP is present at millimolar concentrations in the cell cytosol. Most ATP-activated P2X receptors respond in the micromolar range of ATP concentrations.$^{156}$ Under normal conditions released ATP may only reach micromolar ranges in the vicinity of the zone of release, pointing at auto- and paracrine signaling. However, during systemic inflammation a general increase in plasma ATP levels is present.$^{139}$
Figure 4. Schematic depiction of ATP-release via membrane channels. Activated phospholipase C (PLC) cleaves the membrane bound phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol. Released IP3 binds to the IP3 receptor in the membrane of the endoplasmic reticulum (ER), causing release of ER-stored calcium. This is turn opens Pannexin-channels in the plasma membrane and allows transport of adenosine triphosphate (ATP) out of the cell. Extracellular ATP activates P2X receptors on the cell from which it was released and on neighbouring cells, which results in influx of ions, such as calcium. ATP is degraded by the ectonucleosidase CD39 into adenosine diphosphate (ADP) that binds and activates P2Y channels. ADP is further degraded by CD39 into AMP, which is ultimately cleaved by CD73 into adenosine that binds to P1 receptors.

ATP release via bacterial factors

There are various modes by which bacterial factors may induce release of ATP from host cells. Direct interaction of virulence factors at the cell membrane may stimulate release of ATP by induction of intracellular signaling. In immune cells LPS mediates release of ATP via activation of TLR4.\textsuperscript{157} Other bacterial toxins may allow ATP release by forming a membrane pore. For example, HokB from \textit{Escherichia coli} forms a transmembrane pore leading to ATP leakage.\textsuperscript{158} Similarly, EHEC-hemolysin and leukotoxin A from \textit{Aggregatibacter actinomycetemcomitans} were incorporated in lipid vesicles allowing ATP release via toxin pores.\textsuperscript{159}
Degradation of extracellular ATP

Enzymes that degrade ATP and other purinergic nucleotides are called ectonucleotidases. There are two different ectonucleotidases in humans, namely CD39 and CD73. CD39 and CD73 are membrane bound enzymes that are mainly expressed on vascular endothelial cells and lymphocytes. CD39 cleaves off terminal phosphate groups of ATP and ADP and CD73 cleaves off the final phosphate group of AMP to form adenosine (Figure 4). The expression of CD39 and CD73 is inducible and has been indicated as a mechanism for regulatory T-cells and neutrophils to regulate the immune response. Continuous stimulation with ATP quickly desensitizes P2X receptors. Ectonucleotidases are thus crucial in order to keep purinergic receptors sensitive to stimuli and to regulate the levels of extracellular ATP.

Apyrase is a nucleotidase that cleaves off phosphate groups of ATP and ADP. In contrast to the endogenous membrane bound ectonucleotidases, apyrase is a soluble enzyme. In a murine model for LPS-induced systemic inflammation, excessive levels of ATP was shown to drive tissue damage and increase the risk of mortality in mice and exogenously administrated apyrase displayed a protective effect. One of the focuses of this thesis was to investigate the effect of exogenously administrated apyrase in a mouse model of EHEC infection.

The role of extracellular ATP in blood cells

ATP is involved in platelet aggregation. ATP-release has been suggested to be a major route for auto- and paracrine amplification of calcium influx leading to aggregation. Collagen and thrombin have been shown to induce ATP release from platelets and blockade of the ATP-signaling, either via degradation of ATP or inhibition of the P2X1 receptor, was shown to reduce both the calcium response and platelet aggregation. Platelets from P2X1-deficient mice exhibit reduced adhesion and thrombus formation ex vivo in response to collagen stimulation.

In monocytes ATP release and stimulation plays a major role in the release of IL-1β. ATP comprises an important step in assembly and activation of a complex called the inflammasome. The inflammasome is necessary for IL-1β maturation and release. Both LPS and the complement factor C3a were shown to induce release of IL-1β from monocytes only during simultaneous presence of extracellular ATP. Apyrase treatment and P2X7 receptor blockade inhibited the C3a-induced release of the cytokines. Induction of chemotaxis of neutrophils through IL-8 has been shown to be dependent on stimulation with extracellular ATP. Furthermore, neutrophils
release ATP from the site in which the chemotactic stimulant has a high concentration in order to amplify their own response and increase their motility to reach chemoattractants.\textsuperscript{169}
Extracellular vesicles

Extracellular vesicles comprise a collective name for spherical vesicles that are released from most, if not all, cells in the body. Extracellular vesicles include exosomes, microvesicles and apoptotic bodies. These three classes of vesicles have been separated based on several factors, such as size, cellular markers and specific lipids in their outer leaflet. However, as more knowledge has emerged about extracellular vesicles it has become apparent that the distinction between the different populations is not as clear as was previously thought. Currently the only accurate definition seems to be based on their mechanism of release.

One of the first recorded observations of extracellular vesicles were seen during red blood cell maturation and were believed to be residual waste, discarded by the blood cells. With time they have become regarded as a major means of cell communication with functions in immune response, cellular repair and reprogramming and coagulation, to name a few areas. In this thesis we have been investigating the role of microvesicles in systemic transport of Shiga toxin.

Exosomes

Exosome biogenesis takes place within the cell via inward budding of the membrane of late endosomes. Endosomes containing intraluminal vesicles are referred to as multivesicular bodies. When the multivesicular bodies fuse with the plasma membrane the intraluminal vesicles are released, at which stage they are termed exosomes. Exosomes are generally smaller than the other populations of extracellular vesicles, ranging between 30 and 100 nm in diameter. Exosomes are the most abundant extracellular vesicle in the circulation and may carry various forms of cargo, including microRNAs, mRNAs, lipids and proteins.

Microvesicles

Microvesicles are shed directly by outward budding and scission of the plasma membrane. Microvesicles are larger than exosomes, with a diameter between 100 to 1000 nm. The vesicles are released constitutively but during cellular activation
their release is increased and their contents and composition is altered. As in the case of exosomes, microvesicles have been shown to transport several types of cargo to other cells and also have an important role in coagulation and thrombosis which will be described below.\textsuperscript{177}

**Apoptotic bodies**

Apoptotic bodies are the largest kind of extracellular vesicles with a diameter between 1 and 5 μm. Apoptotic bodies are shed during cellular breakdown of apoptotic cells. Apoptotic bodies may therefore contain genomic DNA and organelles.\textsuperscript{178} Apoptotic bodies may limit inflammatory and apoptotic responses in neighboring cells by phagocytic uptake by immune cells.\textsuperscript{179}

**Release mechanism of microvesicles**

Microvesicles shed from the cell membrane upon activation were the focus of this thesis. The release of microvesicles have been closely linked to cleavage of cortical actin, which separates the actin-mediated anchoring of the plasma membrane, and loss of membrane lipid asymmetry.\textsuperscript{180, 181}

Cells have an asymmetrical distribution of lipids in the plasma membrane whereby phosphatidylserine (PS) and phosphatidylethanolamine (PE) are mainly located on the inner leaflet of the membrane.\textsuperscript{182} This asymmetry is regulated by ATP-dependent flippases and floppases. Flippases are active during normal levels of intracellular calcium and move certain lipids, such as PS and PE, from the outside to the inside of the membrane, against their gradient.\textsuperscript{183} Floppases are inactive during normal intracellular calcium levels and become activated upon increased intracellular calcium levels, allowing them to move lipids from the inside to the outside of the membrane.\textsuperscript{184} A third class of intramembranous lipid-moving enzymes are the scramblases, which are activated in the presence of high levels of intracellular calcium.\textsuperscript{185} During increased cytosolic calcium concentration levels, flippases are inactivated and ceases to internalize PS and PE, while floppases and Scramblases are activated. This leads to translocation of PS and PE to the outer membrane leaflet.\textsuperscript{186}

Lipids in the plasma membrane come in various geometrical shapes, which, in part, dictate their distribution. Focal congregation of certain lipids are known to initiate budding via strict geometrical remodeling of the plasma membrane and are of vital importance for endocytosis.\textsuperscript{187} The same phenomenon has been described for shedding of microvesicles. In a study in a *C. elegans* model it was shown that
the loss of flippase led to a significant increase in shedding of microvesicles. The proposed mechanism was that the lack of inward flipping of PE led to clustering of the inverted cone-shaped lipid, creating highly curved membrane patches, that provided a basis for microvesicle release.  

The plasma membrane is anchored and stabilized by the cortical actin network. In order to release parts of the plasma membrane in the form of microvesicles this actin network needs to be disrupted. During elevated calcium levels certain actin degrading enzymes, such as calpain, are activated. Calpain cleaves actin-anchoring proteins and disrupts the cortical actin-based cytoskeleton.

Several mechanisms may induce budding of microvesicles, but a scission step is also necessary to complete the process. This is mainly mediated via the ESCRT machinery. The ESCRT machinery consists of four different protein complexes, ESCRT-0, -I, -II and –III. The ESCRT machinery is vital in most processes that require scission of membranes that bud away from the cytosol, such as cytokinesis.

**Uptake of microvesicles**

Released microvesicles may be taken up by neighboring or distant cells through a variety of uptake mechanisms including phagocytosis, macropinocytosis, clathrin-dependent and –independent endocytosis and membrane fusion. The mode of uptake depends on several factors, such as the composition and origin of the microvesicle and the type of and level of activation the recipient cell.

The main mechanism of vesicle uptake is phagocytosis. Phagocytosis is a receptor-mediated uptake process whereby the cell extends its membrane and engulfs another cell or larger particle, delivering these to phagosomes in the cell. Phagocytosis of microvesicles is not restricted to immune cells, but the rate of the uptake is dependent on the phagocytic capabilities of the recipient cell. Phagocytosis by immune cells such as monocytes is believed to be largely a clearance mechanism. Microvesicles generally expose PS on their outer membrane leaflet, which is a potent signal for phagocytosis.

Macropinocytosis is a type of phagocytosis but instead of uptake of large particles, several smaller particles or solutes are engulfed and taken up. This has been shown to include uptake of microvesicles.

Microvesicles can also be taken up by clathrin-independent uptake such as caveolin- and lipid raft-mediated uptake. This uptake process is often initiated by receptor binding and has been implicated in endocytosis of extracellular vesicles in both endothelial and epithelial cells.
Clathrin-dependent endocytosis has been shown to play a role in uptake of microvesicles. Clathrin promotes endocytosis following ligand binding to a membrane receptor. During the inward budding of the cell membrane clathrin forms a coat around the vesicle, which is then uncoated and transported to the early endosome. Clathrin-mediated uptake of extracellular vesicles have been shown in bone marrow-derived mesenchymal stromal cells.

Extracellular vesicles may fuse with the plasma membrane and thereby empty their content into the recipient cell. This is, however, believed to be one of the less common uptake pathways of extracellular vesicles, as it requires higher membrane fluidity that is present in acidic environments, which generally only is present in tumors.

The function of microvesicles

Microvesicles have been implicated in numerous cellular processes, such as cell signaling, transfer of RNAs, proteins and receptors leading to coagulation, thrombosis and inflammation. They may also be used as biomarkers for diagnostic purposes and as vehicles for drug-delivery. The most important function of microvesicles is intracellular communication by transport of proteins, lipids, carbohydrates and RNAs. As microvesicles may contain cargo they can transport molecules that would otherwise be phagocytosed or degraded.

Microvesicles can induce cell signaling in recipient cells by membrane-bound ligands that bind directly to receptors on the surface of the target cell. Furthermore, microvesicles may fuse with the target cell membrane. Microvesicles may deliver receptors directly to the plasma membrane of the recipient cell. For example, functional CCR5 or bradykinin B1 receptor may be transferred to recipient cells thus promoting infection or inflammation.

Microvesicles can regulate protein expression in target cells by delivery of microRNAs, which interfere with mRNA translation. In the same fashion, coding mRNAs can be delivered via microvesicles, followed by subsequent translation of the mRNAs into proteins. In a study on microvesicles derived from glioblastoma cells, approximately 27,000 unique mRNAs were found within the vesicle population. Recipient cells were able to translate the mRNAs following vesicle uptake.

Microvesicles have been shown to play an important role in coagulation and thrombosis by several mechanisms. Microvesicles derived from platelets, endothelial cells and monocytes carry tissue factor. Additionally, stimulation of monocytes with LPS increases the amount of tissue factor that these vesicles carry. Tissue factor forms a complex with the activated form of
coagulation factor FVII and constitutes the extrinsic pathway of the coagulation cascade. The Tissue factor/FVIIa catalyzes cleavage and activation of FX. Another mechanisms of microvesicle-mediated coagulation is via exposure of PS. PS is an important activator of factor V and factor VII in the coagulation cascade. Platelet-derived microvesicles have also been shown to bind and recruit platelets to sites of endothelial injury, mediated via binding of matrix components, in a rabbit model.

Microvesicles may promote both pro- and anti-inflammatory responses. ATP-stimulation of LPS-primed monocytic cells was shown to induce shedding of microvesicles containing the pro-inflammatory cytokine IL-1β. Leukocyte-derived microvesicles have been shown to induce release of cytokines from endothelial cells. Extracellular vesicles from platelets can inhibit production of inflammatory cytokines in regulatory T cells.

As microvesicles carry surface markers and cargo from their cell of origin they may serve as biomarkers for disease. This has been exploited for diagnostic purposes in different types of cancer. Microvesicles from glioblastoma cells were shown to contain abundant levels of a certain miRNA that could be correlated with disease progression.

Extracellular vesicles have been implicated in drug-delivery, as they are endogenous, may conceal the drug from the immune system and may cross biological barriers, as the blood-brain barrier.

**Microvesicles in hemolytic uremic syndrome**

Patients with EHEC-induced HUS have been shown to have elevated levels of microvesicles. These microvesicles are derived from platelets, monocytes and neutrophils, determined by labeling with specific markers. Furthermore, the microvesicles exhibited pro-thrombotic properties, as they were positive for tissue factor and PS in the outer membrane leaflet. In vitro studies have shown that whole blood stimulated with Stx2 gave rise to similar results as in HUS patients, with increased levels of microvesicles from platelets, monocytes, neutrophils and also from red blood cells. These vesicles were also positive for tissue factor and exposed PS. The presence of circulating prothrombotic microvesicles likely contributes to the platelet consumption and thrombus formation in HUS patients.

Microvesicles from platelets and monocytes from HUS patients and from Stx-stimulated whole blood carry complement factors C3 and C9. Furthermore, red blood cell-derived microvesicles from HUS patients and Stx-stimulated whole blood are positive for C3, C9 and C5b-9. Whether these complement factors bind to blood cells and are shed via microvesicle release or are deposited after the
vesicles are released is unclear. However, microvesicles may provide a mechanism for clearance of complement factors.

In this thesis the role of microvesicles in HUS has been further explored as potential mediators of disease.
The present investigation

The overall aim of this thesis was to investigate a mechanism of systemic Shiga toxin transport to the kidney and to assess the importance of the toxin receptor and intracellular pathways of toxin signaling. Shiga toxin is of major importance for the virulence of EHEC strains and therefore a better understanding of its transport and signaling is imperative to the development of future therapeutic strategies.

Specific aims

1. To study if Stx is shed within blood cell-derived microvesicles in the systemic circulation, if the toxin retains its toxicity within microvesicles and if this may provide a mechanism for toxin delivery to the kidney.

2. To study if endogenous Gb3 is essential for Shiga toxin, delivered within microvesicles, to exert its cytotoxic effect intracellularly or if Gb3-negative cells can also take up toxin within microvesicles and be affected.

3. To study if Shiga toxin utilizes ATP and purinergic signaling to induce its effect on cells and how blockade of purinergic receptors affects Stx-mediated calcium influx, toxicity and microvesicle release.

4. To study the importance of ATP signaling in EHEC infection \textit{in vivo} by treating EHEC-infected mice with apyrase.

5. To review the role of extracellular vesicles in normal physiology and kidney diseases.
Methods

The following is a brief description of the methods used in the papers included herein. For a more in-depth description, please see the Material and Methods section of each respective paper.

Ethical considerations

Human samples were used in papers I, II, III and IV. The samples were obtained from patients, pediatric controls and healthy adult volunteers with written informed consent from the subjects or their parents. The studies were performed with permission from the Ethics Committee of the Medical Faculty, Lund University.

Animal models were used in papers I, II 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 EU directive for the protection of animals used in science.

Patients and control samples

Samples from 16 children with EHEC-associated HUS, one adult patient with HUS, one adult patient with EHEC infection and four pediatric controls, with conditions that were unrelated with HUS, were available in paper II. In addition, a renal biopsy from a kidney of a 13 year old boy with HUS was available.

Flow cytometry

In papers I, III and IV flow cytometry was used to analyze cells or microvesicles. Flow cytometry is a common research tool that flows cells and particles through a laser. Forward and side scattering of the laser gives information about the volume and granularity of the particle and the method is often combined with fluorescent labelling of the particles that are analyzed. In this thesis flow cytometry was used for antibody-labelled microvesicles, to determine their cellular origin. Stx in microvesicles was labeled in combination with a mild detergent, to allow permeabilization of the microvesicles.

Cell culture and transfection

Several cell lines were used in the studies included in this thesis. Immortalized glomerular endothelial cells were used in paper I, cervical epithelial (HeLa) cells were used in papers II and III, and Chinese hamster ovarian (CHO) and colonic epithelial cells were used in paper II. In paper II, CHO cells were transfected with a plasmid carrying coding DNA for the Gb3 synthase, A4GALT and a resistance
gene. The transfected cells were made into a stable cell line, using negative selection for the cells that did not express the resistance gene. In paper III, HeLa cells were transfected with small interfering RNA to silence the expression of the P2X1 receptor. The transfections were carried out on sub-confluent cells using a liposome-based delivering system.

Transmission electron microscopy
Transmission electron microscopy (TEM) of cell cultures and renal biopsy was used in paper I. In TEM, the sample is irradiated with electrons. Depending on the density of the sample, electrons will pass through in different amounts, resulting in a highly resolved image of the sample. Gold particles coupled to antibodies may be used to label specific antigens in the sample. In paper I, TEM was used to image kidney tissue from a HUS patient and cultured glomerular cells incubated with microvesicles containing Stx. The samples were labelled with gold-conjugated anti-StxB antibodies together with cell-specific markers for microvesicles to enable identification of their parent cells and, in the kidney tissue, for early endosomes and ribosomes.

Viability assay
Viability assays were carried out in papers I, II and III. In paper I the viability of cultured glomerular cells was determined based on detachment of dead cells and subsequent staining with crystal violet of remaining, viable, cells. In papers II and III the viability of cells was measured based on their metabolic activity. Alamar blue was used, a substance that shifts color in response to reduction by cellular NADH and NADPH. The viability measurements were carried out on cells treated with or without the P2X1 inhibitor NF449 and exposed to free Stx or microvesicles with or without Stx.

Protein synthesis assay
In papers I, II, and III the protein synthesis of cultured cells was assayed in cells treated with or without NF449 and exposed to free Stx or microvesicles with or without Stx. In papers I and III protein synthesis was measured by incorporation of radioactively labelled methionine and in paper II a methionine analogue containing an alkyne group, which binds a fluorescent dye. The incorporated methionine or methionine analogue were measured using scintillation or detection of fluorescence, respectively, giving signals proportional to the amount of synthesized proteins.

Caspase activity assay
The caspase activity test was used in paper III. The assay measures the activity of the terminal caspases 3 and 7 by incubating cells with a membrane permeable substrate that is cleaved by activated caspase 3 and 7. When the substrate is
cleaved in gives off a green fluorescence when excited with blue light. Green-fluorescent cells indicate ongoing apoptosis. This assay was used on HeLa cells that had been treated with or without NF449 and incubated with Stx.

**Nanoparticle tracking analysis**

Nanoparticle tracking analysis (NTA) was used for detection of microvesicles in paper II. In NTA, particles are flowed under a camera and the size of the particles is determined based on Brownian motion. Suspensions of microvesicles derived from whole blood that had been stimulated with or without Stx were analyzed in order to determine their size distribution.

**Thin layer chromatography**

In paper II the glycosphingolipid content of HeLa, CHO and DLD-1 cells and blood cell-derived microvesicles was analyzed by thin layer chromatography (TLC). In TLC a mixture of substances, such as lipids, in a solvent are separated on a polar surface, exploiting the capillary force. The mixture is separated based on their solubility and polarity.

**ATP determination**

ATP measurements, using the luciferase activity method, were performed in papers III and IV. Luciferase is an enzyme from fireflies that catalyzes cleavage of ATP in the presence of D-luciferin and oxygen. This reaction leads to emission of light, that is proportional to the amount of ATP that is present. ATP was measured in cell culture supernatants in paper III and in feces from EHEC infected mice in paper IV.

**Calcium imaging**

In paper III HeLa cells and platelets were loaded with Fluo-4. Fluo-4 is cell permeable, but is cleaved once it enters cells, causing it to lose its cell permeability. Fluo-4 is a calcium sensitive fluorescent dye that becomes brighter at high calcium concentrations. Following loading with Fluo-4, HeLa cells were stimulated with Stx and platelets were stimulated with LPS and Stx and calcium influx was measured based on their fluorescence.

**In vivo models**

*In vivo* models were used in paper I, III, and IV. In paper I, III and IV, Balb/c mice were treated with streptomycin, to reduce their normal flora and to improve colonization. The mice were fasted for 16 h followed by inoculation with streptomycin-resistant *E. coli* O157:H7. Weight changes, bacterial colonization and disease symptoms were monitored daily. Upon development of disease symptoms, weight loss of 20% or at the predetermined end-point, the mice were sacrificed and blood, kidneys and intestines were collected.
In paper I and III Balb/c mice were given intraperitoneal injections of Stx2. The mice were monitored and sacrificed as before and blood, kidneys and intestines were collected.

**TUNEL assay**

In paper IV colons taken from EHEC-infected mice that had been treated with apyrase or PBS were stained for apoptotic cells by incorporation of alkyne tagged deoxyuridine triphosphate into fragmented DNA of apoptotic cells, followed by labelling of the alkyne group with a fluorescent probe. The tissues were mounted on slides and analysed by fluorescence microscopy.

**Results and discussion**

**Paper I**

In this study we investigated if circulating microvesicles can contain Stx2 and thereby transport the toxin systemically.

**Results**

Patients with EHEC-associated HUS had elevated levels of circulating microvesicles compared to patients in recovery, as was shown by flow cytometry. These microvesicles carried Stx2 on the vesicle inside, as toxin only could be detected in the presence of a membrane permeabilizing detergent. The microvesicles were derived mainly from platelets, but also from monocytes, neutrophils and red blood cells. Similarly, elevated levels of microvesicles from platelets, neutrophils and monocytes were found in plasma from mice infected with EHEC and these microvesicles also contained Stx2. Infection with an isogenic Stx2-negative bacterial strain displayed similar levels of platelet-derived microvesicles that were negative for the toxin. Intraperitoneal injection of mice with Stx2 also led to elevated levels of platelet-derived microvesicles containing Stx2. Electron microscopy showed that platelet- and leukocyte-derived microvesicles with Stx2 were detected in glomerular and peritubular capillary endothelial cells in a kidney biopsy from a patient with EHEC-associated HUS. In EHEC-infected mice platelet- and leukocyte-derived microvesicles were demonstrated in glomerular and peritubular capillary endothelium and in podocytes and tubular epithelial cells. Furthermore, Stx2-containing microvesicles were observed in glomerular and tubular basement membranes. Stx2 stimulated the release of toxin-containing microvesicles from platelets, monocytes, neutrophils and red blood cells from human whole blood *in vitro*. Immortalized glomerular endothelial cells took up platelet- and leukocyte-derived toxin-
containing microvesicles in vitro. The microvesicles were transported to the early endosome, released the toxin in the cytosol and the toxin was ultimately transported to the ribosomes. In addition, Stx2-contained within blood cell-derived microvesicles was able to induce protein synthesis inhibition and cell death in immortalized glomerular endothelial cells.

Discussion
This study is the first to demonstrate the presence of Stx2 within circulating blood cell-derived microvesicles in patients with HUS and in EHEC-infected mice. Stx2-containing microvesicles were demonstrated in glomerular and peritubular capillary cells in both patients and mice. Murine glomerular endothelial cells in mice are Gb3-negative, thus showing a receptor-independent uptake of Stx2. Stx2-positive microvesicles were taken up by glomerular cells and were shown to locate to the early endosome. Stx2 was released from the microvesicles and subsequently translocated to the ribosomes to induce protein synthesis inhibition and cell death, indicating that Stx2 that is delivered via microvesicles retains its cytotoxic potential and can follow the retrograde transport pathway following intracellular delivery. This study is the first to demonstrate uptake and transport of a bacterial toxin within host blood cell-derived microvesicles, a mechanism that may provide host response evasion of Stx.

These results provide a novel mechanism of systemic Stx transport to kidney cells.

Paper II
The glycosphingolipids Gb3 are crucial for Stx binding, uptake and toxicity. However, paper I demonstrates the presence of Stx2 in Gb3-negative murine glomerular endothelial cells. Here we investigated if Stx2 delivered via microvesicles that were positive for Gb3 could be taken up and have a toxic effect in Gb3-negative cells. Furthermore, we investigated the intracellular transport route of microvesicle-delivered Stx2 in Gb3-positive cells.

Results
Blood cell-derived microvesicles possess Gb3. Microvesicles derived from human whole blood containing Stx2 were highly toxic to CHO cells that had been transfected with Gb3 synthase (CHO-Gb3), but not to Gb3-negative CHO cells that had been transfected with a vector control (CHO-vector). Gb3-negative CHO-native and DLD-1 cells could internalize toxin-positive vesicles but the toxin did not exert a cytotoxic effect in these cells. Stx2 delivered via microvesicles induced protein synthesis inhibition in Gb3-positive CHO-Gb3 and HeLa cells but not in CHO-vector and DLD-1 cells or in HeLa cells that had been treated with an inhibitor of the Gb3-precursor glucosylceramide. CHO-Gb3 cells that were treated with the retrograde transport inhibitor Retro 2.1 were protected against the protein
synthesis inhibition that was induced by Stx2-positive microvesicles. DLD-1 cells that were incubated with exogenous Gb3 were shown to bind free Stx2, but remained resistant to the toxin.

Discussion
This study highlights the importance of Gb3, not only for Stx2 uptake, but also for its intracellular transport and toxicity. The transport of the microvesicle-delivered toxin was shown to go through the retrograde pathway, as indicated by the reduced ribotoxicity following pre-treatment with Retro 2.1, an inhibitor of retrograde transport. Furthermore, we provide a mechanism for the presence of Stx2 in Gb3-negative cells, such as demonstrated in murine endothelial cells in paper I. Importantly, we showed that microvesicles derived from blood cells are positive for Gb3, to which the toxin likely is bound. Despite being Gb3-positive, the microvesicles did not induce toxicity in Gb3-negative cells, indicating that endogenous Gb3 must be present if Stx2 is to retain its toxicity. It is unclear if the uptake of Stx2-containing microvesicles in Gb3-negative cells serves a purpose, but it may be a mechanism through which Stx2 is cleared via degradation in the lysosomes or the vesicles may be transported through the cells via transcytosis.

Paper III
Stx-binding leads to influx of calcium, protein synthesis inhibition, cell death and shedding of microvesicles in different cells. The mechanism of Stx-induced cellular activation is not known. In this paper we investigated cellular release of ATP following stimulation of Stx. Furthermore we investigated if released ATP may act as a second messenger by signaling via purinergic receptors and the role of this in Stx-mediated calcium influx, protein synthesis inhibition, apoptosis and microvesicle release.

Results
Stx1 was shown to induce release of ATP in vitro from HeLa cells and in vivo in a murine model of intraperitoneal injection of Stx2. Stx1-induced calcium influx in HeLa cells was inhibited by both the P2X1 receptor inhibitor NF449 and by the unspecific P2 receptor inhibitor suramin, suggesting involvement of purinergic signaling. In human platelets both Stx1 and Stx2 in combination with LPS induced calcium influx. The influx of calcium was inhibited by treatment with NF449. NF449 did not affect the transport of Stx1 to the ER in HeLa cells, but did protect the cells from Stx1-induced protein synthesis inhibition, caspase activation and Stx1- and Stx2-induced toxicity. The protection from Stx1-induced apoptosis by NF449 was confirmed by P2X1 silencing in HeLa cells leading to reduced caspase activation. Stx1 and Stx2 induced release of toxin-containing microvesicles from HeLa cells and platelets. NF449 inhibited the release of toxin-containing
microvesicles in both HeLa cells and platelets in vitro. Mice infected with EHEC or injected intraperitoneally with Stx2 had elevated levels of platelet-derived microvesicles and microvesicles that contained Stx2 compared to controls. Treatment with suramin lead to significantly lower levels of platelet-derived and Stx2-containing microvesicles. Taken together, Stx induces release of ATP from HeLa cells and in mice. ATP in turn causes calcium influx via purinergic signaling. Inhibition of purinergic signaling is protective against Stx-induced toxicity in HeLa cells and inhibits the release of Stx-containing HeLa cell- and platelet-derived microvesicles.

Discussion

In this study we show for the first time that Stx1 induces release of ATP both in vitro and in vivo. ATP may act as a second messenger and signal via purinergic receptors in an autocrine and paracrine manner. The involvement of purinergic signaling was further strengthened by the abrogation of Stx-induced calcium influx in HeLa cells and platelets by inhibition with NF449 and suramin. Microvesicles may transport and conceal Stx in the circulation. The release of microvesicles may be a mechanism of ridding the cell of the toxin, but in extension leads to increased spread of Stx. Thus, blockade of microvesicle uptake and specific targeting of extracellular ATP may have a therapeutic application during EHEC infections and should be further explored.

Paper IV

Based on our previous observations of ATP release in response to Stx binding in paper III, the importance of systemic purinergic signaling was addressed in this study. EHEC-infected mice were injected with the ATP- and ADP-degrading enzyme apyrase. The mice were monitored with regard to weight loss and development of disease and intestinal tissues from the mice were analyzed for apoptotic markers and mucosal damage. Furthermore, fecal samples form the mice were analyzed for ATP- and Stx2-content.

Results

Mice were inoculated with EHEC and injected intraperitoneally with apyrase or PBS on day 0 and 2 after inoculation. Infected PBS-treated mice developed signs of disease staring three days after inoculation. A significant difference was observed in EHEC-infected mice treated with apyrase, in which development of disease symptoms was observed first five days after inoculation. Apyrase-treatment also had a protective effect with regard to weight loss in the infected mice. Intestinal tissues from mice that had been sacrificed two days after inoculation with EHEC were analyzed for histopathology, goblet cell depletion and apoptosis. EHEC-infected and PBS-treated mice exhibited apoptotic cells on
the luminal side of the intestine, in contrast to apyrase-treated mice. Apyrase-treated mice had significantly more intact goblet cells and significantly less apoptotic cells in the intestine compared to PBS-treated mice. Fecal samples taken one day post-inoculation contained significantly less ATP and exhibited a tendency of lower Stx2 concentrations in apyrase-treated compared to PBS-treated mice, although this effect was not significant.

Discussion

In this study we show that removal of extracellular ATP had a protective effect on mice during EHEC infection. Apyrase delayed the development of disease in the mice by two days and protected intestinal tissues from apoptosis and goblet cell depletion. Even though only partial protection was obtained, this study provides evidence for involvement of extracellular ATP during EHEC infection. The protective effects may also be improved by optimization of the concentration and timing of the apyrase treatment. The mechanism by which apyrase was protective in this study remains to be determined. Apyrase may affect EHEC directly or act via the host-response. The lower fecal ATP levels in apyrase-treated mice indicate that the treatment was able to reach the intestine. A previous study has shown that extracellular ATP is involved in goblet cell depletion \textit{in vitro}.\textsuperscript{216} Apyrase may thus protect goblet cells by degrading ATP and maintain the mucosal barrier. Furthermore, purinergic signaling was shown to be involved in Stx-induced apoptosis of HeLa cells in paper \textbf{III} of this thesis. Apyrase-mediated degradation of ATP may block purinergic signaling in the intestine and could thereby be protective in a similar mechanism in this study.

Paper V

Paper V is a review, in which the role of extracellular vesicles in normal physiology and renal and inflammatory-mediated pathological conditions are described. The review summarizes the literature concerning the biogenesis, release, uptake, function in normal physiology and detection of exosomes, microvesicles and apoptotic bodies. Paper V focuses on the role of extracellular vesicles as biomarkers and promoters of disease in vasculitis and EHEC-induced HUS. Furthermore, the review investigates the role of these vesicles in kidney regeneration and their possibilities as vehicles for drug-delivery.
Conclusions and future perspectives

Conclusions

- Stx2 induces release of blood cell-derived microvesicles that are positive for the toxin. Stx2-containing microvesicles circulate and are delivered to the kidney in EHEC-HUS. Stx2-containing blood cell-derived microvesicles are internalized by renal glomerular cells followed by inhibition of protein synthesis and subsequent cell death.

- Stx2 can be taken up by Gb3-positive and Gb3-negative cells within microvesicles. Stx2 delivered by microvesicles is toxic to Gb3-positive but not to Gb3-negative cells.

- Stx induces the release of ATP and subsequent purinergic receptor activation. Purinergic receptor signaling is involved Stx-mediated calcium influx, microvesicle release, protein synthesis inhibition and apoptosis.

- Removal of extracellular ATP by apyrase degradation is partially protective in EHEC-infected mice and protects the intestine from damage.
Future perspectives

The mechanism of Stx-transfer to the kidney via blood cell-derived microvesicles provides a model for how the toxin reaches and damages the kidney during EHEC-induced HUS. Interventions that target either the release or the uptake of microvesicles during EHEC infections could thus be protective and should be further explored. However, microvesicles have implications in several physiological roles that must be taken into consideration in such studies. The involvement of purinergic signaling in Stx-mediated microvesicle release indicates that targeting of extracellular ATP may inhibit Stx-mediated microvesicle release and could provide a more specific target, as to not interfere with the positive aspects of circulating microvesicles.

Purinergic signaling is also involved in Stx-mediated calcium influx in human platelets. Platelet activation and thrombus formation contributes to the pathogenesis during HUS. Inhibition of purinergic signaling may counteract thrombus formation during EHEC-infection.

The effects of apyrase-treatment during EHEC infections should thus be further studied.
Populärvetenskaplig sammanfattning

Enterohemorrhagisk Escherichia coli (EHEC) är en bakterie som är sjukdomsfördöande i människor. EHEC är en del av normalflora hos idisslare, så som kor, och sprids till människor främst genom förtäring av föröden mat eller dryck. Efter intag färdas bakterien genom mag- och tarmkanalen och fäster sig främst till slemhinnan i tjocktarmen, varpå den börjar växa. EHEC orsakar inflammation och skada i tarmen och kan leda till blodiga diarréer och i vissa fall hemolytiskt uremiskt syndrom, vilket kännetecknas av blodbrist, låga nivåer av blodplättar och akut njurskada och kan vid allvarliga fall leda till död.

EHEC är en icke-invasiv bakterie, vilket betyder att den stannar kvar i tarmen och släpper ifrån ut olika toxiner (gifter) som går in i cirkulationen. Det viktigaste toxinet som släpps ut av bakterien heter Shiga toxin (Stx).

Stx binder till en receptor som heter globotriaosylceramid (Gb3) som finns på vissa celler. Efter att Stx bundit till sin receptor tas toxinet upp av cellen och förstör cellens produktion av äggviteämnen (proteiner), vilket så småningom leder till celldöd. Stx färdas till målorgan, så som njuren och det centrala nervsystem, och orsakar där skador.

Stx kan också binda till och tas upp av olika blodceller i blodbanan utan att orsaka celldöd. När toxinet binder till blodceller påverkas dessa istället genom att producera och frisätta olika proteiner som orsakar inflammation och genom att frisätta mikrovesikler. Mikrovesikler är mycket små blåsor som består av membran som kommer ifrån cellen som de frisatts ifrån.

Det är inte känt hur Stx tar sig till sina målorgan. Toxinet finns endast i mycket små nivåer i blodomloppet. Blodceller som binder Stx kan transporterar runt toxinet i blodet men hur det sen frisläpps från cellen och tar sig till njuren är okänt.

I det första delarbetet undersöktes huruvida mikrovesikler som släpps ut från blodceller innehåller Stx och om dessa mikrovesikler kan tas upp av och orsaka skada i njurceller. Blodplasma från patienter med pågående EHEC-infektion visade sig innehålla mikrovesikler från blodplättar och röda och vita blodceller och en del av dessa vesikler innehöll också toxinet. Dessa vesikler kunde även hittas i njurvävnad både bundna till och inuti celler. En intressant observation var att mikrovesikler med Stx även kunde hittas i celler som saknade receptorn till vilken Stx binder. Mikrovesikler som innehöll Stx togs upp av odlade njurceller och
orsakade skada i dessa. Vi presenterade genom det här delarbetet en modell för hur Stx kan ta sig genom blodbanan till njuren via mikrovesikler och generellt hur bakterier kan använda sig av mikrovesikler för att undgå att upptäckas av kroppens immunförsvar.


Det femte arbetet är en litteraturstudie som sammanfattar betydelsen av olika vesikler som släpps ut från celler, så som mikrovesikler, under normala omständigheter och vid olika njursjukdomar.
Acknowledgements

I would like to thank everyone who, in one way or another, has made this thesis possible.

Diana, min handledare, tack för din ständiga uppmuntran och ditt pådrivande. Utan din kunskap och vägledning hade jag aldrig hittat rätt genom den snåriga akademin. Du har verkligen gjort det möjligt för en yr doktorand att hitta fokus under de här åren.

Anne-lie, min bihandledare, tack för alla samtal, för allt stöd och uppmuntran och framför allt för din vänskap. Du har varit en fantastisk rumskompis som alltid tagit dig tiden att svara på mina helt slumpmässiga frågor.

Ann-charlotte och Ida, tack för all märkliga diskussioner vid morgonkaffet, för alla falska sångduetter i celllabbet, för alla konstiga metoder vi försökte oss på, för ditt ordningssinne som nästan smittade av sig på mig och för att du alltid tagit dig tid för att hjälpa till med både forskning och uppmuntran.

Ashmita, thanks for being a source for optimism and smiles, for all the little talks before leaving the lab, for always being more than happy to drain me on blood and for introducing me to weird podcasts.

Milan, tack för din obotliga optimism under den tiden vi jobbade tillsammans. Jag hoppas mycket på att vi kommer hamna i samma labb igen någon gång.

Ingrid, Sunna, Zivile, Maria och Sebastian, tack till er kära kollegor för många trevliga stunder, roliga samtal, intressanta diskussioner och för att ni hjälpt mig att förstå ”läkarbiten”.

Niklas och Hilda, tack för den visserligen korta, men mycket trevliga, tiden vi har jobbat tillsammans. Jag önskar er ett stort lycka till med er forskning.

Johan, tack för att du handledde mig under min första tid på labbet, för din kunskap och uppmuntran när du jobbade på vårt labb och för hjälp och trevliga samtal i lunchrummet nu när du är vår granne.
Karina, tack för att du har tagit dig tid att svara på mina frågor, lånat ut material, gett tips på labbutrustning och varit så snäll och glad.

Martin, Jill, Linn, Anja, Philaiphon, Abdul, Jennifer and Annika, a big thanks for being such nice neighbors, sharing your knowledge and brightening up the lunch room with your presence.

Eva, tack till dig för att du är en så bra granne, alltid är glad och hjälpsam och för trevliga samtal i fikarummet.

Mattias, Daniel, Ludger and Robin, a big thanks to my co-authors. Without you this thesis would not have been possible.

Vänner, tack för att ni finns där när jag behöver er som mest.

The hockey team, thanks for helping me taking my mind off research now and then and for being such amazing people.


Mamma och Lars, tack för att ni är så fantastiska föräldrar och förebilder och alltid finns till hand oavsett vad.
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