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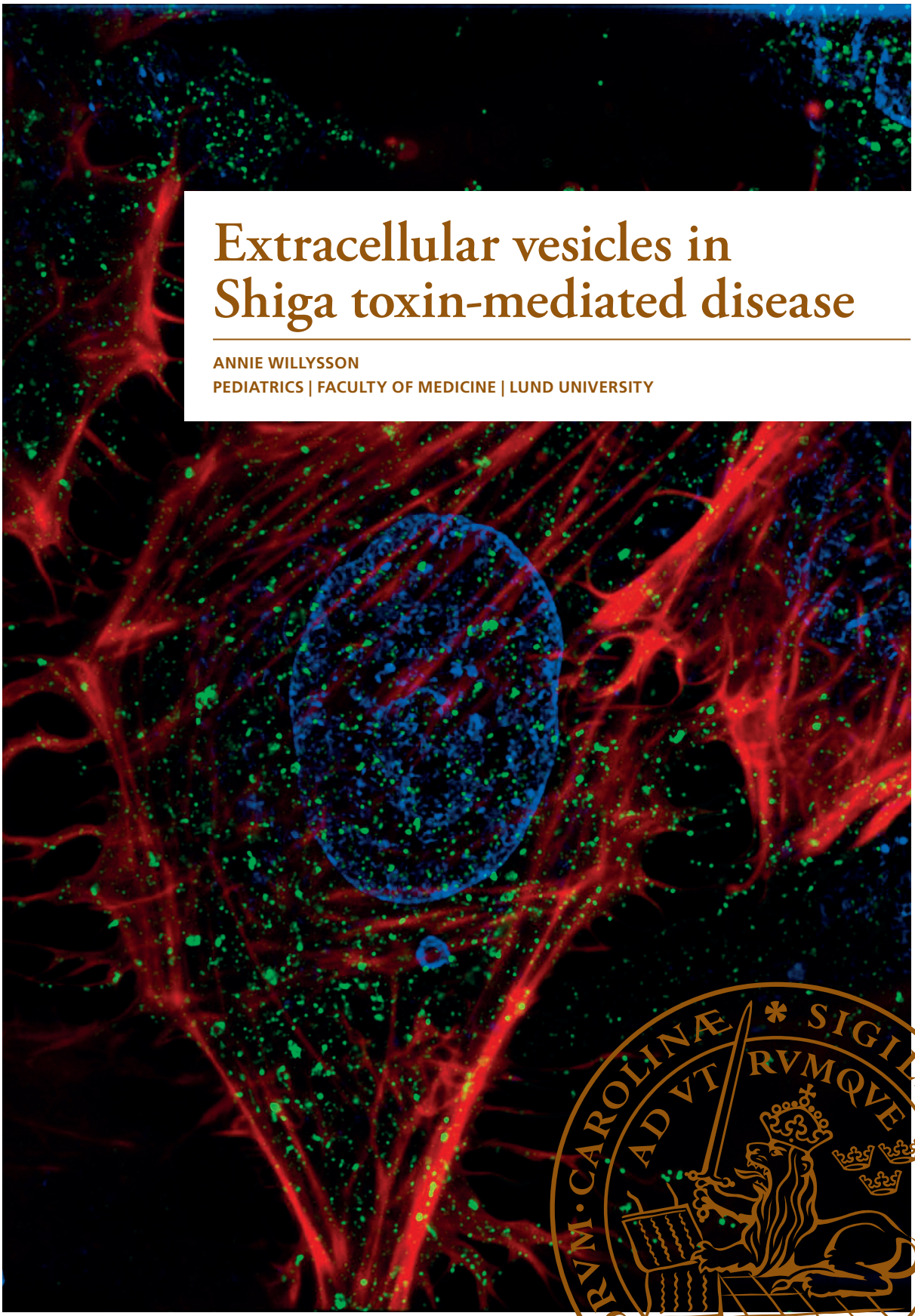

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Extracellular vesicles in Shiga toxin-mediated disease

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Abstract

Extracellular vesicles are released during infectious, inflammatory and prothrombotic conditions. These are membrane-derived vesicles shed by cells during activation, senescence and/or apoptosis. Extracellular vesicles contain and transfer components of the parent cell from which they are shed. During gastrointestinal infection with enterohemorrhagic *Escherichia coli* (EHEC) extracellular vesicles are released from blood cells into the circulation and contain Shiga toxin, the main EHEC virulence factor with potent cytotoxic effects. EHEC can cause hemorrhagic colitis or hemolytic uremic syndrome, characterized by acute kidney injury, thrombocytopenia, and non-immune hemolytic anemia. This PhD thesis addressed mechanisms of release and uptake of Shiga toxin-positive extracellular vesicles and characterized prothrombotic and proinflammatory properties related to vesicles released after Shiga toxin stimulation of blood cells. The thesis also provides methodology for the of these extracellular vesicles.

Paper I shows that once Shiga toxin is taken up by cells it is released within vesicles within minutes and does not undergo retrograde transport. Toxin could bind directly to extracellular vesicles and could transfer from the surface to the inside of the vesicle suggesting sequestration, possibly to evade the host response. This is a novel mechanism by which vesicles could protect their content while in the circulation.

Paper II investigated the importance of the toxin receptor, globotriaosylceramide (Gb3), in recipient cells for the uptake of Shiga toxin-containing extracellular vesicles by the cells and exertion of a cytotoxic effect. Various Gb3-negative and Gb3-positive cells were used showing that the recipient cell must possess endogenous Gb3 for the toxin, taken up in extracellular vesicles, to exert a toxic effect on cell viability.

Paper III investigated the pro-thrombotic and proinflammatory properties of Shiga toxin-induced blood cell-derived extracellular vesicles. These vesicles possessed more tissue factor activity and thrombin-generating activity and induced platelet aggregation. The vesicles contained pro-inflammatory cytokines and induced the release of IL-8 from endothelial cells co-incubated with monocytes.

Paper IV describes isolation procedures of blood cell- and cell culture-derived microvesicles and characterization of the vesicles by various methods including flow cytometry, electron microscopy, nanoparticle tracking, ELISA, proteomics and live cell imaging.

In summary, this thesis characterized important aspects of Shiga toxin-induced extracellular vesicles including their uptake, intracellular effects, release, and properties that can contribute to the development of hemolytic uremic syndrome.

Key words: Extracellular vesicles, Shiga toxin, EHEC, hemolytic uremic syndrome

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Cover photo

Structure illumination microscopy image of Shiga toxin taken up by HeLa cells. Shiga toxin is labelled in green, actin is red and nuclear staining is blue, by the author.

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List of papers

This thesis is based on the following papers:

I. **Willysson A**, Ståhl AL, Gillet D, Barbier J, Cintrat JC, Chambon V, Billet A, Johannes L, Karpman D. Shiga toxin uptake and sequestration in extracellular vesicles is mediated by its B-subunit. *Toxins*. 2020 10;12(7):449.

II. Johansson K, **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. *Front Cell Infect Microbiol.* 2020;10:212.

III. **Willysson A**, Karpman D. Shiga toxin induces release of prothrombotic and proinflammatory extracellular vesicles. *Manuscript*.

IV. **Willysson A**, Ståhl AL, Karpman D. Isolation and characterization of Shiga toxin-associated microvesicles. *Methods Mol Biol*. 2021;2291:207-228.

Paper IV was reprinted with the permission of the publisher.

The following paper has been published but is not included in the thesis:

Villysson A, Tontanahal A, Karpman D. Microvesicle involvement in Shiga toxin-associated infection. *Toxins*. 2017;9(11):376.

Abbreviations

EHEC	Enterohemorrhagic *Escherichia coli*
HUS	Hemolytic uremic syndrome
EVs	Extracellular vesicles
Gb3	Globotriaosylceramide
Gb4	Globotetraosylceramide
PS	Phosphatidylserine
PE	Phosphatidylethanolamine
NF-kB	Nuclear factor kappa B
RANTES	Regulated upon activation, normal T cell expressed and presumably secreted
TNF	Tumor necrosis factor
IL	Interleukin
NTA	Nanoparticle tracking analysis
TEM	Transmission electron microscopy
MAC	Membrane-attack complex
TF	Tissue factor
ER	Endoplasmic reticulum

Abstract

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Introduction

Extracellular vesicles are membrane-encapsulated particles released by cells that contain a bioactive cargo of proteins, lipids and RNAs which can be functionally transferred to other cells [1]. Extracellular vesicles are involved in many biological processes. They partake in intercellular communication which plays a role in homeostasis as well as immune modulation but may also have a role in the development of diseases [2]. Extracellular vesicles can be divided into exosomes, microvesicles and apoptotic bodies based on size. They can also be classified according to their biogenesis and content [3]. Exosomes originate from intracellular multivesicular bodies while microvesicles bud directly from the plasma membrane of the cell [4,5]. Apoptotic bodies are shed by cells during apoptosis. Upon cellular activation, extracellular vesicles are extensively released and may exhibit pro-inflammatory and pro-thrombotic properties [6-8].

Extracellular vesicles were shown to participate in the development of hemolytic uremic syndrome (HUS) associated with enterohemorrhagic *Escherichia coli* (EHEC) infection. These bacteria release Shiga toxin as their main virulence factor [9]. Shiga toxin is an AB5 toxin. It binds by its pentameric B-subunit to a specific receptor, globotriaosylceramide (Gb3), and is taken up by receptor-mediated endocytosis [10]. Intracellularly it is transported via the retrograde route from the trans-Golgi network to the endoplasmic reticulum (ER) followed by translocation of the toxin A-subunit into the cytosol [11,12]. In the cytosol, the enzymatically active A-subunit binds to ribosomes and cleaves an adenine from the ribosomal 60S subunit following inhibition of protein synthesis and eventually cell death [13,14]. In addition to its cytotoxic effect, Shiga toxin can induce cellular activation during which toxin-containing extracellular vesicles may be released from cells [7].

EHEC is a food- and water-borne pathogen that can cause severe disease in humans. EHEC infection can lead to diarrhea and hemorrhagic colitis and in certain cases develop into the life-threatening condition HUS. HUS is characterized by thrombocytopenia, non-immune hemolytic anemia, and acute kidney injury [15]. EHEC is a non-invasive bacterium and releases virulence factors in the intestine where it causes local inflammation [16]. Shiga toxin may thereby come in contact with the bloodstream. Extracellular vesicles play an important role in the development of HUS as they can transport the toxin within the circulation to the target organ, the kidney, where the vesicles are taken up and release their toxic

content [17]. Additionally, their pro-inflammatory and prothrombotic potential may play a role in disease development as will be addressed herein.

In this thesis, aspects of extracellular vesicle pathophysiology relevant for mediating Shiga toxin-associated disease were addressed as well as the methodology used for detection of toxin-containing vesicles. The presence of Shiga toxin within extracellular vesicles could be due to its shedding within vesicles that contain cytosolic components, including Shiga toxin, when they bud off the cell membrane but could also be caused by toxin binding directly to the outer membrane of the vesicle within the circulation, followed by uptake and sequestration within the vesicle. This process could occur in the circulation and promote disease, as the toxin within a vesicle would evade the host response. This was addressed in the first paper of this thesis.

The Gb3 receptor is required to usher the toxin along the retrograde route towards the trans-Golgi network and ER [18]. The second paper of this thesis investigated if the presence of Gb3 in vesicles containing Shiga toxin could suffice to allow retrograde transport and the induction of a cytotoxic effect in cells that take up the toxin within vesicles. This would suggest that toxin-positive vesicles taken up by Gb3-negative cells could have a cytotoxic effect. This aspect was addressed using cells with or without endogenous Gb3.

When Shiga toxin binds to blood cells in the circulation, it induces release of extracellular vesicles which contain additional substances, other than the toxin itself. Vesicles released by toxin stimulation were evaluated for prothrombotic and proinflammatory properties in the third paper of this thesis. Such extracellular vesicles could play a role in the development of disease.

Several methods can be used to detect and quantify Shiga toxin within extracellular vesicles. The fourth paper describes suitable methods for the study of Shiga toxin-associated extracellular vesicles, particularly shed microvesicles.

To-date there are no specific treatments for Shiga toxin-associated HUS. Extracellular vesicles are involved in the development of this severe disease [19]. Improved knowledge about their role in Shiga toxin-mediated disease may enable the development of novel therapeutic strategies.

Extracellular vesicles

Secretory vesicles, known as extracellular vesicles, can be released by most, if not all, cells. Initially, extracellular vesicles were described as a mechanism for the cell to get rid of material during maturation of erythrocytes [20]. Today, extracellular vesicles are known to be much more than just disposal bags. Extracellular vesicles can exchange biologically active compounds between cells – varying from RNA to proteins and lipids - and function as signaling vehicles in homeostasis processes as well as partaking in the pathogenesis of various diseases such as inflammatory diseases [21], infectious diseases [22] and cancer [23].

In this thesis, extracellular vesicle release, uptake and properties in Shiga toxin-related disease were studied. The following section gives an introduction into extracellular vesicle subgroups, the mechanism of microvesicle formation, the vesicle cargo, and cellular uptake mechanisms. It also gives a description of methods for isolation and characterization of extracellular vesicles as well as the role of extracellular vesicles in homeostasis and disease.

Exosomes, microvesicles and apoptotic bodies

Extracellular vesicles can be divided into three main subgroups based on their biogenesis or size; exosomes (small extracellular vesicles), microvesicles (medium-sized extracellular vesicles) and apoptotic bodies (large extracellular vesicles) [3]. The formation of exosomes and microvesicles is presented in Figure 1. Exosomes, which are the most abundant extracellular vesicles within the circulation, originate from the endosomal network. They are generated as intraluminal vesicles within multivesicular bodies and released from cells by fusion of multivesicular bodies with the plasma membrane, upon their release they are called exosomes [24]. Microvesicles are generated by outward budding of the plasma membrane and are overall larger (100-1000 μm [4]) than exosomes (30-150 μm [1]). However, as the size of exosomes and microvesicles overlaps the possibility to separate these two subtypes based on size is limited. Apoptotic bodies are distinct from exosomes and microvesicles as they arise during apoptosis and cell fragmentation and the large vesicles (1-5 μm) contain organelles and/or nuclear fragments [25]. In this thesis, only extracellular vesicles in the size range of shed microvesicles were studied and are therefore the focus of the following section.

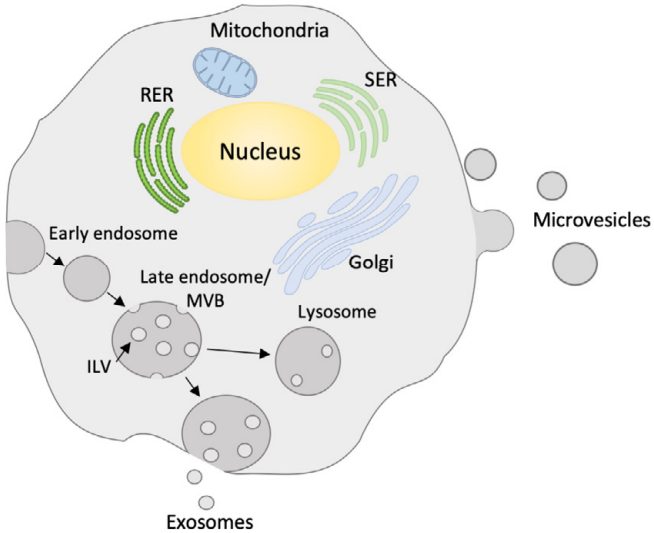

Figure 1: Schematic presentation of exosome and microvesicle formation. Exosomes are generated as intraluminal vesicles (ILVs) by the inward budding of late endosomes leading to formation of multivesicular bodies (MVBs). ILVs are either released into the extracellular space by fusion of MVBs with the plasma membrane or fused with lysosomes and degraded. Upon release of ILVs into the extracellular space they are termed exosomes. Microvesicles are generated by outward budding of the plasma membrane followed by pinching off and release into the extracellular space. RER: rough endoplasmic reticulum, SER: smooth endoplasmic reticulum. This figure was created with Biodraw, Motifolio.

Microvesicle formation

Microvesicles are released by cells under physiological conditions but the release of microvesicles is increased upon cell activation as a consequence of elevated cytosolic calcium levels [6]. During microvesicle formation physiological membrane asymmetry is disrupted. In resting cells, plasma membrane lipids are asymmetrically distributed. Phosphatidylcholine and sphingomyelin are mainly located on the outer leaflet (exposed to the extracellular space) whereas phosphatidylserine (PS) and phosphatidylethanolamine (PE) are located on the inner side of the membrane [26]. This phospholipid asymmetry is regulated by specific intramembraneous lipid-moving enzymes called floppases, flippases and scramblases [27]. The activity of these enzymes is Ca^{2+}-dependent. Flippases are active in resting cells at normal intracellular calcium levels and can move certain phospholipids (e.g. PS and PE) from the outer to the inner membrane leaflet to maintain membrane asymmetry [28]. Floppases can translocate lipids from the inner side to the outer side of the membrane but is only activated upon increased intracellular level of calcium [29]. Scramblases are also activated by increased intracellular levels of calcium and transports lipids in a bidirectional nonspecific

manner [30]. When the intracellular calcium level increases, such as during cell activation, flippases are inactivated whereas floppases and scramblases are activated. This leads to translocation of PE and PS to the outer leaflet of the membrane and disruption of the lipid asymmetry. Thus, the budding microvesicles may carry PE and PS on their outside. Due to the exposure of PS on microvesicles, labelling of PS by annexin is a common strategy for detection of microvesicles in biological samples. However, PS-negative microvesicles have also been found. The biogenesis of PS-negative microvesicles is poorly understood but may involve certain cellular proteins capable of altering the membrane curvature [31,32].

Other calcium-dependent processes involved in microvesicle formation are cytoskeleton reorganization and disruption of the actin network. One calcium-dependent enzyme responsible for cleavage of actin-anchoring proteins is calpain. Calpain is activated when intracellular calcium is elevated and disrupts the actin-based cytoskeleton, resulting in outward protrusion of the membrane and microvesicle formation. Inhibition of calpain significantly reduces the number of released microvesicles [33].

Microvesicle content

A microvesicle consists of a phospholipid bilayer and a cargo that is either carried on/in the vesicle membrane or is enclosed within the vesicle. The cargo can be divided into three main categories: proteins, lipids, and nucleic acids. Some forms of cargo are commonly detected in most microvesicles irrespective of the cellular origin whereas other forms of cargo are cell-specific [4,34]. The cargo also depends on the physiological state of the cell and/or the stimuli that trigger vesicle release (e.g. calcium ionophore, ATP, hypoxia, lipopolysaccharide, bacterial exotoxins, or cytokines) [7,35-38]. An overview of microvesicle content is presented in Figure 2.

Protein content commonly found in microvesicles includes cytoskeletal proteins (such as actin and myosin) [36,39], membrane adhesion proteins (such as integrins, selectins and tetraspanins) [40] and membrane trafficking proteins (such as Rabs, GTPases and syntaxins) [41]. The GTPase ARF6 and Rab22A play a role in selective enrichment of certain contents into microvesicles as well as being enriched in microvesicles themselves [42].

Other proteins that may be found on or within microvesicles include growth factors [43], cytokines (e.g., regulated upon activation, normal T cell expressed and presumably secreted (RANTES), tumor necrosis factor (TNF)-α) [44,45], signaling receptors (e.g., kinin B1-receptor, TNF receptor) [46,47], membrane glycoprotein receptors (e.g., tissue factor, major histocompatibility complex (MHC) class I and II) [7,48], extracellular matrix proteases [49], and bacterial virulence factors (e.g., Shiga toxin or pore forming toxins) [17,50].

Various types of RNAs have been found in microvesicles including mRNA and microRNA (miRNA) and have been shown to be functionally active when horizontally transferred by microvesicles to cells [51,52].

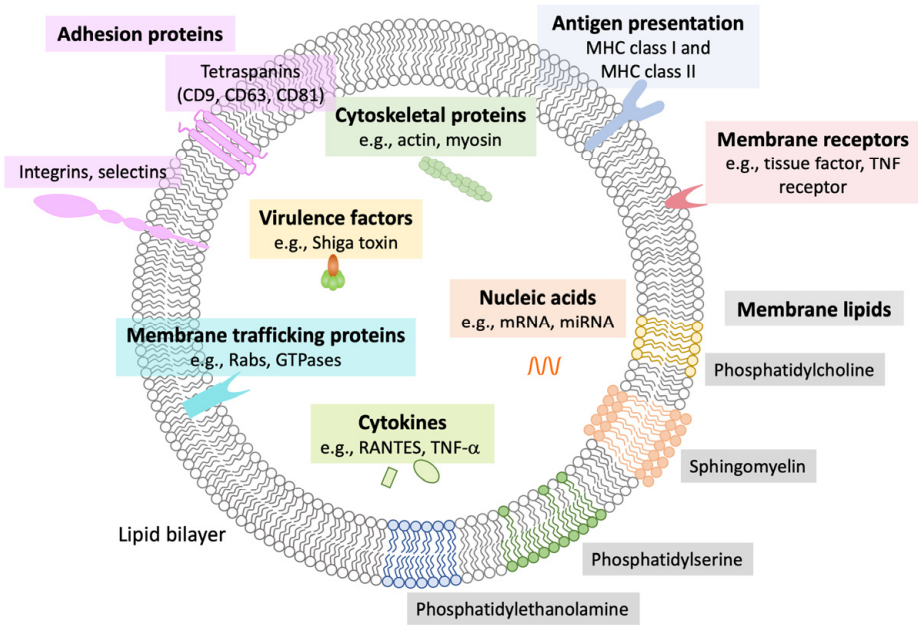

Figure 2: Schematic presentation of a microvesicle and its molecular content. Microvesicles contain lipids, proteins and nucleic acids. The vesicle consists of a lipid bilayer consisting of lipids and membrane-associated proteins. Within the vesicle a variety of proteins and nucleic acids are found and the encapsulated contents are protected from enzymatic degradation by extracellular proteases. MHC: major histocompatibility complex, TNF: tumor necrosis factor, RANTES: regulated upon activation, normal T cell expressed and presumably secreted.

The lipid membrane of circulating microvesicles predominantly contains PS, phosphatidylcholine, sphingomyelin and PE [53,54]. The lipid composition may differ from the plasma membrane of the cell from which the vesicle was released from [55].

Uptake of extracellular vesicles

Released extracellular vesicles can be taken up by nearby or distant cells by endocytosis or fusion with the plasma membrane [56]. Uptake of extracellular vesicles can be achieved by several different pathways; phagocytosis [57], macropinocytosis [58], clathrin-mediated endocytosis [59], caveolin-dependent

endocytosis [60] and lipid raft-mediated uptake [61]. Lipids and proteins enriched on the surface of extracellular vesicles can facilitate cellular uptake by interactions between the extracellular vesicle and the recipient cell [61]. An overview of the different uptake mechanisms and the fate of the vesicle content is presented in Figure 3.

Phagocytosis can be performed by most cells to internalize extracellular materials but is accomplished with higher efficacy by certain immune cells such as macrophages, dendritic cells, monocytes, and neutrophils [62]. Upon binding of extracellular substances to phagocytic receptors (non-opsonic or opsonic) the cell extends its membrane, engulfing the extracellular material to be taken up [63]. During maturation, phagosomes fuse with endosomes and lysosomes creating phagolysosomes in which the internalized material is broken down [62]. Phosphatidylserine located on the outer leaflet of dying cells or on extracellular vesicles is a potential signal for the induction of phagocytosis [61,64].

Macropinocytosis involves plasma membrane extension, subsequent invagination of the membrane followed by fusion of the protrusion with its own membrane and pinching off of the content into the intracellular space [65].

Clathrin-dependent endocytosis involves the formation of clathrin-coated pits that turn into vesicles. This process is initiated by the clustering of endocytic coat proteins on the inner leaflet of the plasma membrane. The assembly of clathrin proteins promotes the membrane to form a vesicle bud. The vesicle in then pinched off into the cytosol, scission is performed by dynamin [66].

Caveolin-dependent endocytosis is similar to clathrin-dependent endocytosis. Instead of clathrin proteins, membrane proteins called caveolins induce membrane invaginations termed caveolae that are subsequently pinched off into the intracellular space by dynamin. Caveolae are domains enriched in caveolins, cholesterol and sphingolipids [67].

Lipid rafts are involved in both clathrin- and caveolae-mediated endocytosis but can also be involved in clathrin- and caveolae-independent uptake. Lipid raft-mediated uptake has also been shown to play a role in cellular uptake of extracellular vesicles and may involve the association of proteins called flotillins with lipids rafts leading to endocytosis [61,68].

Following uptake, endocytic vesicles are delivered to early endosomes in which the extracellular vesicles can either fuse with the endosomal membrane and thereafter expose their content to the cytosol, recycle back to the plasma membrane or be degraded in lysosomes [69-71]. Alternatively, vesicle cargo can also be transported to trans-Golgi network [6].

Extracellular vesicles can also fuse directly with the plasma membrane of the recipient cell. This enables the content of extracellular vesicles to be directly released into the cytosol of the cell [72]. The likelihood of fusion to occur is

determined by several factors including an acidic environment, the degree of binding between the vesicle and the plasma membrane, and the lipid composition of the extracellular vesicle [73].

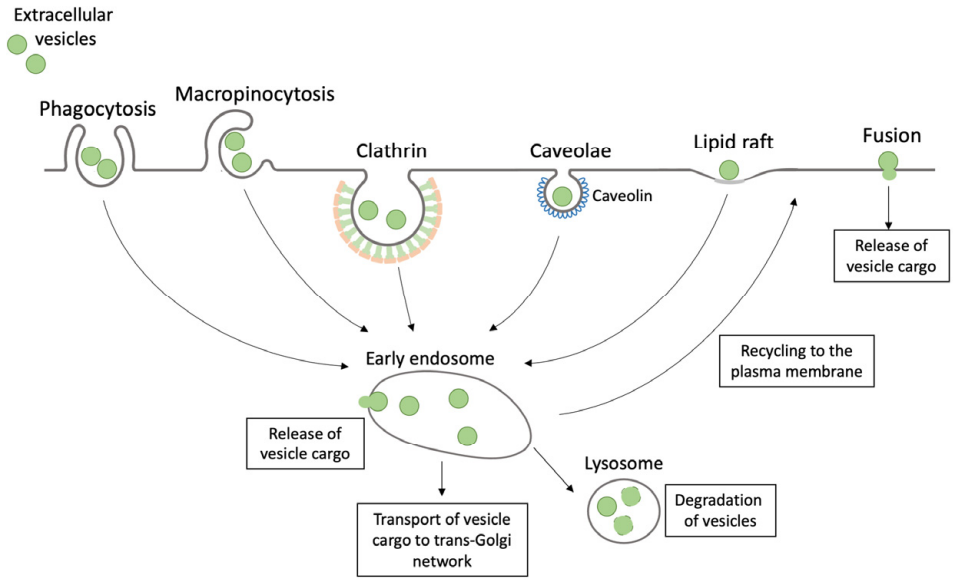

Figure 3: Mechanisms of extracellular vesicle uptake by cells and the fate of vesicular content. Extracellular vesicles can bind to or be taken up by recipient cells by a number of mechanisms. Endocytic vesicles rapidly fuse with early endosomes in which extracellular vesicles release their cargo or alternatively be transported to the trans-Golgi network. Early endosomes can also mature into late endosomes followed by degradation of extracellular vesicles in lysosomes.

Isolation of extracellular vesicles

Isolation of extracellular vesicles can be performed by a variety of methods all with certain advantages or limitations. The use of size- or density-based isolation methods allows for the differentiation of vesicle subpopulations but also some contaminants, such as non-vesicular particles and protein aggregates, based on defined size and density but with overlapping properties [74,75]. The surface proteins and lipids on exosomes and microvesicles are often similar [76], making separation of these two groups of extracellular vesicles difficult based on immunoaffinity-based methods. There is, as yet, no gold-standard method for isolation of extracellular vesicles.

Some of the most commonly used methods include differential centrifugation, ultracentrifugation, density gradient centrifugation, filtration, size-exclusion chromatography, precipitation, and magnetic beads capture. The isolation principles

and the different advantages and limitations for each isolation method are presented in Table 1. Differential centrifugation was used for isolation of extracellular vesicles in the size range of microvesicles in the studies included in this thesis.

Table 1: Methods for isolation of microvesicles and exosomes.

Method	Isolation principle	Advantages/Limitations	Ref
Differential centrifugation/ ultracentrifugation	Centrifugation g-force allows sedimentation of EVs according to size and density.	High yield, easy to perform Low purity, loss of EVs, may cause EV deformation	[77-79]
Density gradient centrifugation	Separation dependent on EV density in a density gradient medium.	High purity, optimized for isolation of exosomes Time consuming, loss of EVs due to incomplete sedimentation, low recovery	[80,81]
Ultrafiltration	Filter membrane with pore diameter smaller than EVs allows smaller components to be filtered away.	Quick, easy Low purity, disruption of EVs	[82,83]
Size-exclusion chromatography	Column with a porous matrix that allows particles larger than the size cutoff to pass through whereas small particles temporarily enter the porous matrix. Large particles therefore elute faster than particles smaller than the size cutoff.	High reproducibility, high purity Time consuming, requires specific equipment	[83,84]
Precipitation	Hydrophilic polymers bind water molecules surrounding the EVs and lower EV solubility forcing them out of solution into a precipitate.	Quick Low purity (co-precipitation of contaminants)	[84]
Magnetic beads capture	Immuno-capture using magnetic beads conjugated to antibodies or proteins with affinity towards EV-specific surface markers. Magnetic force captures EVs bound to the beads whereas other compounds are discarded.	High purity, high specificity Exclusion of EVs	[85]

EVs: extracellular vesicles

Characterization of extracellular vesicles

After isolation of extracellular vesicles, several different methods can be used for their characterization giving information related to size, shape, concentration, and content. Some of the most commonly used methods are summarized in Table 2.

Table 2: Methods for characterization of extracellular vesicles.

Method	Detection range	Principle	Ref
Nanoparticle tracking analysis (NTA)	Size (10 nm-2 µm) concentration 10^7-10^9 particles/mL	Detects single particles by light scattering and determines size and concentration by tracking the particles under Brownian motion.	[86,87]
Dynamic light scattering (DLS)	Size (0.3 nm – 1 µm)	Detects particles by light scattering and estimates the size by fluctuation in scattered light intensity under Brownian motion.	[87]
Flow cytometry	Size > 200 nm concentration 10^6-10^9 particles/mL	Scattered light of EVs flowing through a laser beam relates to the EV size and granularity. Detection of EV antigens can be obtained by labeling with fluorescent antibodies that are detected by fluorescent detectors.	[88]
Electron microscopy (EM)	0.1 nm- µm range	The sample is illuminated by accelerating electrons and the transmitted electrons enter an optical lens and appear as an image	[89]

EVs: Extracellular vesicles.

The size and concentration of extracellular vesicles in a sample can be determined by dynamic light scattering and by nanoparticle tracking analysis. These methods utilize light scattering of a laser beam to detect particles and their random motion in liquid phase, known as Brownian motion [86]. Another method that can be used for quantification of extracellular vesicles is flow cytometry. This method can also be used to differentiate extracellular vesicle subpopulations by using fluorescently labelled antibodies against specific antigens possessed by the vesicles. Flow cytometry is optimized for measuring larger vesicles in the size range of microvesicles [90].

The morphology of extracellular vesicles can be characterized using conventional transmission electron microscope (TEM) or cryo-EM [89]. Conventional TEM includes negative staining, in which the vesicle sample is dried on a carbon-coated grid and the vesicles are visualized by heavy metal staining (Figure 4a) or thin section, in which vesicles are embedded in plastic and sectioned (Figure 4b). In TEM, addition of an antibody can reveal the presence of a specific antigen in the vesicle sample.

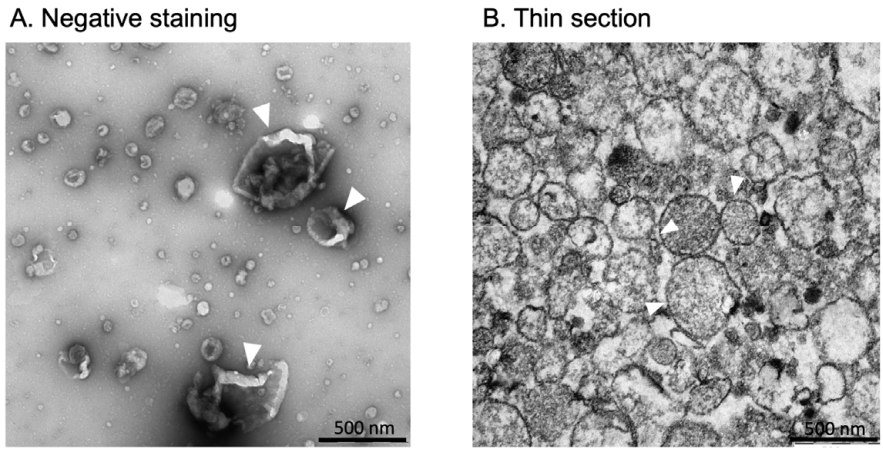

Figure 4: Characterization of extracellular vesicles by transmission electron microscopy. A) Transmission electron microscopy of negatively stained extracellular vesicles reveals the presence of extracellular vesicles (arrow heads) and gives an estimation of the size and amount of isolated extracellular vesicles. B) Thin section of embedded extracellular vesicles enables high contrast visualization of the membrane bilayer of the vesicles (arrowhead).

The protein content of extracellular vesicles can be determined by a variety of methods such as proteomics, enzyme-linked immunosorbent assays (ELISAs) and immunoblotting [91]. Methods for characterization of extracellular vesicles used in the studies included in this thesis include nanoparticle tracking analysis, flow cytometry, proteomics, ELISA and electron microscopy of thin section extracellular vesicles.

The role of extracellular vesicles in homeostasis and disease

Extracellular vesicles are involved in a large number of cellular processes such as immune modulation, coagulation, thrombosis, cell migration and tumorigenesis. In the following section, the role of extracellular vesicles in cell physiology, homeostatic processes, and their contribution to the development of disease will be discussed.

Intercellular communication

Extracellular vesicles are an important means for cell-to-cell communication. Extracellular vesicles can mediate transfer of biologically active substances between cells including RNAs and receptors.

Transfer of RNA by extracellular vesicles

Extracellular vesicles can transfer genetic material such as mRNA and miRNA that may phenotypically alter the recipient cells [52,92]. mRNA and miRNA released from exosomes derived from mouse mast cells were transferred to human mast cells and translated into proteins in the recipient cells [52]. Microvesicles from embryonic stem cells were shown to transfer biologically functional ligands and mRNA to hematopoietic progenitor cells *in vitro* [93]. mRNA delivered by the vesicles was translated into corresponding proteins leading to increase survival and expansion of these cells. Microvesicles released from leukemic bone marrow cells were shown to contain miRNA-21, which is known to be upregulated in several types of tumor cells, and miRNA-29a that were transferred to hematopoietic stem cells *in vitro* resulted in enhanced cell survival [94,95].

Transfer of receptors

Microvesicles possess functional membrane receptors that can be transferred to recipient cells which either lack the receptor or express it at a low level resulting in its increase [46,47]. Microvesicle-mediated receptor transfer can functionally alter the recipient cell [96]. One example is the transfer of human immunodeficiency virus (HIV)-coreceptors CCR5 and C-X-C receptor 4 (CXCR4) important for the virus to enter cells. CCR5 was transferred by microvesicles from Chinese hamster ovary cells or peripheral blood mononuclear cells to receptor-deficient endothelial cells *in vitro* thereby rendering the recipient cells susceptibility to HIV infection [97]. In a similar manner, microvesicles from megakaryocytes or platelets transferred CXCR4 receptor to cells that previously lacked the receptor whereafter the cells could become infected by HIV, as shown *in vitro* [98].

Another example is the transfer of epidermal growth factor receptor (EGFR)vIII by aggressive brain tumor cells. EGFRvIII transferred by microvesicles to more benign cells, previously lacking EGFRvIII, were shown to promote oncogenic transformation *in vitro* [99].

Receptor transfer by microvesicles may also promote inflammation. Platelet microvesicles were shown to transfer glycoprotein IIb/IIIa receptors to neutrophils *in vitro*. When stimulated with granulocyte macrophage colony-stimulating factor (GM-CSF), the transferred glycoprotein IIb/IIa receptor interacted with beta2 integrins resulting in nuclear factor kB (NF-kB) activation [96]. NF-kB is a transcription factor that can induce the expression of a wide range of pro-inflammatory genes and suggests that receptor transfer by microvesicles could have a role in regulating the immune response of neutrophils [100]. Another example is the transfer of the kinin B1-receptor by microvesicles. Neutrophil-derived microvesicles possess functional kinin B1-receptor which was transferred to glomerular endothelial cells thereby promoting glomerular inflammation by the kallikrein-kinin system. Transfer of kinin B1-receptor to glomerular endothelial cells were shown in vasculitis patients *in vivo* and also *in vitro* [46]. The kinin B1-

receptor on microvesicles is involved in neutrophil migration *in vitro* [101] and participates in the development of chronic inflammation.

Extracellular vesicles as a cellular disposal mechanism

Cells can release microvesicles as a mechanism of ridding the cell of harmful substances to maintain homeostasis. One example is the release of the complement membrane-attack complex (MAC) on microvesicles upon complement activation on the cell surface. MAC consist of the complement components: C5b, C6, C7, C8 and C9 and the formation of MAC leads to generation of large pore-like channels in the cell membrane. A sufficient quantity of MAC on the cell surface will eventually lead to lysis of the cell. MAC has been found on microvesicles from activated red blood cells and platelets *in vivo* and its release on the membrane of vesicles could be a mechanism by which the cell survives complement-mediated cell-lysis [102,103]. Another example is the release of active caspase 3, involved in apoptotic pathways, in microvesicles from various cells. Active caspase 3 is not present in resting cells and removal of active caspase 3 by microvesicles, as shown *in vivo* and *in vitro*, has been suggested to increase cell survival [104,105].

In cancer cells, the release of extracellular vesicles can lead to drug resistance [106]. The cancer cell reduces the intracellular level of the drug by exporting it within extracellular vesicles as demonstrated *in vitro* [107].

Virulence factors from bacterial pathogens have been detected in microvesicles and might be released by cells as a protective measure. Some bacteria, such as *Streptococcus pneumonia* and *Streptococcus pyogenes,* produce membrane perforating toxins that form a pore in the plasma membrane resulting in leakage of cytoplasmic proteins and influx of calcium. Instead of leading to cell lysis, the cell can efficiently eliminate the toxin pores by shedding microvesicles as a mechanism of membrane repair to ensure cell survival, as demonstrated *in vitro* [50,108,109].

In patients with HUS, caused by EHEC, microvesicles in the circulation have been shown to contain the main bacterial virulence factor, Shiga toxin [17]. This could be a mechanism by which cells get rid of the toxin in order to decrease the toxin load within the cell.

Immune modulation

Extracellular vesicles play an important role in the modulation of immune responses, especially during infection, injury and inflammation. Neutrophils in the circulation are rapidly recruited to inflamed tissue in response to inflammatory mediators, and extracellular vesicles can contribute to this process. Neutrophils that migrate towards a chemoattractant were shown to release leukotriene B4-containing exosomes. These exosomes could induce activation of resting neutrophils *in vitro*

and increase the chemotactic activity in an autocrine fashion which was dependent on leukotriene B4 receptor activation [110]. In a similar manner, neutrophil-derived microvesicles containing L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) were shown to have chemotactic potential attracting neutrophil migration *in vitro* [111].

Neutrophils have been shown to release neutrophil-derived trails (NDTRs) [8]. NDTRs are highly pro-inflammatory microvesicles released during neutrophil migration from the endothelium into inflamed tissue. NDTRs contain proinflammatory miRNAs and monocyte chemotactic protein-1 (MCP-1) able to induce monocyte recruitment and macrophage polarization *in vitro* suggesting a role of NDTRs in initiating inflammation [8]. Additionally, endothelial cell-derived microvesicles in patients with chronic vascular inflammation possess the kinin B1-receptor and these microvesicles promote neutrophil chemotaxis *in vitro* [101]. Thus, certain microvesicles are chemoattractant for neutrophils.

Microvesicles released from platelets have been shown to exert immunomodulating properties by their ability to activate immune cells as well as endothelial cells and induce cell-to-cell interactions. Platelet microvesicles, containing the inflammatory mediator CCL5 (also called RANTES), were shown to deposit on endothelial cells leading to recruitment of monocytes. This was demonstrated both *in vitro* and by *ex vivo* prefusion of murine carotid arteries [45]. Incorporation of cytokines within extracellular vesicles is a selective process and may be a mechanism of immune modulation [37]. Platelet microvesicles can also deliver arachidonic acid to endothelial cells, monocytes and platelets leading to thromboxane A2 metabolism, cellular activation, increased expression of adhesion molecules on endothelial cells and monocyte adhesion to the endothelium *in vitro* [112,113].

Platelet microvesicles may also play a role in neutrophil aggregation and accumulation. P-selectin located on platelet-derived microvesicles bind to neutrophils via P-selectin glycoprotein ligand-1 (PSGL-1) and serve as a bridge between adherent and circulating neutrophils [114].

Platelet-derived microvesicles released during platelet activation and aggregation induce extensive release of inflammatory cytokines such as interleukin (IL)-1β, TNFα, IL-6 and IL-8 from endothelial cells and the monocytic cell line THP-1 *in vitro.* These microvesicles could also induced the expression of adhesion molecules, such as CD11b by THP-1 cells and intercellular adhesion molecule (ICAM)-1 by endothelial cells, resulting in cell-to-cell adhesion [115].

Thrombosis

When a blood vessel is damaged, the blood forms a blood clot to seal the vessel and prevent blood loss. This process, which maintains the integrity of the vessel, is called hemostasis. Upon vascular damage the endothelium, forming a single cell

layer on the inner wall of the blood vessel, becomes disrupted allowing collagen and tissue factor from the underlying tissue to be exposed to the blood. Collagen induces platelet activation and aggregation whereas tissue factor initiates activation of the extrinsic pathway of the coagulation cascade ultimately leading to thrombin generation. Thrombin converts fibrinogen into fibrin but can also induce platelet activation [116,117]. The recruitment of platelets to the site of injury and the formation of a fibrin meshwork leads to the formation of a blood clot or thrombus.

Microvesicles can promote both coagulation and thrombosis [118]. Under physiological conditions microvesicles in the circulation of healthy individuals induce low-grade thrombin generation and play a role in homeostasis [119]. However, activated platelets are a source of microvesicles with prothrombotic potential. Platelet-derived microvesicles induce platelet adhesion to the endothelium during vascular injury, facilitating thrombus formation [120].

Microvesicles can promote coagulation and thrombosis by the exposure of phosphatidylserine and tissue factor (Figure 5). Phosphatidylserine exposed on the outer leaflet of the microvesicle membrane creates a negatively-charged surface which facilitates assembly of coagulation factors prothrombin, factor Va and factor Xa [121]. This leads to the formation of the prothrombinase complex followed by thrombin generation [122]. Phosphatidylserine is also required for tissue factor, a transmembrane glycoprotein, to bind to factor VII/VIIa. In addition to the high expression of tissue factor in fibroblasts surrounding the blood vessels, tissue factor can also be expressed by monocytes and upregulated upon stimulation with inflammatory stimuli [123-125]. Microvesicles derived from activated monocytes have been shown to possess tissue factor and can be highly procoagulant [126].

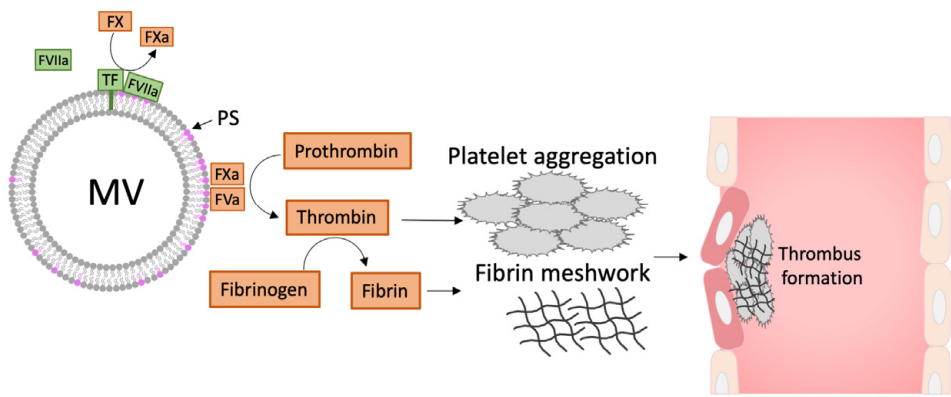

Figure 5: Thrombus formation mediated by monocyte-derived microvesicles. Phosphatidylserine and tissue factor (TF) on microvesicles can be highly prothrombotic by their ability to activate coagulation and induce thrombin generation. Thrombin induces platelet aggregation and converts fibrinogen into fibrin resulting in formation of a fibrin meshwork and clot formation. TF: tissue factor, MV: Microvesicle, PS: phosphatidylserine. This figure was created with Biodraw, Motifolio.

Enterohemorrhagic *Escherichia coli*

Microvesicles have been shown to partake in most aspects of Shiga toxin-mediated disease leading to HUS. Shiga toxin-producing *E. coli* are a particularly virulent subtype of *E. coli*. *E. coli* are gram-negative bacteria and part of the normal bacterial flora in the human gastrointestinal tract. Most strains of *E. coli* are harmless but some strains have obtained specific virulence factors through plasmids, bacteriophages, transposomes and/or pathogenicity islands and have become highly pathogenic causing serious illness in humans [127], such as EHEC. The major reservoir of EHEC is in the intestinal tract of cattle, where it coexists with its host usually without causing disease [128]. In humans, EHEC can cause infection after ingestion of EHEC-contaminated food or water. This may lead to large outbreaks of disease or sporadic cases. EHEC infection can be asymptomatic but may cause watery diarrhea, hemorrhagic colitis (bloody diarrhea) and in some cases develop into the life-threatening condition called HUS [129] described below. EHEC possesses several virulence factors, of which Shiga toxin is considered to be the most important for the pathogenicity of the strain.

Shiga toxin

Shiga toxin is named after the Japanese doctor Kiyoshi Shiga and was originally discovered in *Shigella dysenteriae* [130].

Shiga toxin 1 and 2

Shiga toxins produced by EHEC can be divided into Shiga toxin 1 and 2 of which Shiga toxin 2 is associated with more severe disease in humans [131]. Shiga toxin 1 is subtyped into three variants (Shiga toxin 1a, 1c and 1d) and Shiga toxin 2 into seven variants (Shiga toxin 2a-g) [132]. Shiga toxin 1 differs by one amino acid from the Shiga toxin produced by *S. dysenteriae* while Shiga toxin 2 has only 50-60% sequence homology with Shiga toxin 1 [133]. Shiga toxins are AB5 toxins and belongs to the type II ribosomal-inactivating proteins. A schematic drawing of Shiga toxin is presented in Figure 6. The enzymatically active A-subunit is responsible for the cytotoxic property of the toxin whereas the pentameric B-subunit, composed of

five identical B subunits, is responsible for its binding to the cellular glycosphingolipid receptor, globotriaosylceramide (Gb3) [10,134].

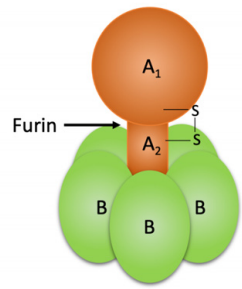

Figure 6: Schematic presentation of the structure of Shiga toxin. Shiga toxin consists of a single A-subunit and a pentameric B-subunit, linked together by non-covalent bonds. The A-subunit can be cleaved by the protease furin into A1 and A2 moieties. After cleavage, A1 and A2 are held together by a disulfide bridge.

The toxin receptor

Shiga toxin binding to its receptor is important for toxin uptake by cells. The binding subunit of Shiga toxin 1 and 2, the B-subunit, binds with high affinity to Gb3 [135]. Shiga toxin can also bind to globotetraosylceramide (Gb4) (the main receptor for Shiga toxin 2e) [10]. The B-subunit of Shiga toxin 1 and 2 binds to the carbohydrate moiety of the Gb3 structure and contains 15 potential binding sites, three in each monomer [134,136,137]. The structure of Gb3 and the synthesis of glycosphingolipids are presented in Figure 7.

Not all cells express Gb3 and even if a cell expresses Gb3 it might not be susceptible to Shiga toxin-induced cytotoxicity. The lipids in the surrounding environment of Gb3 within the plasma membrane and the length and saturation of the fatty acid chain in the Gb3 molecule are factors that affect this outcome [138,139]. The ability of Gb3 to associate and cluster within lipid rafts has been shown to increase Shiga toxin 1 binding and internalization and make the cell more susceptible to Shiga toxin 1 [140]. Lipid rafts, or detergent-resistant membrane regions, are microdomains within the membrane enriched in cholesterol, glycosphingolipids and trans-membrane proteins. The structure of lipid rafts promotes endocytosis and lipid rafts are involved in signal transduction in cells [141]. The association of Gb3 with lipid rafts also affects the intracellular transport after Shiga toxin internalization [142].

Another factor that affects the toxin sensitivity of a cell is the number of Gb3 receptor molecules in the plasma membrane. Lipopolysaccharide and tumor necrosis factor (TNF-α) have been shown to upregulate the expression of Gb3 on certain cells and, hence, increase the number of toxin binding sites [143,144].

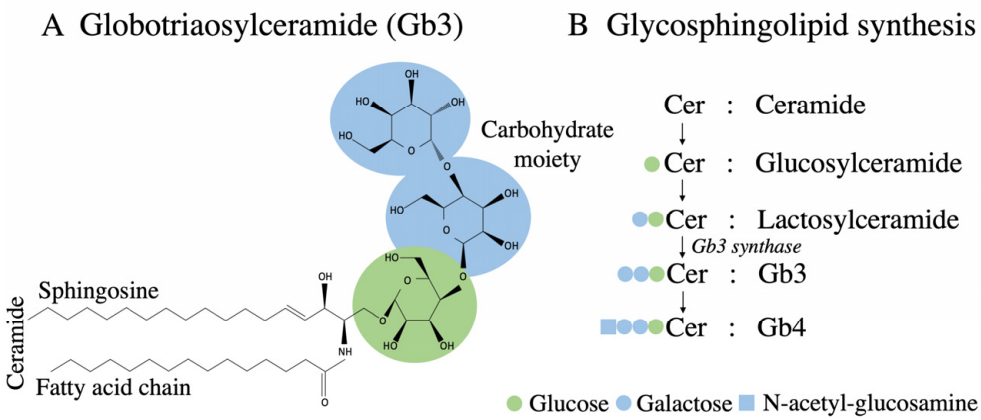

Figure 7: A schematic drawing of the of the Shiga toxin receptor globotriaosylceramide (Gb3) and its synthesis. A) The carbohydrate moiety of Gb3 is composed of one glucose and two galactoses. The glucose molecule is attached to the sphingosine backbone of ceramide. The fatty acid side-chain of ceramide can vary in length by the number of carbons. B) Gb3 is synthesized from ceramide. First, one glucose is attached to ceramide by glucosylceramide synthase resulting in the formation of glucosylceramide. Next, addition of one galactose leads to the formation of lactosylceramide whereafter addition of one more galactose results in the formation of Gb3. Gb3 synthase is responsible for the attachment of galactose and the formation of Gb3. Addition of N-acetyl-glucosamine to Gb3 leads to the formation of Gb4. Gb3: Globotriaosylceramide, Gb4: Globotetraosylceramide, Cer: Ceramide.

Endocytosis and intracellular transport

When Shiga toxin binds to the Gb3 receptor it is mainly taken up by endocytosis. Various endocytic routes have been described for Shiga toxin including clathrin-dependent and clathrin-independent uptake [145,146]. Clathrin-independent endocytosis includes caveolae-mediated endocytosis, tubular formation and other clathrin-independent pathways. During clathrin-dependent endocytosis, Shiga toxin 1 has been shown to promote its own uptake by inducing Syk kinase signaling in cells leading to clathrin phosphorylation and an increased level of clathrin-coated structures [147]. Shiga toxin 1 uptake can also be mediated by tubule formation. Upon binding of the toxin, the assembly of Gb3 receptors induces inward curvature of the plasma membrane, leading to formation of tubular endocytic pits [145]. Release of clathrin-coated vesicles and tubular structures into the cytosol is mediated by dynamin.

After internalization, Shiga toxin-containing vesicles fuse with early endosomes in which sorting of the toxin occurs. For Shiga toxin to induce a cytotoxic effect in cells, it needs to be transported along the retrograde transport, from early endosomes to the trans-Golgi network and further to the endoplasmic reticulum (ER) [12,148]. During Shiga toxin's retrograde transport it is cleaved by furin into A1 and A2 [149]. From the ER, Shiga toxin is transported to the ribosomes in the cytosol where the A1-subunit inhibits protein synthesis by cleaving an adenosine residue from the 60S ribosome [13].

Alternative intracellular routes, besides retrograde transport, have also been described. In toxin-resistant cells in which Gb3 is not localized in lipid rafts, Shiga toxin 1 can be transported to lysosomal compartments leading to its degradation [150]. Additionally, Shiga toxin 1 and 2 can be released within membrane-derived microvesicles. Both toxin-sensitive cells, such as HeLa cells [6], and toxin-resistant cells (resistant to the cytotoxic effects), such as platelets, red blood cells, neutrophils and monocytes, have been shown to release toxin-containing microvesicles [17]. An overview of the intracellular trafficking of Shiga toxin is presented in Figure 8.

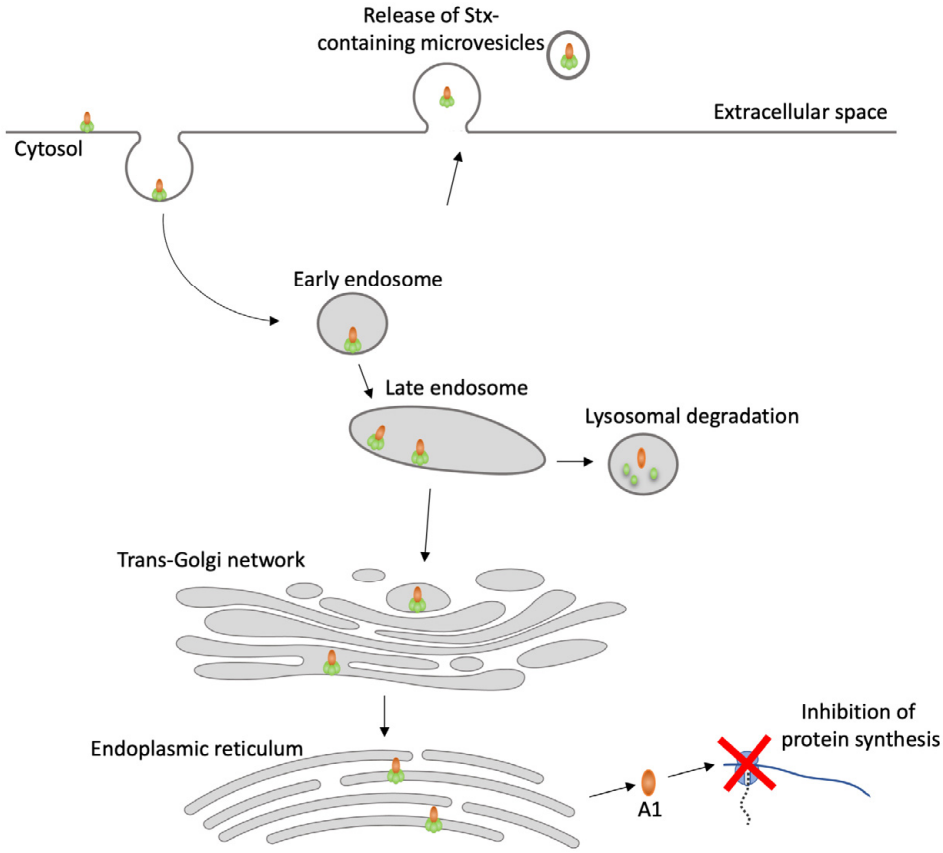

Figure 8: Cellular uptake and intracellular trafficking of Shiga toxin. Shiga toxin is endocytosed by cells after binding to the toxin receptor, Gb3. The toxin undergoes retrograde transport from the endosome to the trans-Golgi network and further to the endoplasmic reticulum (ER). From the ER, Shiga toxin is translocated to the ribosomes in the cytosol where it cleaves an adenine from the 60s rRNA of ribosomes leading to inhibited protein synthesis and eventually cell death. Alternatively, Shiga toxin can be transported to lysosomes where it is broken down or it can be released into the extracellular space within microvesicles shed from the cell membrane. This figure was modified from Biodraw pictures, Motifolio.

Apoptosis

Shiga toxin can induce apoptosis in several cell types such as epithelial and endothelial cells [14,151]. Shiga toxin may induce multiple intracellular signaling pathways leading to the activation of caspase-8 and 9 [14,152]. Both caspases are involved in the activation of caspase-3 and -7 which executes the apoptotic program [153]. Activation of the ER stress response has been demonstrated in human monocytic cells after Shiga toxin 1 uptake [154]. This response is activated when Shiga toxin 1 is recognized in the ER as an incorrectly folded protein by the chaperone Bip [155]. ER stress signaling may further result in activation of apoptotic pathways.

Shiga toxin can also induce the ribotoxic stress response in cells as a consequence of ribosomal damage. Shiga toxin 1-induced stress response may involve recognition of conformational changes in ribosomes that leads to activation of p38 mitogen activated protein kinase [156].

EHEC and the host response

EHEC is a non-invasive bacteria which is not associated with bacteremia [157]. Following colonization of the intestinal mucosa, usually by formation of attaching and effacing lesions, EHEC may induce severe inflammation in the intestine [158]. Virulence factors released by EHEC may gain access to the circulation and play a role in the pathogenesis of HUS [159].

EHEC in the intestine

The intestinal mucosal layer is the major barrier that protects the host against bacterial infection. This layer consists of a mucus layer, produced by goblet cells, and the intestinal epithelium. The initial binding of EHEC is mainly located at the villi of the terminal ileum and the follicle-associated epithelium of Peyer's patches [160,161]. Although most EHEC remain attached to the intestinal mucosa, EHEC can also be taken up by intestinal M cells located in the follicle-associated epithelium and by underlying macrophages in Peyer's patches where they are able to survive and produce Shiga toxin 2 as demonstrated in a murine model *in vivo* and in macrophages *in vitro* [162].

The inflammatory response induced during EHEC colonization of the colon is triggered by recognition of pathogen-associated molecular patterns (PAMPs) [163]. PAMPs are molecules commonly possessed by pathogens, such as lipopolysaccharide (LPS) and flagellin. PAMPs activate cells by binding to pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) [164]. Flagellin, the structural protein of the bacterial flagellum, is recognized by TLR5 and LPS is recognized by TLR4 [165,166]. TLR5 and TLR4 signaling leads to release of cytokines. During EHEC colonization an extensive release of cytokines and chemokines has been reported from human colon epithelium *in vivo* and *in vitro*, such as IL-8 and TNFα [165,167,168]. IL-8 is a potent neutrophil chemoattractant inducing transmigration of neutrophils into the gut. High levels of IL-8 correlate with the risk of developing EHEC-associated HUS in children [169].

Shiga toxin also plays a role in the intestinal inflammation during EHEC infection. In a pediatric patient with EHEC infection, Shiga toxin 1 and 2 were found within intestinal epithelial cells indicating cellular uptake [170]. Shiga toxin 1 and 2 can

bind to human intestinal Paneth cells, located in crypts of the small intestine, which express Gb3, as shown *in vitro* [171]. In mice inoculated with Shiga toxin 2-producing or non-Shiga toxin producing isogenic strains of *E. coli* O157:H7, intestinal mucosal cell damage was strongly associated with the presence of Shiga toxin 2. This indicates that Shiga toxin 2 could be involved in the intestinal cell injury during EHEC infection [158]. Severe inflammation in the intestine during EHEC infection can lead to diarrhea or hemorrhagic colitis. The damaged barrier in the intestine can enable Shiga toxin to leak into the circulation [172]. Shiga toxin has been shown to be involved in the development of hemorrhagic colitis. This was demonstrated in a primate model infected with a Shiga toxin-producing or a toxin-mutant strain of *S. dysenteriae* in which hemorrhagic colitis was only observed when Shiga toxin was present [173].

Shiga toxin in the circulation

Once Shiga toxin is within the circulation it can bind to blood cells by its receptor Gb3. Only minimal amounts of free Shiga toxin have been found in the blood of EHEC patients [174] as the toxin likely circulates bound to blood cells or within blood cell-derived microvesicles [7,17]. Blood cells are resistant to the cytotoxic effects of Shiga toxin which may instead induce cellular activation leading to the release of inflammatory substances and microvesicles [17,175]. The cellular response to Shiga toxin in the circulation is summarized in Figure 9.

Blood cell response to Shiga toxin

Shiga toxin binds to platelets via Gb3 or an alternative glycolipid called 0.03 [176,177]. For binding to occur, the platelets need to be pre-activated and binding of Shiga toxin leads to further platelet activation [176,178]. Thereafter platelets can take up the toxin and aggregate as demonstrated *in vitro* using Shiga toxin 1 [178]. LPS can induce platelet activation *in vitro* by binding to a receptor complex consisting of TLR4 and CD62 [179] and thereby promote Shiga toxin 1 binding [176]. LPS is bound to platelets from pediatric HUS patients [179]. Activated platelets can release pro-inflammatory mediators and promote vascular inflammation [180].

Monocytes also possess Gb3 and can interact with Shiga toxin in the circulation [181,182]. Shiga toxin 1 and 2 have shown to initiate the release of pro-inflammatory cytokines from human monocytes or monocytic THP-1 cells, respectively, such as IL-1ß, IL-6, IL-8 and TNF-α *in vitro* [182,183]. Additionally, THP-1 cells released IL-8, macrophage-derived chemokine (MDC) and RANTES

upon stimulation with Shiga toxin 2, which further induced platelet activation [184]. Shiga toxin 2 can also induce expression of tissue factor in monocytes *in vitro*, especially in the presence of LPS, and in monocytes in complex with platelets [7].

Neutrophils, despite the absence of Gb3, can also bind Shiga toxin. In HUS patients, Shiga toxin 1 or 2 were found on the surface of neutrophils in the circulation [7,174,185]. Shiga toxin 2 has been shown to inhibit spontaneous neutrophil apoptosis [186] and could thereby play a role in the high neutrophil counts that correlate with poor prognosis in EHEC-associated HUS [187].

Shiga toxin can also bind to red blood cells [188]. In red blood cells Gb3 is known as the Pk antigen which is one of three P antigens (termed P1, Pk and P) [189]. Shiga toxin 2 has been shown to induce complement activation on red blood cells and the release of microvesicles from red blood cells *in vitro* possibly contributing to hemolysis [103].

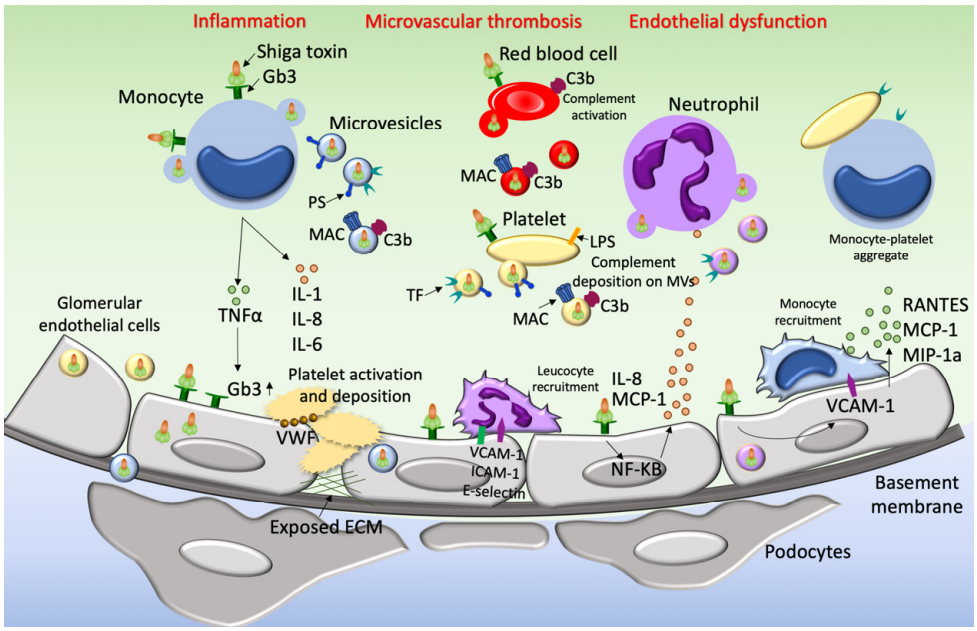

Figure 9: Cellular response to Shiga toxin in a glomerular capillary. Blood cells respond to Shiga toxin by the release of microvesicles, inflammatory and prothrombotic mediators. In glomerular endothelial cells Shiga toxin can induce cytotoxicity, upregulate adhesion factors and induce the release of pro-inflammatory and pro-thrombotic substances leading to recruitment of immune cells, endothelial dysfunction and thrombus formation. TNF: tumor necrosis factor, IL: interleukin, VCAM: vascular cell adhesion molecule-1, ICAM: intercellular adhesion molecule-1, ECM: extracellular matrix, NF-KB: nuclear factor kappa B, RANTES: regulated on activation, normal T cell expressed and secreted, MCP: monocyte chemotactic protein-1, MIP: macrophage inflammatory protein, MVs: microvesicles, LPS: lipopolysaccharide, TF: tissue factor, PS: phosphatidylserine, MAC: membrane attack complex. This figure was created using Biodraw, Motifolio.

Shiga toxin and the kidney

Shiga toxin-induced damage of the renal vasculature is a central event in the pathogenesis of EHEC-induced HUS [190]. Vascular damage leads to a prothrombotic and proinflammatory state.

In EHEC-associated HUS patients, Shiga toxin 2 has been found in kidney cells [17,191,192]. Cells that express the Gb3 receptor in humans include glomerular endothelial cells, podocytes, mesangial cells and proximal tubular epithelial cells [193]. All these cells are sensitive to Shiga toxin [151,194-197]. Human glomerular microvascular endothelial cells were shown to be sensitive to nanomolar of Shiga toxin purified from *S. dysenteriae* [195]. Human podocytes and proximal tubule cells were sensitive to picomolar of Shiga toxin 2 or 1, respectively [196,197].

In addition to the cytotoxic effects of Shiga toxin, it may also contribute to a proinflammatory and prothrombotic state in the kidney by promoting the expression of adhesion proteins and release of cytokines and chemokines from the renal endothelium. This can induce recruitment of immune cells and platelets resulting in thrombus formation [198]. Shiga toxin 2 can induce the expression of E-selectin, ICAM-1 and vascular cell adhesion molecule-1 (VCAM) as well as the release of IL-8, monocyte chemoattractant protein (MCP-1) and stromal cell-derived factor (SDF)-1α in human umbilical vein endothelial cells and human glomerular endothelial cells [199]. This may result in recruitment of leukocytes to the endothelium, formation of platelet-monocyte aggregates and/or platelet aggregation [199-201]. In mice, Shiga toxin 2 injected together with LPS has been shown to induced infiltration of macrophages into the kidney which was dependent on the release of MCP-1, RANTES and macrophage inflammatory protein (MIP)-1α in the kidney. Inhibition of these chemokines lead to decreased macrophage infiltration [202]. The release of proinflammatory substances can exacerbate Shiga toxin-mediated cytotoxicity. For example, TNF-α increases the expression of Gb3 rendering human umbilical vein endothelial cells more sensitive to Shiga toxin 1-induced cytotoxicity [144].

Shiga toxin 1 can also induce the release of von Willebrand factor (VWF) from human umbilical vein endothelial cells resulting in platelet activation and their deposition on the endothelium *in vitro* [203]. VWF mediates platelet adhesion in response to endothelial injury and exposure of the subendothelium [204].

EHEC-associated hemolytic uremic syndrome

EHEC-infection is the main cause of HUS usually associated with a prodrome of gastrointestinal symptoms including watery diarrhea, bloody diarrhea and/or abdominal pain [129]. Within 5-13 days after the debut of diarrhea about 15% of patients will develop HUS [205,206]. Shiga toxin is a crucial factor in the development of EHEC-HUS [207] since other bacteria such as enteropathogenic *E. coli* (EPEC), similar to EHEC in their expression of virulence factors but lacking expression of Shiga toxin [127], are not associated with HUS and bacteria other than *E. coli* such as *S. dysenteriae* [208] or *Citrobacter freundii* [209] can cause HUS when they express Shiga toxin.

HUS is characterized by the simultaneously occurrence of thrombocytopenia (low platelet counts), non-immune hemolytic anemia and acute kidney injury [206]. Extrarenal involvement such as affection of the central nervous system may also occur [210].

The main target organs in HUS are the kidney and the brain. The characteristic pathological lesion in the microvasculature of the kidney is termed thrombotic microangiopathy [211]. Thrombotic microangiopathy is characterized by endothelial cell swelling and thickening, detachment from the basement membrane and thrombus formation in the renal capillaries resulting in occlusion of the vessel [212]. Severe tubular damage occurs as well [151].

Low platelet counts are associated with the risk of developing HUS [213] and poor prognosis in HUS [214]. The underlying mechanism of thrombocytopenia is platelet activation and consumption on damaged endothelium [180]. Children with EHEC-associated HUS have impaired laboratory parameters related to thrombosis and fibrinolysis [215-217]. During the prodromal phase children who later developed HUS were shown to have increased plasma levels of prothrombin fragment 1+2, plasminogen activator (t-PA) antigen, t-PA-plasminogen-activator inhibitor type 1 complex and D-dimer, indicating ongoing endothelial activation and thrombus generation [218,219]. The endothelial damage during HUS leads to the exposure of subendothelial matrix proteins such as VWF, collagen and fibrinogen and results in platelet adhesion and activation [220].

Non-immune hemolytic anemia, characteristic of HUS, is associated with fragmented red blood cells (schistocytes) [212]. The hemolysis is not mediated by antibody binding to red blood cells. The mechanism by which the red blood cells are damaged is suggested to be a consequence of red blood cell passage through partially occluded vessels due to thrombi. The narrow path through the vessels leads to mechanical disruption of the cells and formation of schistocytes. Another possible explanation is that Shiga toxin induces complement-mediated hemolysis of red blood cells. Shiga toxin 2 binding to red blood cells has been shown to induce complement activation and deposition on red blood cells leading to hemolysis [103].

Microvesicles in EHEC-associated hemolytic uremic syndrome

Microvesicles are involved in all aspects of EHEC-HUS including the transport of Shiga toxin to the kidney [17], thrombosis [7] and hemolysis [103]. HUS patients have elevated circulating microvesicles during the acute phase of disease [7,17,221]. These microvesicles originate from platelets, monocytes, neutrophils and red blood cells and were shown to contain Shiga toxin 2 [17]. In one HUS patient, blood cell-derived microvesicles containing Shiga toxin 2 were detected within the renal cortex [17]. Likewise, mice infected with EHEC showed elevated levels of blood cell-derived microvesicles that also contained Shiga toxin 2. Blood cell-derived microvesicles containing Shiga toxin 2 were found within the kidney cells of EHEC-infected mice including the glomerular and peritubular capillary endothelium, podocytes and tubular epithelial cells. *In vitro* experiments showed elevated levels of blood cell-derived microvesicles when whole blood was stimulated with Shiga toxin 2. These vesicles also contained Shiga toxin 2 [17]. Shiga toxin 2 was shown to retain its toxicity within the microvesicles as demonstrated in glomerular endothelial cells after uptake of Shiga toxin 2-containing microvesicles resulting in inhibition of proteins synthesis [17]. These data demonstrated that Shiga toxin 2 could be transported into the kidney within microvesicles.

HUS patients also have elevated levels of microvesicles possessing tissue factor and phosphatidylserine indicating that the microvesicles have pro-thrombotic properties. This could also be demonstrated *in vitro* when whole blood was incubated with Shiga toxin 2 resulting in an increased levels of microvesicles possessing tissue factor and phosphatidylserine [7,17].

The mechanism by which Shiga toxin 1 and 2 induce release of microvesicles has been studied in HeLa cells. Shiga toxin 1 was shown to induce release of extracellular ATP followed by purinergic P2X receptor activation and calcium influx leading to the shedding of microvesicles [6]. The P2X1 receptor antagonist, NF449, inhibited the release of microvesicles from HeLa cells and platelets when stimulated with Shiga toxin 1 or 2 *in vitro*. In a similar manner, a nonselective P2X antagonist, suramin, decreased the release of platelet-derived microvesicles in EHEC-infected mice [6]. Likewise, suramin and PPADS (also a P2X antagonist) decreased the number of microvesicles released from red blood cells stimulated with Shiga toxin 2 *in vitro* and resulted in reduced complement-mediated hemolysis

[103]. Taken together, these data show that Shiga toxin 1 and 2 induce release of microvesicles from cells in a P2X receptor-dependent manner.

In EHEC-HUS patients complement C3 and C9 were detected on blood cell-derived microvesicles from platelets and monocytes [222]. This could also be reproduced *in vitro* upon Shiga toxin 2 stimulation of whole blood resulting in complement deposition of C3 and C9 on released blood cell-derived microvesicles [222]. Likewise, EHEC-HUS patients (both adults and children) exhibit complement deposits on red blood cells and on red blood cell-derived microvesicles. C3 but not C5b-9 was detected on red blood cells while C5b-9 was detected on microvesicles from red blood cells [103]. This suggests that either the microvesicles or the cells of origin were exposed to complement activation during HUS. Cells with complement deposits may release microvesicles in order to maintain cellular integrity and avoid complement-mediated lysis.

The present investigation

The overall aim of this thesis was to study the release, uptake, and properties of Shiga toxin-mediated extracellular vesicles and their detection. Extracellular vesicles have been implicated in the pathogenesis of Shiga toxin-mediated disease as Shiga toxin is transported within extracellular vesicles to the kidney. It is therefore of importance to study the mechanism by which extracellular vesicles may contribute to the pathogenesis of this disease.

Specific aims

1. To investigate the mechanism by which Shiga toxin 1B is released from cells within vesicles and by which the toxin is taken up and sequestered within extracellular vesicles.
2. To investigate the importance of endogenous Gb3 receptors in recipient cells for the uptake of Shiga toxin 2 within extracellular vesicles and for exertion of a cytotoxic effect.
3. To investigate prothrombotic and proinflammatory properties of blood cell-derived extracellular vesicles released after stimulation with Shiga toxin 2.
4. To provide detailed methodology for the isolation and characterization of Shiga toxin 1 and 2-associated extracellular vesicles.

Methods

In the following section, a brief description of the materials and methods used in the papers within this thesis will be presented. For more detailed information, see the material and methods section of each individual paper. The size range of the extracellular vesicles used in the papers within this thesis corresponds to the size of shed microvesicles, however, due to the overlap in size between exosomes and microvesicles the term extracellular vesicles will be exclusively used in the following section.

Ethical considerations

Blood samples from adult healthy volunteers were used in papers **I**, **II**, **III** and **IV** with informed written consent from all subjects. The use of blood from healthy volunteers was approved by the Ethical review board, Lund University.

Shiga toxin

Shiga toxin 1, Shiga toxin 1B-subunit and Shiga toxin 2 were used. In paper **I**, Shiga toxin 1B-subunit was conjugated to Alexa-488, 1.4 nm nanogold or biotin (Stx1B-SS-biotin). In papers **II** and **III**, Shiga toxin 2 was used and in paper **IV** both Shiga toxin 1 and 2 were used. Shiga toxin 2 conjugated to Alexa-555 or Alexa-488 was used in paper **II.**

Cells

HeLa cells which are cervical epithelial were used in papers **I**, **II** and **IV**.

Red blood cells were used from an anonymous donor with a P_1^k phenotype. This phenotype is known to possess high amounts of Gb3 on red blood cells resulting in high levels of Shiga toxin binding compared to other phenotypes [188]. Red blood cells were used in paper **I**.

Chinese hamster ovary (CHO) cells lack Gb3 expression. CHO cells were transfected with a plasmid encoding the Gb3 synthase (A4GALT) resulting in Gb3 expression. CHO cells were used in paper **II**.

Colonic epithelial cells (DLD-1), which lack Gb3 expression, were used in paper **II**.

Platelet-rich plasma was used in paper **III.**

Human umbilical vein endothelial cell line EA.hy 926 is a fusion between primary human umbilical vein cells and a lung epithelial cell line A549. EA.hy 926 cells were used in paper **III**.

A human monocytic cell line, THP-1 cells, were used in paper **III.**

Conditionally immortalized glomerular endothelial cells were used in paper **IV**.

In papers **I** and **II** HeLa cells and DLD-1 cells, respectively, were fluorescently labelled with a plasma membrane dye (CellMask deep red) to visualize the plasma membrane.

Retro 2.1

Retro-2.1 is a compound that blocks the retrograde trafficking of Shiga toxin 1 and 2 between the early endosomes and the trans-Golgi network and thereby protects the cell from the cytotoxic action of Shiga toxin [223-225]. In papers **I** and **II**, HeLa cells and CHO cells, respectively, were treated with Retro-2.1 to inhibit retrograde trafficking of Shiga toxin. Retro-2.1 was used in paper **I** to study the intracellular route of Shiga toxin 1B-subunit prior to release in extracellular vesicles. In paper **II,** Retro 2.1 was used to study if Shiga toxin 2 that was taken up by transfected Gb3-positive CHO cells, within extracellular vesicles, underwent retrograde transport to induce inhibition of protein synthesis.

Exogenous Gb3

In paper **II**, Gb3 was introduced in CHO cells and DLD-1 cells by adding liposomes containing Gb3, phosphatidylethanolamine and phosphatidylserine into the cell medium. Following exogenously inserted Gb3 into the plasma membrane the cytotoxicity of Shiga toxin 2 was studied.

PPMP

In papers **I** and **II** HeLa cells were treated with PPMP (D-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol) which is a glucosylceramide synthase inhibitor that reduces the expression of Gb3 in cells.

Stimulation with Shiga toxin and release of extracellular vesicles

In paper **I**, extracellular vesicles were obtained from HeLa cells stimulated with Shiga toxin 1B 10 ng/mL for 0-120 min. In paper **IV**, Shiga toxin 1 or 2 stimulation of HeLa cells, at a concentration of 200 ng/mL for 40 min, was described. In papers **II**, **III** and **IV**, blood cell-derived extracellular vesicles were obtained from whole blood stimulated with Shiga toxin. Freshly collected citrated blood was incubated with Shiga toxin 1 (paper **IV**) or Shiga toxin 2, both at 200 ng/mL for 40 min (papers **II**, **III** and **IV**).

Isolation of extracellular vesicles

Differential centrifugation (presented in Figure 10) was used for isolation of extracellular vesicles from whole blood and/or cell culture medium in papers **I**, **II**, **III** and **IV**. The protocol was initiated by a low-speed centrifugation step to discard cells. In papers I and IV, extracellular vesicles were isolated from HeLa cells. HeLa cells were pelleted at 800 g for 10 min. In papers II, III and IV, extracellular vesicles were isolated from blood cells that were pelleted at 1500 g for 15 min. The cell-free

supernatant was then collected and centrifuged at 10000 g for 10 min to pellet cell debris. To obtain a extracellular vesicle-enriched suspension, the supernatant was further centrifuged at 20000 g for 40 min whereafter the supernatant was discarded leaving 150 µl of extracellular vesicle-enriched suspension.

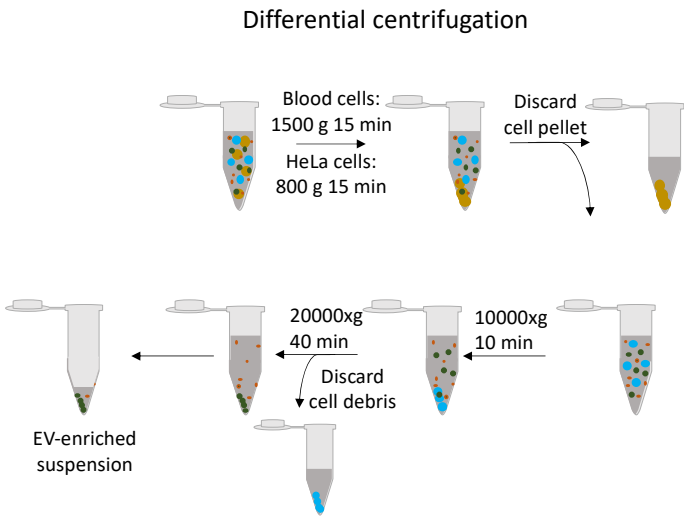

Figure 10: Schematic presentation of extracellular vesicle isolation by differential centrifugation. A low centrifugation g-force was applied to pellet blood cells or HeLa cells followed by a centrifugation step in which cell debris was discarded. A extracellular vesicle-enriched suspension containing extracellular vesicles in the size range of microvesicles were obtained by centrifugation at 20000 g. EV: extracellular vesicle. This figure was created using Biodraw, Motifolio.

Analysis of extracellular vesicles by flow cytometry

Flow cytometry was used in papers **I** and **IV** to analyze extracellular vesicles. In paper **I**, Shiga toxin 1B-subunit fluorescently labeled with Alexa-488 was incubated with HeLa cells to stimulate the release of extracellular vesicles. After isolation of extracellular vesicles they were labeled with anti-CD44 antibody conjugated to phycoerythrin (PE). CD44 is expressed on the surface of HeLa cells and was used to identify extracellular vesicles. The number of fluorescent extracellular vesicles were then detected in the flow cytometer. In paper **IV**, Shiga toxin 1 or 2-positive extracellular vesicles released from toxin-stimulated whole blood or cultured cells were detected by flow cytometry. To enable detection of Shiga toxin located within extracellular vesicles, the vesicles were fixed in 1% paraformaldehyde, washed and permeabilized with 0.1% saponin to allow the anti-Shiga toxin antibody to gain access to the vesicle. Following washing, a secondary fluorescent antibody was added and detection was performed in a CyFlow Cube 8 flow cytometer.

Microscopy

The various types of microscopy used in this thesis includes structure illumination microscopy (papers **I**, **II** and **IV**), confocal microscopy (papers **I** and **IV**), conventional fluorescent microscopy (paper **II**) and transmission electron microscopy (papers **I** and **IV**). Live cell imaging of shed vesicles from the membrane of cells were studied in paper **I**. Confocal microscopy is an adequate microscopy technique to visualize the cell membrane because it is possible to control the field depth of the cell and it provides a high spatial resolution compared to conventional wide-field microscopy. Structure illumination microscopy offers high resolution imaging of live cells and vesicles released from cells. In Paper **II**, z-stack images of cells by structure illumination microscopy were used to provide information at variable depths within the cell which can reveal intracellular uptake of extracellular vesicles. Images taken by structure illumination microscopy compared to conventional microscopy are shown in Figure 11.

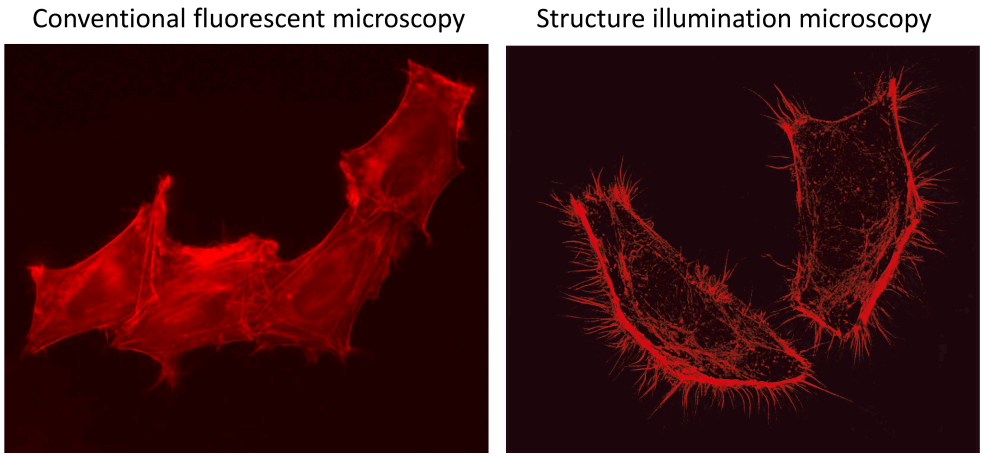

Figure 11: A comparison between conventional fluorescent microscopy (CFM) and structure illumination microscopy (SIM). HeLa cells with fluorescent actin were imaged by CFM and SIM. SIM gave a higher resolution image of actin compared to CFM.

Transmission electron microscopy was used in paper **I** to visualize Shiga toxin 1B-subunit on or within extracellular vesicles and in paper **IV** to visualize uptake of Shiga toxin 2-containing extracellular vesicles by cultured conditionally immortalized glomerular endothelial cells. In paper **I**, Shiga toxin 1B was conjugated to 1.4 nm nanogold particles before incubation with extracellular vesicles. The vesicles were then pelleted onto an agarose gel and embedded in Polybed to enable thin sectioning of the vesicle pellet. Prior to imaging, the sections were treated with gold enhancement in order to enable visualization of the nanogold conjugated to Shiga toxin 1B. In paper **IV,** glomerular endothelial cells that had been incubated with Shiga toxin 2-containing microvesicles were pelleted, fixed,

embedded and sectioned. Shiga toxin 2 was detected in glomerular endothelial cells by labeling Shiga toxin 2 with an anti-Shiga toxin 2 antibody followed by addition of a secondary antibody that detected the Shiga toxin antibody which was conjugated to a 5 nm gold particle.

Enzyme-linked immunosorbent assay (ELISA)

In papers **I, II** and **IV** in-house ELISAs were used to measure the concentration of Shiga toxin 1B (biotinylated), Shiga toxin 1 or Shiga toxin 2 in extracellular vesicles. In paper **III,** a commercially available IL-8 ELISA (R&D) was used to detect IL-8 released by cells during incubation with Shiga toxin 2-induced extracellular vesicles from whole blood.

Thin layer chromatography

In papers **I** and **II** thin layer chromatography (TLC) was used to determine the glycosphingolipid content of cells and extracellular vesicles. Lipids from cells or extracellular vesicles were solubilized by adding methanol followed by sonication of the samples to ensure disruption of the plasma membrane. This procedure was followed by mild saponification, a process of ester bond cleavage between glycerol and fatty acids of triglycerides resulting in generation of free fatty acids and glycerol (soluble in water). To remove proteins and nucleic acids in the samples a set mixture of methanol-chloroform-water was added. This procedure is called Folch partition and results in the generation of different layers that separate the water-soluble substances from the lipids. The lipid mixtures were further loaded onto a TLC plate consisting of a silica gel coated on aluminum foil, in the stationary phase. Then a solvent mixture, the mobile phase, was absorbed onto the plate by capillary force separating the lipids based on solubility and polarity. Glycosphingolipids separated on the gel were visualized by adding orcinol reagent. Alternatively, a Shiga toxin overlay was carried out by incubating the plate with Shiga toxin 2 followed by detection of Shiga toxin with an anti-Shiga toxin 2 antibody.

Nanoparticle tracking analysis

In papers **I**, **II** and **IV** nanoparticle tracking analysis was used to estimate the size range and concentration of all particles in samples containing extracellular vesicles. Nanoparticle tracking analysis is a quick and easy technique to determine size and concentration of submicrometer particles in a solution and can provide information about changes in e.g., vesicle release and uptake, when comparing different experimental conditions. A disadvantage with this analysis is that extracellular vesicles cannot be distinguished from non-extracellular vesicle particles.

Membrane translocation assay

A membrane translocation assay (illustrated in Figure 6A of paper **I**) was used to study if Shiga toxin 1B-subunit could translocate from the outside to the inside of

extracellular vesicles. In this assay, vesicles were incubated with biotinylated Shiga toxin 1B-subunit (Stx1B-SS-biotin) whereafter exposed biotin was cleaved off by a plasma membrane impermeable substance called sodium 2-mercaptoethane sulfonate (MESNA) which reduces the SS-bond between Shiga toxin 1B and biotin. The extracellular vesicles were then washed and lysed and the amount of Stx1B-SS-biotin was detected by ELISA thereby quantifying the amount of Stx1B that was internalized. In certain samples, extracellular vesicles were treated with methyl-β-cyclodextrin, which removes cholesterol from the membrane [226], or chlorpromazine, a well-known inhibitor of clathrin-dependent endocytosis [227], before incubation with Stx1B-SS-biotin.

Cell metabolism assay

In paper **II** a cellular metabolism assay was used to determine the cell viability after incubation with Shiga toxin 2 or extracellular vesicles with or without Shiga toxin 2. Viable cells were determined based on their metabolic activity using alamar blue. Alamar blue is the non-fluorescent oxidation-reduction indicator resazurin. When it is taken up be a viable cell, the reagent is reduced to resorufin, a highly fluorescent compound, due to the cellular metabolic reduction occurring in growing cells.

Protein synthesis assay

In paper **II**, protein synthesis was determined in cells after treatment with free Shiga toxin 2 or with Shiga toxin-containing extracellular vesicles. In this assay, the incorporation of a methionine analogue that binds a fluorescent dye was measured. The fluorescent signal from the cells correlates to the amount of synthesized proteins.

Tissue factor activity

Tissue factor activity of isolated extracellular vesicles were determined by a commercially available assay which measure the generation of FXa. By adding FVIIa, a tissue factor/FVIIa complex is formed that converts FX into FXa. This assay was used in paper **III**.

Procoagulant activity

The procoagulant activity of isolated extracellular vesicles were measured by a commercially available assay that uses the amount of exposed phosphatidylserine on the surface of the extracellular vesicles to assess thrombin generation. By adding coagulation factors Va and Xa to the vesicle samples, FVa/FXa complexes will be formed upon binding to phosphatidylserine that in the presence of calcium cleaves prothrombin into thrombin. This assay was used in paper **III**.

Platelet aggregometry

Platelet aggregates were measured in paper **III** to determine the ability of extracellular vesicles isolated from Shiga toxin 2-stimulated whole blood to induce aggregation. The light transmitted through a sample of platelet-rich-plasma was measured and compared with platelet-poor-plasma which corresponds to 100% light transmission. When platelet aggregation occurs, the sample will absorb less light resulting in more light being transmitted.

Measurement of cytokines in extracellular vesicles

Multiplex immunoassay was used in paper **III** to detect up to 27 cytokines in extracellular vesicles from Shiga toxin-stimulated or unstimulated whole blood. In this method a set of colored beads is used, each distinct bead coated with an antibody against one specific cytokine. The beads are incubated with the sample allowing cytokine binding to the beads. Then a second antibody towards the cytokines, labeled with a fluorescent reporter dye, is added. Analysis by flow cytometry enables detection of the colored beads in one channel and determination of the reporter dye in another channel in which the intensity of the reporter dye determines the concentration of the cytokine.

Liquid chromatography - Mass Spectrometry (LC-MS)

In paper **IV**, the protein content in isolated extracellular vesicles from Shiga toxin 2-stimulated or unstimulated blood cells was analyzed by liquid chromatography-mass spectrometry. In this assay compounds in a sample are separated based on their chemical properties by their interaction with the stationary and mobile phase. After separation, the compounds are inserted into the mass spectrometer which separates molecules based on their mass-to-charge ratio.

Results

Paper I

In this paper we investigated the mechanisms of Shiga toxin 1B uptake and release within extracellular vesicles and Shiga toxin 1B sequestration in extracellular vesicles.

Results

HeLa cells and red blood cells were shown to release Shiga toxin 1B-positive extracellular vesicles within minutes after stimulation with Shiga toxin 1B, detected by live cell imaging. This indicates that Shiga toxin 1B is quickly released within extracellular vesicles from cells after toxin binding. Flow cytometry measurement of Shiga toxin 1B-containing extracellular vesicles revealed that HeLa cells released

extracellular vesicles continuously for up to 40 min whereafter the levels decreased. In contrast, HeLa cells treated with Retro-2.1 continued to release toxin-positive extracellular vesicles even after 40 min. Extracellular vesicles released by HeLa cells were shown to contain Shiga toxin 1B-subunit both on the inside and on the outside of the vesicles.

HeLa cell-derived extracellular vesicles were shown to contain Gb3 indicating their ability to bind toxin to their outer membrane. Shiga toxin 1B was shown to bind directly to extracellular vesicles in the absence of cells and extracellular vesicles from PPMP-treated HeLa cells exhibited significantly lower Shiga toxin 1B binding. Electron microscopy of sectioned blood cell-derived extracellular vesicles that had been incubated with Shiga toxin 1B conjugated with 1.4 nm nanogold (in the absence of cells) revealed that Shiga toxin 1B was located on the membrane and within the vesicles. This result indicated that Shiga toxin 1B could be sequestered within extracellular vesicles. The ability of Shiga toxin 1B to translocate from the membrane surface to the inside of extracellular vesicles was investigated by a translocation assay showing that a fraction of the Shiga toxin 1B was translocated to the inside of extracellular vesicles, a process that was enhanced by treating the vesicles with methyl-β-cyclodextrin or chlorpromazine indicating that the translocation was affected by extracting cholesterol or by making the membrane more permeable, respectively.

The results of this study suggest that Shiga toxin 1B is released by cells, in extracellular vesicles, within minutes after binding to HeLa cells or red blood cells and that the release is continuous without the toxin undergoing retrograde transport. The results also showed that Shiga toxin 1B can bind to released extracellular vesicles, in the absence of cells, in a Gb3-dependent manner and that the toxin could translocate from the outside to the inside of the vesicle membrane, a novel mechanism for bacterial toxin to be sequestered in host cell-derived vesicles.

Paper II

The Gb3 receptor is important for Shiga toxin-mediated uptake and toxicity [228]. In a previous publication from our group using the EHEC-infected mouse model Shiga toxin 2 was demonstrated in mouse glomerular endothelial cells within blood cell-derived extracellular vesicles [17]. This observation was of particular interest because murine glomerular endothelial cells lack the Gb3 toxin receptor [229,196] and suggested that the toxin could possibly affect cells that lack Gb3 when taken up within extracellular vesicles. This aspect was addressed in this study. We investigated if Shiga toxin 2-containing extracellular vesicles, that were Gb3-positive, could interact with and induce cytotoxicity in a variety of Gb3-negative cells. The intracellular route of Shiga toxin 2 delivered by extracellular vesicles was also studied.

Results

Blood cell-derived extracellular vesicles containing Shiga toxin 2 were highly cytotoxic in CHO cells that had been transfected with Gb3 synthase as determined by the cells' metabolic activity and inhibition of protein synthesis. These extracellular vesicles were demonstrated to be taken up by Gb3-negative CHO cells or Gb3-negative DLD-1 intestinal cells, however, without inducing cytotoxicity. Similar results were obtained using PPMP-treated HeLa cells, with reduced Gb3 expression, in which blood cell-derived toxin-containing extracellular vesicles did not induce inhibition of protein synthesis compared to untreated HeLa cells. In Gb3-synthase transfected CHO cells, the retrograde transport inhibitor Retro-2.1 protected the cells from the cytotoxic effect of Shiga toxin 2 which was delivered within extracellular vesicles.

DLD-1 cells and CHO cells, both lacking the Gb3 toxin receptor, that were incubated with exogenous Gb3 in liposomes were shown to bind free Shiga toxin 2, however, without an effect on cell metabolism. For this reason, experiments with toxin-positive extracellular vesicles were not carried out.

The results of this paper suggest that Shiga toxin 2 can be taken up by cells within extracellular vesicles regardless of if the cell expresses Gb3 or not. However, in order for Shiga toxin 2 introduced within extracellular vesicles to induce cytotoxicity in the cells, endogenous expression of Gb3 in the recipient cells is a prerequisite.

Paper III

Shiga toxin 2 induces release of extracellular vesicles that exhibit pro-thrombotic potential by exposing phosphatidylserine and tissue factor [7]. In paper III we investigated the pro-thrombotic and pro-inflammatory properties of Shiga toxin 2-positive vesicles released from blood cells that could thereby contribute to the pathogenesis of Shiga toxin-mediated disease.

Results

Extracellular vesicles released upon Shiga toxin 2 stimulation of whole blood were shown to possess tissue factor activity as they converted coagulation FX into FXa. The vesicles also exhibited enhanced thrombin generation capacity by increased exposure of phosphatidylserine which creates a binding site for FVa and FXa. FVa/FXa complexes cleave prothrombin into thrombin. These vesicles induced platelet aggregation, which extracellular vesicles released from unstimulated blood did not. This indicates that extracellular vesicles released from blood stimulated with Shiga toxin 2 can trigger thrombosis.

Extracellular vesicles released from whole blood stimulated with Shiga toxin 2 exhibited pro-inflammatory attributes. The vesicles induced release of IL-8 from

endothelial cells (EA.hy 926 cells) co-incubated with monocytes (THP-1 cells). The release of IL-8 was not demonstrated when the cells were incubated with each cell separately. Four proinflammatory cytokines: eotaxin, IL-9, MIP-1β and RANTES, were significantly increased in extracellular vesicles released from whole blood stimulated with Shiga toxin 2, compared to vesicles in unstimulated samples.

The results suggest that Shiga toxin 2 induced release of extracellular vesicles from blood cells that attained prothrombotic and proinflammatory properties. These extracellular vesicles supported thrombin generation and induced platelet aggregation. They also promoted the release of IL-8 from endothelial cells co-incubated with monocytes and contained elevated levels of proinflammatory cytokines.

Paper IV

In this paper various methods were used to isolate and characterize extracellular vesicles released after stimulation of HeLa cells or whole blood with Shiga toxin 1 or 2. These methods can specifically be used for purification of vesicles such as differential centrifugation, their quantification such as flow cytometry and nanoparticle tracking analysis and their imaging such as confocal microscopy, structure illumination microscopy and transmission electron microscopy. Furthermore, methodology for the characterization of their content was described including Shiga toxin 1 and 2 ELISAs, and proteomics analysis.

Discussion

Extracellular vesicles play an important role in intercellular communication. They transfer components from their cell of origin that can phenotypically alter the recipient cells and affect normal physiological processes [52,96]. They can also function as a disposal mechanism by which cells release harmful substances. Their capability to concentrate cellular contents, during shedding of the vesicle from the cell [42], can contribute to their role in pathophysiology and the development of disease. Extracellular vesicles play a major role in the pathogenesis of EHEC-associated HUS. In this thesis I focused on extracellular vesicles induced by stimulation of cells with Shiga toxin, their mechanism of release and uptake in cells as well as the mechanism by which Shiga toxin interacts with vesicles. The thesis provides insight into the properties of these vesicles that may promote disease, and describes methodology used for the detection of vesicles and their content.

Upon incubation with cells, Shiga toxin is taken up by Gb3-mediated endocytosis and the toxin can be packaged into and shed within extracellular vesicles in minutes,

as demonstrated in **Paper I**. The cells, probably in an attempt to rid themselves of toxin, release toxin within vesicles. The toxin would otherwise undergo retrograde transport to the trans-Golgi network followed by the ER and from there to the cytosolic ribosomes where it inhibits protein synthesis and induces cell death. Instead, it is immediately shed within vesicles. Shedding of toxin in vesicles was increased in the presence of Retro 2.1, a substance that inhibits retrograde transport of Shiga toxin [225]. The release of toxin within vesicles is presumably crucial for the survival of the cells but this process can be harmful for the organism as a whole. Furthermore, we could show that extracellular vesicles possess the Shiga toxin Gb3 receptor allowing toxin binding, and that a fraction of the toxin is then taken up from the exterior of the vesicle. These two processes, shedding from toxin-simulated cells and direct binding to and sequestration in vesicles, allow the toxin to circulate within vesicles in the bloodstream and thereby be protected from the immune response. Within plasma the effects of Shiga toxin 2 can be partially inhibited [230], possibly by an interaction with the plasma protein amyloid P [231]. Shiga toxin sequestration within extracellular vesicles may enable the toxin to escape this inhibition and maintain its toxicity.

When Shiga toxin-positive vesicles interact with recipient cells the vesicles are taken up into early endosomes and thereafter release their toxic content. The toxin can then undergo retrograde transport and harm the recipient cell [17]. Several questions arise from these findings. It is unclear why some cells shed the toxin in vesicles while others allow the toxin to undergo retrograde transport that will ultimately destroy the cell. Which cellular protective mechanisms determine whether the toxin will be shed in vesicles or not? Also, it is unclear why certain cells will take up the toxin within extracellular vesicles, such as renal cells, while others do not, as the mechanism of tissue tropism could explain why the kidneys are specifically affected during EHEC infection.

The presence of Gb3 on cells is essential for Shiga toxin-mediated toxicity. Mice deficient in Gb3 [229] or cells that lack Gb3 [232] remain unaffected by the toxin's inhibitory effect on protein synthesis. Shiga toxin binds to Gb3 located within lipid rafts and is thereby taken up by cells and exerts cytotoxic effects [233]. In bovine intestinal epithelial cells, where the Gb3 is not localized in lipid rafts, these effects could not be demonstrated [234]**.** However, toxin can be visualized within Gb3-negative cells, as shown for T84 intestinal cells that are Gb3-negative [170], in which uptake by macropinocytosis was suggested**.**

Our group has previously shown that Gb3-negative cells can take up Shiga toxin 2 within extracellular vesicles. This was demonstrated in a mouse model of EHEC infection in which toxin-positive vesicles were detected in murine glomerular endothelial cells [17] that have been shown to be Gb3-negative [196]. This finding prompted the study presented in **Paper II** in which the significance of endogenous

Gb3 in cells for the uptake of toxin-positive vesicles and exertion of toxicity was investigated. The findings suggest that recipient cells can take up toxin-positive vesicles regardless of Gb3 expression, but for the toxic effect to occur the Gb3 receptor needs to be expressed by the recipient cell. Shiga toxin enters the cell by receptor-mediated endocytosis bound to Gb3. Gb3 is required for retrograde transport [150,235,236]. The Gb3 can be taken up via extracellular vesicles, as we showed the vesicles were Gb3-positive. All the same, the Gb3 in the vesicle membrane is insufficient to target the toxin towards retrograde transport and raises the question of if the Gb3 in vesicles is localized within lipid rafts and if the toxin follows a predestined route determined by the cell membrane composition, specifically detergent-resistant regions, that may differ from the composition of the vesicle membrane. Another remarkable aspect previously shown was that toxin-positive extracellular vesicles within Gb3-negative murine glomerular endothelial cells did not always release their content. Some vesicles transferred through the cells to the basement membrane and from there towards podocytes [17] suggesting that components of the recipient cell may signal to the vesicle to empty its contents. This aspect remains to be investigated.

Extracellular vesicles contribute to the development of EHEC-associated HUS by transporting the toxin to the kidney [17]. Our group has previously reported that circulating extracellular vesicles during this condition have deposits of complement C3 and C9 [103,222], tissue factor and phosphatidylserine [7]. They are also released from red blood cells during hemolysis exhibiting complement deposits and thereby indicate that complement may partake in the hemolytic process [102]. In **Paper III** we further investigated the role of blood cell-derived extracellular vesicles in this condition by investigating the prothrombotic and proinflammatory potential of the vesicles released after incubation of whole blood with Shiga toxin 2. The released vesicles possess excess tissue factor and thrombin-generating activity as well the capacity to induce platelet aggregation. This can explain how vesicles reaching the kidney can induce thrombotic microangiopathy. As described in the paper, we used a given volume of extracellular vesicles in suspension and thus the amount of vesicles in the Shiga toxin 2-stimulated sample would presumably be higher. Therefore, we cannot determine if each individual vesicle possesses more prothrombotic potential or if this is due to an increased number of vesicles. Nonetheless, the effect can contribute to the pathogenesis of capillary occlusion due to thrombi, hemolysis and endothelial damage in the kidney.

We further investigated the pro-inflammatory potential of blood cell-derived extracellular vesicles. We found that the vesicles released after stimulation of blood cells with Shiga toxin 2 possessed a proinflammatory cytokine profile including elevated levels of eotaxin, RANTES, MIP-1ß, and IL-9. In the presence of endothelial cells and THP-1 monocytes we demonstrated increased IL-8 release. IL-8 has been demonstrated in children with HUS and correlated to the severity of

disease [169,237]. It is plausible that circulating blood cell-derived vesicles contribute to the release of IL-8 from endothelial cells and monocytes [115] and that the released IL-8 would promote inflammation by recruiting inflammatory cells. Interestingly, incubation of the extracellular vesicles released from Shiga toxin-stimulated blood cells with endothelial cells alone or monocytic cells alone was insufficient to induce IL-8 release. This may be related to the quantity and concentration of vesicles which may differ from the *in vivo* setting.

Taken together, blood cell-derived extracellular vesicles seem to play a crucial role in the development of all aspects of HUS and particularly thrombotic microangiopathy. The delivery of toxin to its target organ, the kidney, and exertion of thrombotic and inflammatory effects at the site of injury, can lead to endothelial cell injury, platelet aggregation and thrombocytopenia due to consumption of platelets in microthrombi as well as neutrophil recruitment with release of harmful proteases and proinflammatory cytokines. Microthrombi occlude glomerular capillaries leading to ischemic damage, and the toxin, delivered in vesicles, has a direct cytotoxic effect on glomerular and tubular cells [17,151,212]. These effects combined with inflammation will lead to renal failure.

The importance of extracellular vesicles for the development of this condition led us to **Paper IV** which describes the various methods used for detection, quantification, and determination of vesicle content. Of note, freeze-thawing affected the localization of Shiga toxin in vesicles, which is important to consider. Following our group's studies on this topic other groups have shown that patients have elevated extracellular vesicles during HUS [221,238]. It is therefore important to determine the appropriate methodology for detection of extracellular vesicles. The international society for extracellular vesicles (ISEV) has updated its recommendations and protocols in 2018 [3] for a more uniform use of extracellular vesicles in research.

Ultimately, the goal of these studies will be to find an appropriate treatment for HUS. Patients are currently treated by supportive care and careful hydration during the prodromal phase of diarrhea and once renal failure, thrombocytopenia and hemolytic anemia develop. There is no specific treatment for HUS [239]. Antibiotic therapy during the prodromal phase may worsen the outcome, as more toxin can be released from the bacteria [240]. Once HUS has developed antibiotics may be beneficial in some cases [241], but this finding is uncertain and requires more investigation. As extracellular vesicles seem to be involved in most aspects of disease blocking their release or uptake may have beneficial effects. Blocking their release may, however, have detrimental effects as vesicle shedding rids cells of injurious substances. Blocking their uptake may therefore be more promising and could temporarily inhibit toxin uptake by the kidney. In line with this, a recent study from our group showed that annexin A5, that binds phosphatidylserine on the

vesicle membrane, induced excess phagocytosis of extracellular vesicles and delayed the onset of disease in EHEC-infected mice [242]. Plasma exchange would remove extracellular vesicles from the circulation. This treatment was effective in a few cases of HUS [243] but is generally not beneficial or recommended [241], most probably because by the time the patient presents renal failure is already fulminant. Thus, the aspect of whether blocking or removing extracellular vesicles during HUS is effective remains to be determined.

One unexpected finding in this thesis was that Shiga toxin bound to the surface of the vesicle membrane could translocate to the inside of the vesicle as shown in **Paper I**. To my knowledge the transfer of a naturally occurring protein from a vesicle surface to its inside has not been described before. Extensive studies are focused on bioengineering extracellular vesicles as drug delivery systems. For these purposes small molecules, drug substances and small interfering RNAs (siRNAs) are used [244-246]. Various methods have been described for drug loading into vesicles, including preloading in which the content is incorporated into cells that shed vesicles or post-loading in which drugs either integrate into the vesicle membrane or are incorporated by pore formation or permeabilization of the membrane. Cholesterol-conjugated siRNAs were also described as a therapeutic option [244]. These therapeutic systems may prove very promising. Shiga toxin induces apoptosis and was suggested as a treatment for certain types of cancer such as astrocytoma [247,248]. Gb3 is elevated in various types of cancer such as pancreatic, ovarian, breast, testicular, renal, among others, and Shiga toxin has been shown to effectively target cancer cells [249]. The finding that Shiga toxin could translocate naturally into extracellular vesicles could be utilized to target vesicles towards specific cancer cells.

In this thesis Shiga toxin 1 B subunit, Shiga toxin 1 and Shiga toxin 2 were used. Shiga toxin 1 B subunit was used to study the binding interaction of Shiga toxin 1 without the enzymatic cytotoxic effect of the A subunit. We had access to various conjugates of the B subunit of Shiga toxin 1 (**Paper I** Table 1) which we did not have for Shiga toxin 2. There may be important differences between Shiga toxin 1 and 2, for example in their interaction with the glycolipid receptor [138,250]. Shiga toxin 2 is strongly associated with clinical isolates of EHEC that cause HUS [251], whereas Shiga toxin 1 is not. All the same, Shiga toxin 1 exhibits a similar mode of action *in vitro* in cell models.

An interesting aspect that was found in all studies included herein is the donor dependent response to Shiga toxin. Blood from different donors responded differently to Shiga toxin as demonstrated by release of extracellular vesicles. It is unclear why some donors release an excess number of extracellular vesicles when their blood is stimulated with Shiga toxin whereas others do not and if this finding would correlate with increased clearance of Shiga toxin from the bloodstream of

such individuals. One study has shown that children with P1 antigen expression on red blood cells were protected against HUS compared to P1-negative children. The P1 antigen could promote binding of Shiga toxin to red blood cells which may thereby clear the toxin from the circulation [252].

As described in the methods a variety of cells were used in the studies included in this thesis. Some of the cells were chosen because of their explicit susceptibility to the cytotoxic effects of Shiga toxin, such as HeLa cells [152]. These cells have also been extensively studied regarding the toxin's retrograde transport [253]. Other cells were chosen because they naturally lack Gb3 (CHO cells and DLD-1 cells). Two types of endothelial cells were used, EA.hy 926 a cultured HUVEC cell line as well as immortalized glomerular endothelial cells, the latter to study the effects of the toxin in its relevant clinical setting. For the same purpose studies were carried out using blood cells, red blood cells and platelets, as well as extracellular vesicles released from toxin-stimulated whole blood. *In vitro* cell models cannot mimic all aspects of a clinical disease but the cells used were deemed suitable for the experimental settings described.

In summary, this thesis investigated the importance of extracellular vesicles in EHEC-associated HUS and could show that vesicles containing toxin are released from affected cells shortly after stimulation, the toxin can also bind to the surface of vesicles and be internalized and thereby circulate in the bloodstream. Toxin within vesicles is harmful to cells when the vesicles are taken up and release their cargo. For this to occur the recipient cell must possess the toxin's Gb3 receptor. The vesicles released from blood cells upon Shiga toxin stimulation are both prothrombotic and pro-inflammatory. Thus, extracellular vesicles released by Shiga toxin stimulation possess detrimental properties and should be targeted as a therapeutic strategy.

Conclusions

- Shiga toxin can be associated with extracellular vesicles by two separate mechanisms. Shiga toxin can either bind to cells followed by rapid release of toxin-containing extracellular vesicles without undergoing retrograde transport. Alternatively, Shiga toxin can bind directly to extracellular vesicles even in the absence of cells and translocate from the outside to the inside of the vesicle. The latter mechanism would allow its sequestration within the host membrane and thereby evasion of an immune response during its transfer within the circulation.

- Shiga toxin 2 released in extracellular vesicles can be taken up by Gb3-negative and Gb3-positive cells but is only toxic to cells that endogenously express Gb3. Shiga toxin 2 delivered to Gb3-positive cells by extracellular vesicles retains its toxicity and undergoes retrograde transport thereby inducing a cytotoxic effect within the cell.

- Shiga toxin 2 induces the release of pro-thrombotic and pro-inflammatory extracellular vesicles from blood cells.

Populärvetenskaplig sammanfattning på svenska

Enterohemorragisk *Escherichia coli* (EHEC) är en bakterie som kan orsaka tarminfektion hos människor. EHEC är vanligt förekommande i tarmfloran hos framför allt nötkreatur och kan spridas till oss människor genom förtäring av förorenad mat och vatten. Efter intag av EHEC kan den färdas genom mag-tarmsystemet och slutligen fästa vid framför allt tjocktarmen. Här koloniseras bakterien och symtom kan uppstå efter ca 3–7 dagar som ofta innefattar buksmärtor och diarréer som ibland kan vara blodiga. I 10–15 % av fallen kan även den livshotande sjukdomen, hemolytisk uremisk syndrom (HUS), uppkomma. HUS kännetecknas av blodbrist, låga nivåer av blodplättar och akut njursvikt. EHEC är icke-invasiv, vilket innebär att den stannar kvar i tarmen och orsakar sjukdom genom att frisätta giftiga ämnen som kallas för bakterietoxiner. Dessa kan komma in i blodbanan och orsaka sjukdom. Bakteriens främsta toxin heter Shigatoxin (Stx).

Stx binder till en specifik receptor, globotriasylceramid (Gb3), som finns på vissa av kroppens celler. När Stx binder till receptorn tas den upp av cellen och kan därefter genomgå en väldigt specifik intracellulär transport. Under transporten förändras toxinets struktur som resulterar i att Stx kan förstöra cellens maskineri för att producera proteiner. I och med att maskineriet är nödvändigt för en cells överlevnad kommer detta i sin tur leda till att cellen dör.

När Stx cirkulerar i blodbanan binder det till cellerna i blodet och tas upp, dock utan att orsaka celldöd i dessa celler. Istället aktiveras cellerna och börjar frisläppa membranblåsor från cellmembranet som kallas för mikrovesikler. Mikrovesikler har många viktiga funktioner i kroppen, bland annat genom att förmedla biologiskt material mellan olika celler som ett sätt att kommunicera. Vår forskargrupp har tidigare visat att Stx cirkulerar i blodbanan inuti mikrovesikler från blodceller och att detta används som ett transportsätt för toxinet att nå och tas upp av cellerna i njuren som i sin tur leder till celldöd i njurcellerna.

I det första delarbetet studerades hur Stx kommer in i mikrovesikler. Dels undersöktes hur snabbt Stx frisläpptes av celler, inuti mikrovesikler, och om det krävdes en specifik intracellulär transport av toxinet innan den frisläpptes. I detta arbete undersöktes även om Stx kan binda till fria mikrovesikler, utan närvaro av en cell. Vi observerade att Stx släpps ut, redan efter 5 min, i vesikler efter cellulärt

upptag. En annan intressant observation var att Stx kan binda till redan fria mikrovesikler och omplaceras från att vara bunden på utsidan av vesiklen, till att befinna sig på insidan. Vi tror att detta kan vara ett sätt för Stx att gömma sig från kroppens försvarssystem och på så sätt kunna transporteras gömd inuti mikrovesikler till njuren där den sedan kan orsaka skada.

I det andra delarbetet studerades om Stx, som har blivit frisläppt i mikrovesikler, även kan vara giftigt för celler som saknar toxinreceptorn. I normala fall är toxinreceptorn helt avgörande för Stx att kunna tas upp av cellen och orsaka celldöd men är detta även fallet för Stx som finns i mikrovesikler? I detta projekt skapades celler med och utan Gb3 och när Stx tillsattes till dessa celler orsakade toxinet endast celldöd i cellerna med Gb3. När mikrovesikler innehållande Stx istället tillsattes till cellerna kunde vi observera att dessa togs upp av celler oberoende om cellerna innehöll Gb3 eller inte. Dock var inte toxinet giftigt för cellerna som inte hade Gb3. Detta innebär att mikrovesikler som innehåller Stx kan tas upp av celler men att toxinet bara kan utföra sin skadliga handling om cellen innehåller Gb3.

I det tredje delarbetet utreddes om Stx kan stimulera celler i blodet att frisläppa mikrovesikler med ett specifikt innehåll som gynnar uppkomsten av inflammation och bildning av blodproppar. Vi fann att mikrovesikler som frisläpptes från blodceller i närvaro av Stx innehöll ökad mängd av inflammationsframkallande substanser jämför med mikrovesikler från ostimulerade celler. Vi kunde också mäta en ökad nivå av vissa komponenter som gynnar uppkomsten av blodproppar. I detta delarbete visar vi att Stx gynnar frisläppning av mikrovesikler innehållande substanser som kan bidra till ett förvärrat sjukdomstillstånd.

I det fjärde arbetet beskrivs det i detalj olika metoder för framrening och karakterisering av Stx-associerade mikrovesikler. Arbetet innehåller flera metoder som har använts i ovanstående delarbeten och som har utvecklats för att rena fram mikrovesikler från blod och cellkulturer. Det beskriver även flera metoder för att mäta Stx inuti mikrovesikler samt hur antalet, storleken och innehållet på de framrenade mikrovesiklerna kan analyseras.

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References

1. Colombo M, Raposo G, Thery C (2014) Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol, 30: 255-289.

2. Alberro A, Iparraguirre L, Fernandes A, Otaegui D (2021) Extracellular vesicles in blood: sources, effects, and applications. Int J Mol Sci, 22.

3. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R *et al.* (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles, 7: 1535750.

4. Raposo G, Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol, 200: 373-383.

5. Yáñez-Mó M, Siljander PR, Andreu Z, Zavec AB, Borràs FE, Buzas EI *et al.* (2015) Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles, 4: 27066.

6. Johansson KE, Ståhl AL, Arvidsson I, Loos S, Tontanahal A, Rebetz J *et al.* (2019) Shiga toxin signals via ATP and its effect is blocked by purinergic receptor antagonism. Sci Rep, 9: 14362.

7. Ståhl AL, Sartz L, Nelsson A, Békássy ZD, Karpman D (2009) Shiga toxin and lipopolysaccharide induce platelet-leukocyte aggregates and tissue factor release, a thrombotic mechanism in hemolytic uremic syndrome. PLoS One, 4: e6990.

8. Youn YJ, Shrestha S, Lee YB, Kim JK, Lee JH, Hur K *et al.* (2021) Neutrophil-derived trail is a proinflammatory subtype of neutrophil-derived extracellular vesicles. Theranostics, 11: 2770-2787.

9. Boerlin P, McEwen SA, Boerlin-Petzold F, Wilson JB, Johnson RP, Gyles CL (1999) Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. J Clin Microbiol, 37: 497-503.

10. Lingwood CA, Law H, Richardson S, Petric M, Brunton JL, De Grandis S *et al.* (1987) Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin *in vitro*. J Biol Chem, 262: 8834-8839.

11. Sandvig K, Skotland T, van Deurs B, Klokk TI (2013) Retrograde transport of protein toxins through the Golgi apparatus. Histochem Cell Biol, 140: 317-326.

12. Sandvig K, Garred O, Prydz K, Kozlov JV, Hansen SH, van Deurs B (1992) Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature, 358: 510-512.

13. Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K (1988) Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur J Biochem, 171: 45-50.

14. Tesh VL (2010) Induction of apoptosis by Shiga toxins. Future Microbiol, 5: 431-453.

15. Desch K, Motto D (2007) Is there a shared pathophysiology for thrombotic thrombocytopenic purpura and hemolytic-uremic syndrome? J Am Soc Nephrol, 18: 2457-2460.

16. Karpman D, Ståhl AL (2014) Enterohemorrhagic *Escherichia coli* Pathogenesis and the Host Response. Microbiol Spectr, 2.

17. Ståhl AL, Arvidsson I, Johansson KE, Chromek M, Rebetz J, Loos S *et al.* (2015) A novel mechanism of bacterial toxin transfer within host blood cell-derived microvesicles. PLoS Pathog, 11: e1004619.

18. Falguières T, Mallard F, Baron C, Hanau D, Lingwood C, Goud B *et al.* (2001) Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes. Mol Biol Cell, 12: 2453-2468.

19. Villysson A, Tontanahal A, Karpman D (2017) Microvesicle involvement in Shiga toxin-associated infection. Toxins 9.

20. Pan BT, Johnstone RM (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*: selective externalization of the receptor. Cell, 33: 967-978.

21. Buzas EI, György B, Nagy G, Falus A, Gay S (2014) Emerging role of extracellular vesicles in inflammatory diseases. Nat Rev Rheumatol, 10: 356-364.

22. Schorey JS, Harding CV (2016) Extracellular vesicles and infectious diseases: new complexity to an old story. J Clin Invest, 126: 1181-1189.

23. Chang WH, Cerione RA, Antonyak MA (2021) Extracellular vesicles and their roles in cancer progression. Methods Mol Biol, 2174: 143-170.

24. Hessvik NP, Llorente A (2018) Current knowledge on exosome biogenesis and release. Cell Mol Life Sci, 75: 193-208.

25. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol, 35: 495-516.

26. Bretscher MS (1972) Asymmetrical lipid bilayer structure for biological membranes. Nature New Biol, 236: 11-12.

27. Daleke DL (2003) Regulation of transbilayer plasma membrane phospholipid asymmetry. J Lipid Res, 44: 233-242.

28. Tang X, Halleck MS, Schlegel RA, Williamson P (1996) A subfamily of P-type ATPases with aminophospholipid transporting activity. Science, 272: 1495-1497.

29. Hamon Y, Broccardo C, Chambenoit O, Luciani MF, Toti F, Chaslin S *et al.* (2000) ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. Nat Cell Biol, 2: 399-406.

30. Yang H, Kim A, David T, Palmer D, Jin T, Tien J *et al.* (2012) TMEM16F forms a Ca2+-activated cation channel required for lipid scrambling in platelets during blood coagulation. Cell, 151: 111-122.

31. Wehman AM, Poggioli C, Schweinsberg P, Grant BD, Nance J (2011) The P4-ATPase TAT-5 inhibits the budding of extracellular vesicles in *C. elegans* embryos. Curr Biol, 21: 1951-1959.

32. Farsad K, De Camilli P (2003) Mechanisms of membrane deformation. Curr Opin Cell Biol, 15: 372-381.

33. Fox JE, Austin CD, Reynolds CC, Steffen PK (1991) Evidence that agonist-induced activation of calpain causes the shedding of procoagulant-containing microvesicles from the membrane of aggregating platelets. J Biol Chem, 266: 13289-13295.

34. Cocucci E, Racchetti G, Meldolesi J (2009) Shedding microvesicles: artefacts no more. Trends Cell Biol, 19: 43-51.

35. Alexy T, Rooney K, Weber M, Gray WD, Searles CD (2014) TNF-α alters the release and transfer of microparticle-encapsulated miRNAs from endothelial cells. Physiol Genomics, 46: 833-840.

36. Bernimoulin M, Waters EK, Foy M, Steele BM, Sullivan M, Falet H *et al.* (2009) Differential stimulation of monocytic cells results in distinct populations of microparticles. J Thromb Haemost, 7: 1019-1028.

37. Barnes BJ, Somerville CC (2020) Modulating cytokine production via select packaging and secretion from extracellular vesicles. Frontiers in immunology, 11: 1040.

38. Aatonen MT, Ohman T, Nyman TA, Laitinen S, Grönholm M, Siljander PR (2014) Isolation and characterization of platelet-derived extracellular vesicles. J Extracell Vesicles, 3.

39. Dozio V, Sanchez JC (2017) Characterisation of extracellular vesicle-subsets derived from brain endothelial cells and analysis of their protein cargo modulation after TNF exposure. J Extracell Vesicles, 6: 1302705.

40. Crescitelli R, Lässer C, Szabó TG, Kittel A, Eldh M, Dianzani I *et al.* (2013) Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J Extracell Vesicles, 2.

41. Sandvig K, Llorente A (2012) Proteomic analysis of microvesicles released by the human prostate cancer cell line PC-3. Mol Cell Proteomics, 11: M111.012914.

42. Muralidharan-Chari V, Clancy J, Plou C, Romao M, Chavrier P, Raposo G *et al.* (2009) ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. Curr Biol, 19: 1875-1885.

43. Oszvald Á, Szvicsek Z, Sándor GO, Kelemen A, Soós A, Pálóczi K *et al.* (2020) Extracellular vesicles transmit epithelial growth factor activity in the intestinal stem cell niche. Stem Cells, 38: 291-300.

44. Fitzgerald W, Freeman ML, Lederman MM, Vasilieva E, Romero R, Margolis L (2018) A system of cytokines encapsulated in extracellular vesicles. Sci Rep, 8: 8973.

45. Mause SF, von Hundelshausen P, Zernecke A, Koenen RR, Weber C (2005) Platelet microparticles: a transcellular delivery system for RANTES promoting monocyte recruitment on endothelium. Arterioscler Thromb Vasc Biol, 25: 1512-1518.

46. Kahn R, Mossberg M, Ståhl AL, Johansson K, Lopatko Lindman I, Heijl C *et al.* (2017) Microvesicle transfer of kinin B1-receptors is a novel inflammatory mechanism in vasculitis. Kidney Int, 91: 96-105.

47. Baj-Krzyworzeka M, Majka M, Pratico D, Ratajczak J, Vilaire G, Kijowski J *et al.* (2002) Platelet-derived microparticles stimulate proliferation, survival, adhesion, and chemotaxis of hematopoietic cells. Exp Hematol, 30: 450-459.

48. Zeng F, Morelli AE (2018) Extracellular vesicle-mediated MHC cross-dressing in immune homeostasis, transplantation, infectious diseases, and cancer. Semin Immunopathol, 40: 477-490.

49. Nawaz M, Shah N, Zanetti BR, Maugeri M, Silvestre RN, Fatima F *et al.* (2018) Extracellular vesicles and matrix remodeling enzymes: the emerging roles in extracellular matrix remodeling, progression of diseases and tissue Repair. Cells, 7.

50. Romero M, Keyel M, Shi G, Bhattacharjee P, Roth R, Heuser JE *et al.* (2017) Intrinsic repair protects cells from pore-forming toxins by microvesicle shedding. Cell Death Differ, 24: 798-808.

51. Kim KM, Abdelmohsen K, Mustapic M, Kapogiannis D, Gorospe M (2017) RNA in extracellular vesicles. Wiley Interdiscip Rev RNA, 8.

52. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol, 9: 654-659.

53. Weerheim AM, Kolb AM, Sturk A, Nieuwland R (2002) Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography. Anal Biochem, 302: 191-198.

54. Larson MC, Woodliff JE, Hillery CA, Kearl TJ, Zhao M (2012) Phosphatidylethanolamine is externalized at the surface of microparticles. Biochim Biophys Acta, 1821: 1501-1507.

55. Pollet H, Conrard L, Cloos AS, Tyteca D (2018) Plasma membrane lipid domains as platforms for vesicle biogenesis and shedding? Biomolecules, 8.

56. Asare-Werehene M, Nakka K, Reunov A, Chiu C-T, Lee W-T, Abedini MR *et al.* (2020) The exosome-mediated autocrine and paracrine actions of plasma gelsolin in ovarian cancer chemoresistance. Oncogene, 39: 1600-1616.

57. Rudt S, Müller RH (1993) *In vitro* phagocytosis assay of nano- and microparticles by chemiluminescence. III. Uptake of differently sized surface-modified particles, and its correlation to particle properties and *in vivo* distribution. Eu J Pharm Sci, 1: 31-39.

58. Costa Verdera H, Gitz-Francois JJ, Schiffelers RM, Vader P (2017) Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis. J Control Release, 266: 100-108.

59. Tian T, Zhu YL, Zhou YY, Liang GF, Wang YY, Hu FH *et al.* (2014) Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. J Biol Chem, 289: 22258-22267.

60. Nanbo A, Kawanishi E, Yoshida R, Yoshiyama H (2013) Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells. J Virol, 87: 10334-10347.

61. Mulcahy LA, Pink RC, Carter DR (2014) Routes and mechanisms of extracellular vesicle uptake. J Extracell Vesicles, 3.

62. Uribe-Querol E, Rosales C (2020) Phagocytosis: Our Current Understanding of a Universal Biological Process. Frontiers in immunology, 11: 1066.

63. Freeman SA, Grinstein S (2014) Phagocytosis: receptors, signal integration, and the cytoskeleton. Immunol Rev, 262: 193-215.

64. Segawa K, Nagata S (2015) An apoptotic 'eat me' signal: phosphatidylserine exposure. Trends Cell Biol, 25: 639-650.

65. Bloomfield G, Kay RR (2016) Uses and abuses of macropinocytosis. J Cell Sci, 129: 2697-2705.

66. Kaksonen M, Roux A (2018) Mechanisms of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol, 19: 313-326.

67. Anderson RG (1998) The caveolae membrane system. Annu Rev Biochem, 67: 199-225.

68. Otto GP, Nichols BJ (2011) The roles of flotillin microdomains--endocytosis and beyond. J Cell Sci, 124: 3933-3940.

69. Joshi BS, de Beer MA, Giepmans BNG, Zuhorn IS (2020) Endocytosis of extracellular vesicles and release of their cargo from endosomes. ACS Nano, 14: 4444-4455.

70. Jovic M, Sharma M, Rahajeng J, Caplan S (2010) The early endosome: a busy sorting station for proteins at the crossroads. Histol Histopathol, 25: 99-112.

71. Mellman I (1996) Endocytosis and molecular sorting. Annu Rev Cell Dev Biol, 12: 575-625.

72. Prada I, Meldolesi J (2016) Binding and fusion of extracellular vesicles to the plasma membrane of their cell targets. Int J Mol Sci, 17.

73. Maas SL, Breakefield XO, Weaver AM (2017) Extracellular vesicles: unique intercellular delivery vehicles. Trends Cell Biol, 27: 172-188.

74. Liangsupree T, Multia E, Riekkola ML (2021) Modern isolation and separation techniques for extracellular vesicles. J Chromatogr A, 1636: 461773.

75. Konoshenko MY, Lekchnov EA, Vlassov AV, Laktionov PP (2018) Isolation of extracellular vesicles: general methodologies and latest trends. Biomed Res Int, 2018: 8545347.

76. Skotland T, Sagini K, Sandvig K, Llorente A (2020) An emerging focus on lipids in extracellular vesicles. Adv Drug Deliv Rev, 159: 308-321.

77. Théry C, Amigorena S, Raposo G, Clayton A (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol, Chapter 3: Unit 3 22.

78. Cvjetkovic A, Lötvall J, Lässer C (2014) The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. J Extracell Vesicles, 3.

79. Momen-Heravi F, Balaj L, Alian S, Mantel PY, Halleck AE, Trachtenberg AJ *et al.* (2013) Current methods for the isolation of extracellular vesicles. Biol Chem, 394: 1253-1262.

80. Théry C, Amigorena S, Raposo G, Clayton A (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol, Chapter 3: Unit 3.22.

81. Baranyai T, Herczeg K, Onódi Z, Voszka I, Módos K, Marton N *et al.* (2015) Isolation of exosomes from blood plasma: qualitative and quantitative comparison of ultracentrifugation and size exclusion chromatography methods. PLoS One, 10: e0145686.

82. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK (2012) Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. Kidney Int, 82: 1024-1032.

83. Coumans FAW, Brisson AR, Buzas EI, Dignat-George F, Drees EEE, El-Andaloussi S *et al.* (2017) Methodological guidelines to study extracellular vesicles. Circ Res, 120: 1632-1648.

84. Gámez-Valero A, Monguió-Tortajada M, Carreras-Planella L, Franquesa M, Beyer K, Borràs FE (2016) Size-exclusion chromatography-based isolation minimally alters extracellular Vesicles' characteristics compared to precipitating agents. Sci Rep, 6: 33641.

85. Nakai W, Yoshida T, Diez D, Miyatake Y, Nishibu T, Imawaka N *et al.* (2016) A novel affinity-based method for the isolation of highly purified extracellular vesicles. Sci Rep, 6: 33935.

86. Dragovic RA, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJ, Hole P *et al.* (2011) Sizing and phenotyping of cellular vesicles using nanoparticle tracking analysis. Nanomedicine, 7: 780-788.

87. Xu R, Greening DW, Zhu HJ, Takahashi N, Simpson RJ (2016) Extracellular vesicle isolation and characterization: toward clinical application. J Clin Invest, 126: 1152-1162.

88. Nolan JP, Duggan E (2018) Analysis of individual extracellular vesicles by flow cytometry. Methods Mol Biol, 1678: 79-92.

89. Cizmar P, Yuana Y (2017) Detection and Characterization of Extracellular Vesicles by Transmission and Cryo-Transmission Electron Microscopy. Methods Mol Biol, 1660: 221-232.

90. Hartjes TA, Mytnyk S, Jenster GW, van Steijn V, van Royen ME (2019) Extracellular vesicle quantification and characterization: common methods and emerging approaches. Bioengineering 6: 6010007.

91. Serrano-Pertierra E, Oliveira-Rodríguez M, Matos M, Gutiérrez G, Moyano A, Salvador M *et al.* (2020) Extracellular vesicles: current analytical techniques for detection and quantification. Biomolecules, 10.

92. Ratajczak MZ, Ratajczak J (2016) Horizontal transfer of RNA and proteins between cells by extracellular microvesicles: 14 years later. Clin Transl Med, 5: 7.

93. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P *et al.* (2006) Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia, 20: 847-856.

94. Razmkhah F, Soleimani M, Mehrabani D, Karimi MH, Amini Kafi-Abad S, Ramzi M *et al.* (2017) Leukemia microvesicles affect healthy hematopoietic stem cells. Tumour Biol, 39: 1010428317692234.

95. Feng YH, Tsao CJ (2016) Emerging role of microRNA-21 in cancer. Biomed Rep, 5: 395-402.

96. Salanova B, Choi M, Rolle S, Wellner M, Luft FC, Kettritz R (2007) Beta2-integrins and acquired glycoprotein IIb/IIIa (GPIIb/IIIa) receptors cooperate in NF-kappaB activation of human neutrophils. J Biol Chem, 282: 27960-27969.

97. Mack M, Kleinschmidt A, Brühl H, Klier C, Nelson PJ, Cihak J *et al.* (2000) Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. Nat Med, 6: 769-775.

98. Rozmyslowicz T, Majka M, Kijowski J, Murphy SL, Conover DO, Poncz M *et al.* (2003) Platelet- and megakaryocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV. Aids, 17: 33-42.

99. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A *et al.* (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol, 10: 619-624.

100. Liu T, Zhang L, Joo D, Sun SC (2017) NF-κB signaling in inflammation. Signal Transduct Target Ther, 2: 17023-.

101. Mossberg M, Ståhl AL, Kahn R, Kristoffersson AC, Tati R, Heijl C *et al.* (2017) C1-inhibitor decreases the release of vasculitis-like chemotactic endothelial microvesicles. J Am Soc Nephrol, 28: 2472-2481.

102. Sims PJ, Faioni EM, Wiedmer T, Shattil SJ (1988) Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. J Biol Chem, 263: 18205-18212.

103. Arvidsson I, Ståhl AL, Hedström MM, Kristoffersson AC, Rylander C, Westman JS *et al.* (2015) Shiga toxin-induced complement-mediated hemolysis and release of complement-coated red blood cell-derived microvesicles in hemolytic uremic syndrome. J Immunol, 194: 2309-2318.

104. Abid Hussein MN, Nieuwland R, Hau CM, Evers LM, Meesters EW, Sturk A (2005) Cell-derived microparticles contain caspase 3 *in vitro* and *in vivo*. J Thromb Haemost, 3: 888-896.

105. Böing AN, Stap J, Hau CM, Afink GB, Ris-Stalpers C, Reits EA *et al.* (2013) Active caspase-3 is removed from cells by release of caspase-3-enriched vesicles. Biochim Biophys Acta, 1833: 1844-1852.

106. Jorfi S, Inal JM (2013) The role of microvesicles in cancer progression and drug resistance. Biochem Soc Trans, 41: 293-298.

107. Guerra F, Paiano A, Migoni D, Girolimetti G, Perrone AM, De Iaco P *et al.* (2019) Modulation of RAB7A protein expression determines resistance to cisplatin through late endocytic pathway impairment and extracellular vesicular secretion. Cancers 11.

108. Wolfmeier H, Radecke J, Schoenauer R, Koeffel R, Babiychuk VS, Drücker P *et al.* (2016) Active release of pneumolysin prepores and pores by mammalian cells undergoing a *Streptococcus pneumoniae* attack. Biochim Biophys Acta, 1860: 2498-2509.

109. Wolfmeier H, Schoenauer R, Atanassoff AP, Neill DR, Kadioglu A, Draeger A *et al.* (2015) Ca^{2+}-dependent repair of pneumolysin pores: A new paradigm for host cellular defense against bacterial pore-forming toxins. Biochim Biophys Acta, 1853: 2045-2054.

110. Majumdar R, Tavakoli Tameh A, Arya SB, Parent CA (2021) Exosomes mediate LTB4 release during neutrophil chemotaxis. PLoS Biol, 19: e3001271.

111. Nolan S, Dixon R, Norman K, Hellewell P, Ridger V (2008) Nitric oxide regulates neutrophil migration through microparticle formation. Am J Pathol, 172: 265-273.

112. Barry OP, Praticò D, Savani RC, FitzGerald GA (1998) Modulation of monocyte-endothelial cell interactions by platelet microparticles. J Clin Invest, 102: 136-144.

113. Barry OP, Pratico D, Lawson JA, FitzGerald GA (1997) Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. J Clin Invest, 99: 2118-2127.

114. Forlow SB, McEver RP, Nollert MU (2000) Leukocyte-leukocyte interactions mediated by platelet microparticles under flow. Blood, 95: 1317-1323.

115. Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J (2001) High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. Atherosclerosis, 158: 277-287.

116. Bach RR (1988) Initiation of coagulation by tissue factor. CRC Crit Rev Biochem, 23: 339-368.

117. Davey MG, Lüscher EF (1966) The action of thrombin on platelet proteins. Thromb Diath Haemorrh Suppl, 20: 283-287.

118. Nomura S, Shimizu M (2015) Clinical significance of procoagulant microparticles. J Intensive Care, 3: 2.

119. Berckmans RJ, Nieuwland R, Böing AN, Romijn FP, Hack CE, Sturk A (2001) Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost, 85: 639-646.

120. Merten M, Pakala R, Thiagarajan P, Benedict CR (1999) Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein IIb/IIIa-dependent mechanism. Circulation, 99: 2577-2582.

121. Bevers EM, Williamson PL (2016) Getting to the outer leaflet: physiology of phosphatidylserine exposure at the plasma membrane. Physiol Rev, 96: 605-645.

122. Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV *et al.* (2007) Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. Thromb Haemost, 97: 425-434.

123. Drake TA, Ruf W, Morrissey JH, Edgington TS (1989) Functional tissue factor is entirely cell surface expressed on lipopolysaccharide-stimulated human blood monocytes and a constitutively tissue factor-producing neoplastic cell line. J Cell Biol, 109: 389-395.

124. Hiller E, Saal JG, Riethmüller G (1977) Procoagulant activity of activated monocytes. Haemostasis, 6: 347-350.

125. Mandal SK, Pendurthi UR, Rao LV (2006) Cellular localization and trafficking of tissue factor. Blood, 107: 4746-4753.

126. Satta N, Toti F, Feugeas O, Bohbot A, Dachary-Prigent J, Eschwege V *et al.* (1994) Monocyte vesiculation is a possible mechanism for dissemination of membrane-associated procoagulant activities and adhesion molecules after stimulation by lipopolysaccharide. J Immunol, 153: 3245-3255.

127. Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol, 2: 123-140.

128. Beutin L, Geier D, Steinrück H, Zimmermann S, Scheutz F (1993) Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. J Clin Microbiol, 31: 2483-2488.

129. Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev, 11: 142-201.

130. Trofa AF, Ueno-Olsen H, Oiwa R, Yoshikawa M (1999) Dr. Kiyoshi Shiga: discoverer of the dysentery bacillus. Clin Infect Dis, 29: 1303-1306.

131. Neupane M, Abu-Ali GS, Mitra A, Lacher DW, Manning SD, Riordan JT (2011) Shiga toxin 2 overexpression in *Escherichia coli* O157:H7 strains associated with severe human disease. Microb Pathog, 51: 466-470.

132. Shridhar PB, Siepker C, Noll LW, Shi X, Nagaraja TG, Bai J (2017) Shiga toxin subtypes of non-O157 *Escherichia coli* serogroups isolated from cattle feces. Front Cell Infec Microbiol, 7: 121-121.

133. Newland JW, Strockbine NA, Neill RJ (1987) Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II. Infect Immun, 55: 2675-2680.

134. Stein PE, Boodhoo A, Tyrrell GJ, Brunton JL, Read RJ (1992) Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*. Nature, 355: 748-750.

135. Lindberg AA, Brown JE, Strömberg N, Westling-Ryd M, Schultz JE, Karlsson KA (1987) Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. J Biol Chem, 262: 1779-1785.

136. Ling H, Boodhoo A, Hazes B, Cummings MD, Armstrong GD, Brunton JL *et al.* (1998) Structure of the Shiga-like toxin I B-pentamer complexed with an analogue of its receptor Gb3. Biochemistry, 37: 1777-1788.

137. Jacobson JM, Yin J, Kitov PI, Mulvey G, Griener TP, James MN *et al.* (2014) The crystal structure of Shiga toxin type 2 with bound disaccharide guides the design of a heterobifunctional toxin inhibitor. J Biol Chem, 289: 885-894.

138. Kiarash A, Boyd B, Lingwood CA (1994) Glycosphingolipid receptor function is modified by fatty acid content. Verotoxin 1 and verotoxin 2c preferentially recognize different globotriaosyl ceramide fatty acid homologues. J Biol Chem, 269: 11138-11146.

139. Legros N, Pohlentz G, Steil D, Kouzel IU, Liashkovich I, Mellmann A *et al.* (2018) Membrane assembly of Shiga toxin glycosphingolipid receptors and toxin refractiveness of MDCK II epithelial cells. J Lipid Res, 59: 1383-1401.

140. Spilsberg B, Llorente A, Sandvig K (2007) Polyunsaturated fatty acids regulate Shiga toxin transport. Biochem Biophys Res Commun, 364: 283-288.

141. Kasahara K, Sanai Y (1999) Possible roles of glycosphingolipids in lipid rafts. Biophysical Chemistry, 82: 121-127.

142. Hanashima T, Miyake M, Yahiro K, Iwamaru Y, Ando A, Morinaga N *et al.* (2008) Effect of Gb3 in lipid rafts in resistance to Shiga-like toxin of mutant Vero cells. Microb Pathog, 45: 124-133.

143. Louise CB, Obrig TG (1992) Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells *in vitro*. Infect Immun, 60: 1536-1543.

144. van de Kar NC, Monnens LA, Karmali MA, van Hinsbergh VW (1992) Tumor necrosis factor and interleukin-1 induce expression of the verocytotoxin receptor globotriaosylceramide on human endothelial cells: implications for the pathogenesis of the hemolytic uremic syndrome. Blood, 80: 2755-2764.

145. Romer W, Berland L, Chambon V, Gaus K, Windschiegl B, Tenza D *et al.* (2007) Shiga toxin induces tubular membrane invaginations for its uptake into cells. Nature, 450: 670-675.

146. Torgersen ML, Lauvrak SU, Sandvig K (2005) The A-subunit of surface-bound Shiga toxin stimulates clathrin-dependent uptake of the toxin. FEBS J, 272: 4103-4113.

147. Utskarpen A, Massol R, van Deurs B, Lauvrak SU, Kirchhausen T, Sandvig K (2010) Shiga toxin increases formation of clathrin-coated pits through Syk kinase. PLoS One, 5: e10944.

148. Sandvig K (2001) Shiga toxins. Toxicon, 39: 1629-1635.

149. Garred O, van Deurs B, Sandvig K (1995) Furin-induced cleavage and activation of Shiga toxin. J Biol Chem, 270: 10817-10821.

150. Falguieres T, Mallard F, Baron C, Hanau D, Lingwood C, Goud B *et al.* (2001) Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes. Mol Biol Cell, 12: 2453-2468.

151. Karpman D, Håkansson A, Perez MT, Isaksson C, Carlemalm E, Caprioli A *et al.* (1998) Apoptosis of renal cortical cells in the hemolytic-uremic syndrome: *in vivo* and *in vitro* studies. Infect Immun, 66: 636-644.

152. Fujii J, Matsui T, Heatherly DP, Schlegel KH, Lobo PI, Yutsudo T *et al.* (2003) Rapid apoptosis induced by Shiga toxin in HeLa cells. Infect Immun, 71: 2724-2735.

153. Lee MS, Cherla RP, Tesh VL (2010) Shiga toxins: intracellular trafficking to the ER leading to activation of host cell stress responses. Toxins, 2: 1515-1535.

154. Lee SY, Lee MS, Cherla RP, Tesh VL (2008) Shiga toxin 1 induces apoptosis through the endoplasmic reticulum stress response in human monocytic cells. Cell Microbiol, 10: 770-780.

155. Johannes L, Römer W (2010) Shiga toxins--from cell biology to biomedical applications. Nat Rev Microbiol, 8: 105-116.

156. Smith WE, Kane AV, Campbell ST, Acheson DW, Cochran BH, Thorpe CM (2003) Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. Infect Immun, 71: 1497-1504.

157. McKee ML, O'Brien AD (1995) Investigation of enterohemorrhagic *Escherichia coli* O157:H7 adherence characteristics and invasion potential reveals a new attachment pattern shared by intestinal *E. coli*. Infect Immun, 63: 2070-2074.

158. Békássy ZD, Calderon Toledo C, Leoj G, Kristoffersson A, Leopold SR, Perez MT *et al.* (2011) Intestinal damage in enterohemorrhagic *Escherichia coli* infection. Pediatr Nephrol, 26: 2059-2071.

159. Karch H (2001) The role of virulence factors in enterohemorrhagic *Escherichia coli* (EHEC)--associated hemolytic-uremic syndrome. Semin Thromb Hemost, 27: 207-213.

160. Phillips AD, Navabpour S, Hicks S, Dougan G, Wallis T, Frankel G (2000) Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. Gut, 47: 377-381.

161. Chong Y, Fitzhenry R, Heuschkel R, Torrente F, Frankel G, Phillips AD (2007) Human intestinal tissue tropism in *Escherichia coli* O157 : H7--initial colonization of terminal ileum and Peyer's patches and minimal colonic adhesion *ex vivo*. Microbiology (Reading), 153: 794-802.

162. Etienne-Mesmin L, Chassaing B, Sauvanet P, Denizot J, Blanquet-Diot S, Darfeuille-Michaud A *et al.* (2011) Interactions with M cells and macrophages as key steps in the pathogenesis of enterohemorrhagic *Escherichia coli* infections. PLoS One, 6: e23594.

163. Lewis SB, Cook V, Tighe R, Schüller S (2015) Enterohemorrhagic *Escherichia coli* colonization of human colonic epithelium *in vitro* and *ex vivo*. Infect Immun, 83: 942-949.

164. Rivera A, Siracusa MC, Yap GS, Gause WC (2016) Innate cell communication kick-starts pathogen-specific immunity. Nat Immunol, 17: 356-363.

165. Miyamoto Y, Iimura M, Kaper JB, Torres AG, Kagnoff MF (2006) Role of Shiga toxin versus H7 flagellin in enterohaemorrhagic *Escherichia coli* signalling of human colon epithelium *in vivo*. Cell Microbiol, 8: 869-879.

166. Calderon Toledo C, Rogers TJ, Svensson M, Tati R, Fischer H, Svanborg C *et al.* (2008) Shiga toxin-mediated disease in MyD88-deficient mice infected with *Escherichia coli* O157:H7. Am J Pathol, 173: 1428-1439.

167. Thorpe CM, Hurley BP, Lincicome LL, Jacewicz MS, Keusch GT, Acheson DW (1999) Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. Infect Immun, 67: 5985-5993.

168. Jung HC, Eckmann L, Yang SK, Panja A, Fierer J, Morzycka-Wroblewska E *et al.* (1995) A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J Clin Invest, 95: 55-65.

169. Westerholt S, Hartung T, Tollens M, Güstrau A, Oberhoffer M, Karch H *et al.* (2000) Inflammatory and immunological parameters in children with haemolytic uremic syndrome (HUS) and gastroenteritis-pathophysiological and diagnostic clues. Cytokine, 12: 822-827.

170. Malyukova I, Murray KF, Zhu C, Boedeker E, Kane A, Patterson K *et al.* (2009) Macropinocytosis in Shiga toxin 1 uptake by human intestinal epithelial cells and transcellular transcytosis. Am J Physiol Gastrointest Liver Physiol, 296: G78-92.

171. Schüller S, Heuschkel R, Torrente F, Kaper JB, Phillips AD (2007) Shiga toxin binding in normal and inflamed human intestinal mucosa. Microbes Infect, 9: 35-39.

172. Schüller S (2011) Shiga toxin interaction with human intestinal epithelium. Toxins 3: 626-639.

173. Fontaine A, Arondel J, Sansonetti PJ (1988) Role of Shiga toxin in the pathogenesis of bacillary dysentery, studied by using a Tox- mutant of *Shigella dysenteriae* 1. Infect Immun, 56: 3099-3109.

174. Brigotti M, Tazzari PL, Ravanelli E, Carnicelli D, Rocchi L, Arfilli V *et al.* (2011) Clinical relevance of Shiga toxin concentrations in the blood of patients with hemolytic uremic syndrome. Pediatr Infect Dis J, 30: 486-490.

175. Lee MS, Tesh VL (2019) Roles of Shiga toxins in immunopathology. Toxins 11.

176. Ghosh SA, Polanowska-Grabowska RK, Fujii J, Obrig T, Gear AR (2004) Shiga toxin binds to activated platelets. J Thromb Haemost, 2: 499-506.

177. Cooling LL, Walker KE, Gille T, Koerner TA (1998) Shiga toxin binds human platelets via globotriaosylceramide (Pk antigen) and a novel platelet glycosphingolipid. Infect Immun, 66: 4355-4366.

178. Karpman D, Papadopoulou D, Nilsson K, Sjögren AC, Mikaelsson C, Lethagen S (2001) Platelet activation by Shiga toxin and circulatory factors as a pathogenetic mechanism in the hemolytic uremic syndrome. Blood, 97: 3100-3108.

179. Ståhl AL, Svensson M, Mörgelin M, Svanborg C, Tarr PI, Mooney JC *et al.* (2006) Lipopolysaccharide from enterohemorrhagic *Escherichia coli* binds to platelets through TLR4 and CD62 and is detected on circulating platelets in patients with hemolytic uremic syndrome. Blood, 108: 167-176.

180. Karpman D, Manea M, Vaziri-Sani F, Ståhl AL, Kristoffersson AC (2006) Platelet activation in hemolytic uremic syndrome. Semin Thromb Hemost, 32: 128-145.

181. Geelen JM, van der Velden TJ, van den Heuvel LP, Monnens LA (2007) Interactions of Shiga-like toxin with human peripheral blood monocytes. Pediatr Nephrol, 22: 1181-1187.

182. van Setten PA, Monnens LA, Verstraten RG, van den Heuvel LP, van Hinsbergh VW (1996) Effects of verocytotoxin-1 on nonadherent human monocytes: binding

characteristics, protein synthesis, and induction of cytokine release. Blood, 88: 174-183.

183. Brandelli JR, Griener TP, Laing A, Mulvey G, Armstrong GD (2015) The effects of Shiga toxin 1, 2 and their subunits on cytokine and chemokine expression by human macrophage-like THP-1 cells. Toxins 7: 4054-4066.

184. Guessous F, Marcinkiewicz M, Polanowska-Grabowska R, Keepers TR, Obrig T, Gear AR (2005) Shiga toxin 2 and lipopolysaccharide cause monocytic THP-1 cells to release factors which activate platelet function. Thromb Haemost, 94: 1019-1027.

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

186. Liu J, Akahoshi T, Sasahana T, Kitasato H, Namai R, Sasaki T *et al.* (1999) Inhibition of neutrophil apoptosis by verotoxin 2 derived from *Escherichia coli* O157:H7. Infect Immun, 67: 6203-6205.

187. Fernandez GC, Gomez SA, Ramos MV, Bentancor LV, Fernandez-Brando RJ, Landoni VI *et al.* (2007) The functional state of neutrophils correlates with the severity of renal dysfunction in children with hemolytic uremic syndrome. Pediatr Res, 61: 123-128.

188. Bitzan M, Richardson S, Huang C, Boyd B, Petric M, Karmali MA (1994) Evidence that verotoxins (Shiga-like toxins) from *Escherichia coli* bind to P blood group antigens of human erythrocytes *in vitro*. Infect Immun, 62: 3337-3347.

189. Spitalnik PF, Spitalnik SL (1995) The P blood group system: biochemical, serological, and clinical aspects. Transfus Med Rev, 9: 110-122.

190. Petruzziello-Pellegrini TN, Moslemi-Naeini M, Marsden PA (2013) New insights into Shiga toxin-mediated endothelial dysfunction in hemolytic uremic syndrome. Virulence, 4: 556-563.

191. Uchida H, Kiyokawa N, Horie H, Fujimoto J, Takeda T (1999) The detection of Shiga toxins in the kidney of a patient with hemolytic uremic syndrome. Pediatr Res, 45: 133-137.

192. Chaisri U, Nagata M, Kurazono H, Horie H, Tongtawe P, Hayashi H *et al.* (2001) Localization of Shiga toxins of enterohaemorrhagic *Escherichia coli* in kidneys of paediatric and geriatric patients with fatal haemolytic uraemic syndrome. Microb Pathog, 31: 59-67.

193. Williams JM, Boyd B, Nutikka A, Lingwood CA, Barnett Foster DE, Milford DV *et al.* (1999) A comparison of the effects of verocytotoxin-1 on primary human renal cell cultures. Toxicol Lett, 105: 47-57.

194. Simon M, Cleary TG, Hernandez JD, Abboud HE (1998) Shiga toxin 1 elicits diverse biologic responses in mesangial cells. Kidney Int, 54: 1117-1127.

195. Obrig TG, Louise CB, Lingwood CA, Boyd B, Barley-Maloney L, Daniel TO (1993) Endothelial heterogeneity in Shiga toxin receptors and responses. J Biol Chem, 268: 15484-15488.

196. Psotka MA, Obata F, Kolling GL, Gross LK, Saleem MA, Satchell SC *et al.* (2009) Shiga toxin 2 targets the murine renal collecting duct epithelium. Infect Immun, 77: 959-969.

197. Hughes AK, Stricklett PK, Kohan DE (1998) Cytotoxic effect of Shiga toxin-1 on human proximal tubule cells. Kidney Int, 54: 426-437.

198. Reitsma PH, Versteeg HH, Middeldorp S (2012) Mechanistic view of risk factors for venous thromboembolism. Arterioscler Thromb Vasc Biol, 32: 563-568.

199. Zoja C, Angioletti S, Donadelli R, Zanchi C, Tomasoni S, Binda E *et al.* (2002) Shiga toxin-2 triggers endothelial leukocyte adhesion and transmigration via NF-kappaB dependent up-regulation of IL-8 and MCP-1. Kidney Int, 62: 846-856.

200. Eisenhauer PB, Jacewicz MS, Conn KJ, Koul O, Wells JM, Fine RE *et al.* (2004) *Escherichia coli* Shiga toxin 1 and TNF-alpha induce cytokine release by human cerebral microvascular endothelial cells. Microb Pathog, 36: 189-196.

201. Guessous F, Marcinkiewicz M, Polanowska-Grabowska R, Kongkhum S, Heatherly D, Obrig T *et al.* (2005) Shiga toxin 2 and lipopolysaccharide induce human microvascular endothelial cells to release chemokines and factors that stimulate platelet function. Infect Immun, 73: 8306-8316.

202. Keepers TR, Gross LK, Obrig TG (2007) Monocyte chemoattractant protein 1, macrophage inflammatory protein 1 alpha, and RANTES recruit macrophages to the kidney in a mouse model of hemolytic-uremic syndrome. Infect Immun, 75: 1229-1236.

203. Morigi M, Galbusera M, Binda E, Imberti B, Gastoldi S, Remuzzi A *et al.* (2001) Verotoxin-1-induced up-regulation of adhesive molecules renders microvascular endothelial cells thrombogenic at high shear stress. Blood, 98: 1828-1835.

204. Savage B, Shattil SJ, Ruggeri ZM (1992) Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb-IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. J Biol Chem, 267: 11300-11306.

205. Tarr PI, Neill MA, Clausen CR, Watkins SL, Christie DL, Hickman RO (1990) *Escherichia coli* O157:H7 and the hemolytic uremic syndrome: importance of early cultures in establishing the etiology. J Infect Dis, 162: 553-556.

206. Karpman D, Loos S, Tati R, Arvidsson I (2017) Haemolytic uraemic syndrome. J Intern Med, 281: 123-148.

207. Karmali MA, Petric M, Lim C, Fleming PC, Arbus GS, Lior H (1985) The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J Infect Dis, 151: 775-782.

208. Taylor CM (2008) Enterohaemorrhagic *Escherichia coli* and *Shigella dysenteriae* type 1-induced haemolytic uraemic syndrome. Pediatr Nephrol, 23: 1425-1431.

209. Tschape H, Prager R, Streckel W, Fruth A, Tietze E, Bohme G (1995) Verotoxinogenic *Citrobacter freundii* associated with severe gastroenteritis and cases of haemolytic uraemic syndrome in a nursery school: green butter as the infection source. Epidemiol Infect, 114: 441-450.

210. Khalid M, Andreoli S (2019) Extrarenal manifestations of the hemolytic uremic syndrome associated with Shiga toxin-producing *Escherichia coli* (STEC HUS). Pediatr Nephrol, 34: 2495-2507.

211. Zoja C, Morigi M, Remuzzi G (2001) The role of the endothelium in hemolytic uremic syndrome. J Nephrol, 14 Suppl 4: S58-62.

212. Ruggenenti P, Noris M, Remuzzi G (2001) Thrombotic microangiopathy, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. Kidney Int, 60: 831-846.

213. McKee RS, Schnadower D, Tarr PI, Xie J, Finkelstein Y, Desai N *et al.* (2020) Predicting Hemolytic Uremic Syndrome and Renal Replacement Therapy in Shiga Toxin-producing *Escherichia coli*-infected Children. Clin Infect Dis, 70: 1643-1651.

214. Robson WL, Fick GH, Wilson PC (1988) Prognostic factors in typical postdiarrhea hemolytic-uremic syndrome. Child Nephrol Urol, 9: 203-207.

215. Nevard CH, Jurd KM, Lane DA, Philippou H, Haycock GB, Hunt BJ (1997) Activation of coagulation and fibrinolysis in childhood diarrhoea-associated haemolytic uraemic syndrome. Thromb Haemost, 78: 1450-1455.

216. van de Kar NC, van Hinsbergh VW, Brommer EJ, Monnens LA (1994) The fibrinolytic system in the hemolytic uremic syndrome: *in vivo* and *in vitro* studies. Pediatr Res, 36: 257-264.

217. Van Geet C, Proesmans W, Arnout J, Vermylen J, Declerck PJ (1998) Activation of both coagulation and fibrinolysis in childhood hemolytic uremic syndrome. Kidney Int, 54: 1324-1330.

218. Aronson DL, Stevan L, Ball AP, Franza BR, Jr., Finlayson JS (1977) Generation of the combined prothrombin activation peptide (F1-2) during the clotting of blood and plasma. J Clin Invest, 60: 1410-1418.

219. Chandler WL, Jelacic S, Boster DR, Ciol MA, Williams GD, Watkins SL *et al.* (2002) Prothrombotic coagulation abnormalities preceding the hemolytic-uremic syndrome. N Engl J Med, 346: 23-32.

220. Savage B, Saldívar E, Ruggeri ZM (1996) Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell, 84: 289-297.

221. Ge S, Hertel B, Emden SH, Beneke J, Menne J, Haller H *et al.* (2012) Microparticle generation and leucocyte death in Shiga toxin-mediated HUS. Nephrol Dial Transplant, 27: 2768-2775.

222. Ståhl AL, Sartz L, Karpman D (2011) Complement activation on platelet-leukocyte complexes and microparticles in enterohemorrhagic *Escherichia coli*-induced hemolytic uremic syndrome. Blood, 117: 5503-5513.

223. Gupta N, Pons V, Noel R, Buisson DA, Michau A, Johannes L *et al.* (2014) (S)-N-Methyldihydroquinazolinones are the Active Enantiomers of Retro-2 Derived Compounds against Toxins. ACS Med Chem Lett, 5: 94-97.

224. Forrester A, Rathjen SJ, Daniela Garcia-Castillo M, Bachert C, Couhert A, Tepshi L *et al.* (2020) Functional dissection of the retrograde Shiga toxin trafficking inhibitor Retro-2. Nat Chem Biol, 16: 327-336.

225. Stechmann B, Bai SK, Gobbo E, Lopez R, Merer G, Pinchard S *et al.* (2010) Inhibition of retrograde transport protects mice from lethal ricin challenge. Cell, 141: 231-242.

226. Zidovetzki R, Levitan I (2007) Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. Biochim Biophys Acta, 1768: 1311-1324.

227. Wang LH, Rothberg KG, Anderson RG (1993) Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. J Cell Biol, 123: 1107-1117.

228. Pudymaitis A, Armstrong G, Lingwood CA (1991) Verotoxin-resistant cell clones are deficient in the glycolipid globotriosylceramide: differential basis of phenotype. Arch Biochem Biophys, 286: 448-452.

229. Okuda T, Tokuda N, Numata S, Ito M, Ohta M, Kawamura K *et al.* (2006) Targeted disruption of Gb3/CD77 synthase gene resulted in the complete deletion of globo-series glycosphingolipids and loss of sensitivity to verotoxins. J Biol Chem, 281: 10230-10235.

230. Bitzan M, Klemt M, Steffens R, Müller-Wiefel DE (1993) Differences in verotoxin neutralizing activity of therapeutic immunoglobulins and sera from healthy controls. Infection, 21: 140-145.

231. Kimura T, Tani S, Matsumoto Yi Y, Takeda T (2001) Serum amyloid P component is the Shiga toxin 2-neutralizing factor in human blood. J Biol Chem, 276: 41576-41579.

232. Waddell T, Cohen A, Lingwood CA (1990) Induction of verotoxin sensitivity in receptor-deficient cell lines using the receptor glycolipid globotriosylceramide. Proc Natl Acad Sci U S A, 87: 7898-7901.

233. Lingwood CA, Binnington B, Manis A, Branch DR (2010) Globotriaosyl ceramide receptor function - where membrane structure and pathology intersect. FEBS Lett, 584: 1879-1886.

234. Hoey DE, Sharp L, Currie C, Lingwood CA, Gally DL, Smith DG (2003) Verotoxin 1 binding to intestinal crypt epithelial cells results in localization to lysosomes and abrogation of toxicity. Cell Microbiol, 5: 85-97.

235. Falguiéres T, Romer W, Amessou M, Afonso C, Wolf C, Tabet JC *et al.* (2006) Functionally different pools of Shiga toxin receptor, globotriaosyl ceramide, in HeLa cells. FEBS J, 273: 5205-5218.

236. Bielaszewska M, Ruter C, Bauwens A, Greune L, Jarosch KA, Steil D *et al.* (2017) Host cell interactions of outer membrane vesicle-associated virulence factors of enterohemorrhagic *Escherichia coli* O157: Intracellular delivery, trafficking and mechanisms of cell injury. PLoS Pathog, 13: e1006159.

237. Fitzpatrick MM, Shah V, Trompeter RS, Dillon MJ, Barratt TM (1992) Interleukin-8 and polymorphoneutrophil leucocyte activation in hemolytic uremic syndrome of childhood. Kidney Int, 42: 951-956.

238. Varrone E, Carnicelli D, Brigotti M (2021) Extracellular vesicles and renal endothelial cells: a fatal attraction in hemolytic uremic syndrome. Am J Pathol, 191: 795-804.

239. Karpman D (2012) Management of Shiga toxin-associated *Escherichia coli*-induced haemolytic uraemic syndrome: randomized clinical trials are needed. Nephrol Dial Transplant, 27: 3669-3674.

240. Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI (2000) The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. N Engl J Med, 342: 1930-1936.

241. Menne J, Nitschke M, Stingele R, Abu-Tair M, Beneke J, Bramstedt J *et al.* (2012) Validation of treatment strategies for enterohaemorrhagic *Escherichia coli* O104:H4 induced haemolytic uraemic syndrome: case-control study. BMJ, 345: e4565.

242. Tontanahal A, Arvidsson I, Karpman D (2021) Annexin induces cellular uptake of extracellular vesicles and delays disease in *Escherichia coli* O157:H7 Infection. Microorganisms, 9.

243. Colic E, Dieperink H, Titlestad K, Tepel M (2011) Management of an acute outbreak of diarrhoea-associated haemolytic uraemic syndrome with early plasma exchange in adults from southern Denmark: an observational study. Lancet, 378: 1089-1093.

244. O'Loughlin AJ, Mäger I, de Jong OG, Varela MA, Schiffelers RM, El Andaloussi S *et al.* (2017) Functional delivery of lipid-conjugated siRNA by extracellular vesicles. Mol Ther, 25: 1580-1587.

245. Wiklander OPB, Brennan M, Lötvall J, Breakefield XO, El Andaloussi S (2019) Advances in therapeutic applications of extracellular vesicles. Sci Transl Med, 11.

246. Walker S, Busatto S, Pham A, Tian M, Suh A, Carson K *et al.* (2019) Extracellular vesicle-based drug delivery systems for cancer treatment. Theranostics, 9: 8001-8017.

247. Arab S, Murakami M, Dirks P, Boyd B, Hubbard SL, Lingwood CA *et al.* (1998) Verotoxins inhibit the growth of and induce apoptosis in human astrocytoma cells. J Neurooncol, 40: 137-150.

248. Arab S, Rutka J, Lingwood C (1999) Verotoxin induces apoptosis and the complete, rapid, long-term elimination of human astrocytoma xenografts in nude mice. Oncol Res, 11: 33-39.

249. Lingwood C (2021) Therapeutic uses of bacterial subunit toxins. Toxins 13.

250. Gallegos KM, Conrady DG, Karve SS, Gunasekera TS, Herr AB, Weiss AA (2012) Shiga toxin binding to glycolipids and glycans. PLoS One, 7: e30368.

251. Friedrich AW, Bielaszewska M, Zhang WL, Pulz M, Kuczius T, Ammon A *et al.* (2002) *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J Infect Dis, 185: 74-84.

252. Taylor CM, Milford DV, Rose PE, Roy TC, Rowe B (1990) The expression of blood group P1 in post-enteropathic haemolytic uraemic syndrome. Pediatr Nephrol, 4: 59-61.

253. Mallard F, Johannes L (2003) Shiga toxin B-subunit as a tool to study retrograde transport. Methods Mol Med, 73: 209-220.