Extracellular vesicles in inflammatory bowel disease: small particles, big players

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Abstract

Extracellular vesicles are nanovesicles released by many cell types into the extracellular space. They are important mediators of intercellular communication, enabling the functional transfer of molecules from one cell to another. Moreover, their molecular composition reflects the physiological status of the producing cell and tissue. Consequently, these vesicles have been involved in many (patho)physiological processes such as immunomodulation and intestinal epithelial repair, both key processes involved in inflammatory bowel disease. Given that these vesicles are present in many body fluids, they also provide opportunities for diagnostic, prognostic and therapeutic applications. In this review, we summarize functional roles of extracellular vesicles in health and disease, with a focus on immune regulation and intestinal barrier integrity, and review recent studies on extracellular vesicles and inflammatory bowel disease. We also elaborate on their clinical potential in inflammatory bowel disease.

1. Introduction

Inflammatory bowel disease (IBD), a group of chronic immune-mediated diseases consisting of ulcerative colitis (UC) and Crohn's disease (CD), has experienced an increased incidence and prevalence over the last decade, indicating its emergence as a global disease.[1] Affecting approximately 0.3% of the European population and now becoming an increased burden also in newly industrialized countries, IBD has a large economic and social impact.[2,3]

A large part of the etiology and exact pathology of IBD remains unknown, but its polygenic and multifactorial nature is well known.[4] Interaction of different genetic, microbiome, and environmental factors with the immune system gives IBD its complex character. A key element in immune regulation is the balance between immune suppression and stimulation, while responding correctly to environmental triggers. This regulation is disturbed in IBD patients, resulting in inflammation and compromised integrity of the intestinal barrier.

An important clinical issue is the delayed diagnosis of IBD, especially for CD, with the median time between symptoms and diagnosis being nine months, and sometimes more than two years. This delay often is associated with an increase in complications and risk of needing intestinal surgery.[5] Diagnosis of IBD is based on a combination of clinical, biochemical, stool, endoscopic, and histological investigations.[6] However, besides endoscopy, a "gold standard" to establish the diagnosis of IBD does not exist. The current biomarkers used for diagnosis and assessment of disease activity are blood C-reactive protein (CRP) and faecal calprotectin.[7] Elevated CRP levels however are not specific to intestinal inflammation, and also do not necessarily correlate with active disease. Vice versa, a low concentration of CRP can be seen in patients with active IBD.[8] The neutrophil-specific calprotectin is more specific of intestinal inflammation, although not for IBD-caused inflammation. A second challenge in IBD is dealing with the heterogeneity in clinical phenotypes. IBD patients show variable disease courses and complications, such as intestinal strictures, fistulas, and even extraintestinal manifestations.[9] Being able to accurately and consistently distinguish between patients is of great importance for the clinical follow-up of the patient, e.g. to decide on treatment options and on the intensity of follow-up. The current treatment options are generally categorized in four classes: aminosalicylates, corticosteroids, immunomodulators, and biologics.[10,11] These can be applied in either a more conventional step-up strategy (gradually stepping up to more potent drugs as needed), or a "top-down" approach that starts with early combined immunosuppression and de-escalation if possible. Several new drugs targeting different pathways (cell adhesion molecule inhibitors, anti-IL-12/23, small molecules etc) are currently being tested in clinical trials, or have recently entered the market.[10] With this growing therapeutic armamentarium patient stratification based on treatment

response would help to pick the ideal drug for a patient. Despite the broad range of therapeutic agents, many patients do not respond to current treatment, or lose response over time [12], leading to 10-20% of UC patients and 50% of CD patients needing surgery within 10 years after diagnosis.[13] Taken together, there is a need for new, non-invasive markers aiding in the diagnosis, prognosis and monitoring of IBD, and for new therapeutic targets.

Extracellular vesicles (EVs) are small lipid bilayer-enclosed spheres used by cells to transfer small molecules to other cells. As such, EVs are present in many body fluids, and are thus easily accessible to be used for diagnostic, prognostic and therapeutic applications. They have been shown to have an important role in immune processes and intestinal barrier integrity, and in disease pathophysiology. In this review, we first discuss biogenesis and content of EVs, and then summarize their role in immune regulation and intestinal barrier integrity, and review recent studies on EVs and IBD. Finally, we will elaborate on the diagnostic, prognostic and therapeutic potential of EVs for IBD, as summarized in Figure 1.

2. Extracellular vesicles

EVs are lipid bilayer membrane vesicles released by cells and are critical in the communication between cells. They were first described in the early 1980s in a study investigating the fate of the transferrin receptor. The receptor was found to be present on newly discovered vesicles released from reticulocytes, later to be named EVs.[14] Where EVs were first solely considered as a medium to dispose of undesired components of a cell, their importance in intercellular communication has now become clear.[15,16] It was indeed seen that circulating EVs contain nucleic acids and other molecules (cargo), and that they are guided to specific target cells via membrane receptors.[17] Together with their ability to travel long distances, EVs can impact multiple organ systems. Once they arrive at their destination, EVs are either internalized by the target cell with subsequent release of their contents, or they adhere to the surface and induce signaling. Since their discovery, EVs have been found to be secreted by numerous cell types, both in normal and pathological situations, and to be present in different biological fluids (see **Table 1** and **Figure 1A** for some examples).

Especially in relation to regulation of the immune system, the role of EVs and their content has been hypothesized to be bigger than initially expected. Previous studies have highlighted the role of extracellular vesicles in chronic immune diseases such as rheumatoid arthritis and diabetes mellitus.[18,19] In these cases, EVs have even been proposed to be 'the missing link between autoimmunity and inflammation'. Also, studies have been performed in the context of cancer, where

EVs and EV cargo have been shown to fulfill an important role in early detection of cancer and as tumor therapy.[20,21] A number of clinical trials using EVs have been completed for different indications.

It should be noted that broadly three subgroups of EVs are distinguished, based on their size and origin: apoptotic bodies, microvesicles/ectosomes, and exosomes.[22] Exosomes are the smallest and most homogenous in size (30-150 nm in diameter) and density compared with microvesicles and apoptotic bodies ranging from 100-1000 nm and 500-5000 nm respectively. Apoptotic bodies and microvesicles originate from the plasma membrane, while exosomes are formed by creating an endosome. These EV subgroups have many overlapping properties, and isolating and characterizing individual subtypes is a substantial challenge in the field.[23] In literature the different EV terms are variably used. For this reason we will in this review refer to 'extracellular vesicles' as a general term, unless specifically stated otherwise.

3. EV biogenesis

Apoptotic bodies originate from blebbing of the plasma membrane during apoptosis, while microvesicles are directly formed from the plasma membrane.[24] Their biogenesis has been previously described in detail elsewhere.[25] Most research on EV biogenesis has however focused specifically on exosomes.

In contrast to apoptotic bodies and microvesicles, exosomes originate from endosomes. The early endosome matures into a late endosome, a process in which multiple intraluminal vesicles are formed by inward budding of the late endosomal membrane (also see **Figure 1B**). These late endosomes with intraluminal vesicles, called multivesicular bodies, can either fuse with lysosomes to be degraded (degradative multivesicular bodies), or can undergo fusion with the plasma membrane of the cell (exocytic multivesicular bodies) and release their intraluminal vesicles, referred to as exosomes.[26]

Exosomal biogenesis can occur in an ESCRT (endosomal-sorting complexes required for transport) -dependent or an ESCRT-independent pathway. The ESCRT-dependent pathway involves four ESCRT (0-III) proteins and their associated proteins, for instance VSP4, VTA1, and ALIX.[27] The different functions of the ESCRT-complexes are elaborately reviewed in Hurley et al.[28] Ceramide and tetraspanins are involved in the ESCRT-independent exosomal biogenesis pathway, but their exact function in exosomal biogenesis is not yet fully understood. Ceramide is a highly abundant sphingolipid in eukaryotic cells, and is formed by hydrolyzation of sphingomyelin by neutral sphingomyelinase (n-Smase).[29] Sphingomyelin stabilizes the endosomal membrane, but after its cleavage, ceramide induces spontaneous negative curvature to the membrane, hereby forming intraluminal vesicles. Tetraspanins, a transmembrane protein family including CD9, CD63, CD37, CD81 and CD82, also

influence membrane curvature and protrusion activity,[30] but additionally play a role in sorting of the EV cargo, discussed later in this review. The multivesicular bodies formed by these different pathways require microtubules for transport to the plasma membrane, and Rab27a/b (small GTPases) for docking at the plasma membrane.[31] Next the multivesicular body fuses with the plasma membrane aided by the SNARE protein complex specialized in mediating the fusion of vesicles to their target membrane, and finally the exosomes are released.

4. EV membrane composition and molecular cargo

In the beginning of EV research, exosomes were simply defined as the 'content' released by multivesicular bodies, and found to be enriched in specific proteins from the parental cell from which the EV was secreted.[32] Studies since have shown that in different pathological conditions – such as exposure to hypoxia, acidic environments or inflammatory signals – the content of EVs can be altered.[27] This induced change in EV content and also in number of EVs, is why they may serve as 'snapshots' of diseased cells.[33]

The cargo and membrane composition of EVs is complex, consisting of proteins, nucleic acids, lipids, and metabolites (see **Figure 2** for an overview).

- Proteins

Both intraluminal and transmembrane proteins are loaded into EVs from the parental cell. A distinction is often made between two sets of proteins: proteins common to all EVs, and proteins specific to parental cellular functions.

EVs are typically enriched with transmembrane proteins such as tetraspanins and syndecans. While syndecans are mostly known in relation to exosome biogenesis, the role of tetraspanins is more diverse (e.g. EV biogenesis, cargo sorting and antigen presentation).[34,35] Other transmembrane proteins include MHC-I and MHC-II, which are abundantly expressed on EVs secreted from antigen presenting cells. In EVs originating from dendritic cells, besides MHC-I and MHC-II, the costimulatory factor CD86 is present. Another example of cell-specific proteins are the integrins expressed on the surface of EVs. The specific integrins depend on the cell type the EVs were secreted from, e.g. α4β1 when secreted from reticulocytes, or αMβ2 when secreted from dendritic cells.[36] Exosomes derived from intestinal epithelial cells (IEC) have been shown to carry the IEC specific marker A33 in their membranes.[37]

Common intraluminal proteins include heat-shock proteins (e.g. HSP90 and HSP70), the ESCRT machinery with their related proteins, and cytoskeletal proteins such as actin.[36] IEC-derived exosomes

also contain antimicrobial peptides such as cathelicidin-37 and beta-defensin 2.[38] In a proteomic study on serum exosomes isolated from dextran sodium sulfate (DSS) induced acute colitis mice, 56 differentially expressed proteins were found, many of which were acute phase proteins and immunoglobulins.[39]

- Nucleic acids

DNA, mRNAs of short length, and several non-coding RNAs (e.g. miRNA, tRNA, rRNA) can be loaded into EVs. This provides them with a more stable environment shielded from degradation compared to free circulating nucleic acids. Single-stranded DNA as well as double-stranded and mitochondrial DNA have been found in EVs, although their functional significance remains somewhat unknown.[40] When RNAs present in EVs are transferred to target cells they can there take part in gene expression and protein synthesis, hereby altering protein production in the recipient cell (**Figure 1B**).[17] An example is the non-coding RNA miR-29a. miR-29a is transported by EVs and contributes to intestinal function and barrier integrity by downregulation of glutamine synthetase, aquaporin (AQP), NF-κB repressing factor (NKRF) and Claudin-1 causing increased epithelial permeability.[38,41,42] Similar as with proteins in and on EVs, EVs are also expected to have different subsets of miRNA depending on their tissue origin and cell-state. In general, the composition of nucleic acids in microvesicles is more representative of the parental cell than in exosomes. [43]

- Lipids

Compared to the parental cell, EVs are heavily enriched in specific lipids, such as phosphatidylserine, cholesterol, and the above-mentioned sphingomyelin and ceramide. The presence of several phospholipids in the EV membrane provides rigidity, and ensures great stability in physiological conditions *in vivo*.[44] Similar to tetraspanins forming tetraspanin-enriched microdomains with lipids and other transmembrane proteins, several lipids in EV membranes assemble as lipid rafts.[34] These lipid rafts, typically rich in sphingolipids and cholesterol, ensure the considerable detergent-resistance of EVs.[27]

- Metabolites

Another important component of the EVs cargo are metabolites. In a study comparing metabolites from urinary EVs and EVs from platelets, versus metabolites from their cell of origin, 55 metabolites could be quantified. Many of these were found to be commonly present in EVs arising from these different tissues but some were tissue specific. Additionally this study found that metabolites belonging to the nucleotide and spermidine pathways (glucuronate, D-ribose 5-phosphate and isobutyryl-L-carnitine) were found enriched in EVs from normal samples compared to EVs from pre-

prostatectomy samples of prostate cancer patients.[45] In addition, metabolomic analysis of serum samples exposed to hepatocyte EVs showed that EVs also have metabolic activity. Royo et al. for example found quantitative changes in over 90 different metabolites in serum samples after exposure to EVs, several of which are involved in oxidative stress metabolism and endothelial function. This suggests that EVs contain functional enzymes and are metabolically active.[46]

EVs and their parental cells have some differences in their protein-, lipid-, metabolite and nucleic acid profile. This suggests the existence of a specific mechanism to load the EV cargo. The components involved in the sorting of proteins into EVs are the same as those important in exosomal biogenesis. For example, ESCRT proteins also play a role in the sorting of cargo into the intraluminal vesicles. Ubiquitination of the proteins being sorted seems to be of importance for the recognition by ESCRT-complexes.[47] Besides, ceramide does not only induce membrane curvature for formation of intraluminal vesicles, but also functions in an ESCRT-independent, lipid-based mechanism of cargo sorting into intraluminal vesicles. Ceramide requires continuous activation of Gi-coupled sphingosine-1-phosphate (S1P) receptors on multivesicular bodies for this.[48] Another ESCRT-independent mechanism is the tetraspanin-based sorting of cargo into EVs. Tetraspanin-enriched microdomains can interact with different proteins of the parental cell thereby regulating their sorting into the vesicles.[49]

Although the mechanism is not fully understood, research into EV RNA sorting has identified several RNA binding proteins such as Annexin A2[50], KRAS[51], SYNCRIP[52], and ribonucleoprotein A2B1 (hnRNPA2B1)[53] to be involved in this process. In the latter study by Villarroya-Beltri et al, the authors analyzed a subset of miRNAs that were more abundant in EVs than in their parental cell. They identified conserved motifs on EV miRNAs, which were recognized by hnRNPA2B1, hereby controlling their loading into EVs.

Taken together, the cargo loaded into EVs is carefully selected by several involved proteins, and is most likely a selective representation of the cell of origin and disease state.

5. Functional roles of extracellular vesicles

EVs facilitate transport of various molecules over long distances inside the body, which points to their involvement in many biological activities, including some of interest in the context of IBD. These include the modulation of immune responses, and maintaining and restoring intestinal barrier integrity. EVs can be released from both the apical and basolateral side of intestinal epithelial cells.

Due to the small size of certain EVs, they can migrate through the pores of the intestinal lining.[54] There they can fulfill their role as intercellular communicators and execute their different functions.

- Immunomodulation

EVs can modulate the immune response, for example by antigen presentation and immune cell activation. As mentioned above, some EVs carry MHC-I and MHC-II and their costimulatory molecules on their surface. EVs thus can transfer antigenic information between dendritic cells in the form of intact antigens or peptide-MHC complexes, after which the naïve dendritic cells will also be competent for immune cell stimulation.[55] Besides this indirect form of antigen presentation, EVs can mediate direct antigen presentation to T cells.[56] In addition, Zitvogel et al. showed that EVs isolated from cultured mouse dendritic cells primed T cell responses, both *in vitro* and *in vivo*.[57] Dendritic cell-derived EVs are also able to promote natural killer cell activation and proliferation.[58] Other immune cells that have been shown to secrete EVs include mast cells[59], neutrophils[60], B cells[61], and T cells.[62] Regarding the latter, EVs released from $CD4^+$ T cells promote B cell activation and proliferation, and antibody production.[63] EVs released by different innate immune cell types (mast cells, macrophages, neutrophils, natural killer cells…) thus have the capacity to influence adaptive immune responses (T and B cells), and vice versa, either by directly targeting these cells or indirectly by targeting dendritic cells.[64]

Together with dendritic cells and B cells, macrophages are considered among the most potent antigen presenting cells. Moreover, macrophages are essential for the maintenance of intestinal homeostasis.[65] Several studies have shown that macrophage activity is modulated by EVs. Mitsuhashi et al. found that EVs derived from the colonic luminal fluid of IBD patients contained high mRNA and protein levels of several inflammatory cytokines, and promoted macrophage migration.[66] Also, serum exosomes isolated from the DSS-induced acute colitis mouse model could activate macrophages.[39]

Regulatory T cells (Tregs) are critical in immune homeostasis, through active control of innate and adaptive immune responses.[67] Dysfunction of Tregs is associated with a failure of intestinal tolerance, and contributes to the pathogenesis of IBD.[68] EVs derived from Tregs were shown to induce other T cells to develop into the Treg phenotype.[69] Furthermore, both the development and proliferation of Tregs could be stimulated by EVs isolated from breast milk in a dose-dependent manner.[70]

Another important role of EVs in the innate immune response is their ability to regulate complement activity, hereby contributing to the pro- and anti-inflammatory immune balance, extensively reviewed in Karasu et al.[71] Since imbalance in the intestinal complement system has a large influence on

intestinal barrier function,[72] the involvement of EVs in this system could have a severe impact on intestinal inflammation.

- Restitution of the intestinal barrier

With intestinal inflammation and the subsequent breakdown of the intestinal barrier leading to epithelial dysfunction, a critical component in the recovery phase of IBD is restoration of the intestinal epithelial barrier and regaining gastrointestinal function. EVs and their components have shown to aid this process, implying also a significant clinical potential of EVs in IBD (see below).

An interesting protein in this regard is Annexin-1 (ANXA1), a calcium-dependent phospholipid binding protein which is known for its anti-inflammatory and inflammation-resolving properties.[73] Earlier studies indeed demonstrated the ability of this protein to resolve inflammation by binding to formyl peptide receptors (FPRs),[74] and to stimulate intestinal mucosal wound repair.[75] ANXA1 has also been shown to be upregulated in both newly diagnosed and late-stage CD, and in post-operative recurrent CD.[76] Leoni et al. found that ANXA1 is released in EVs, and that this release is increased in inflammatory conditions: incubation of epithelial cells with pro-inflammatory cytokines IL-1β, IFN-γ, and TNF-α led to an increased ANXA1 release in EVs.[77] To support this *in vitro* data, serum samples of IBD patients and healthy individuals were also analyzed, finding increased numbers of ANXA1-containing EVs in patients with active IBD compared to healthy individuals.

Another study has shown that increased release of EVs containing high levels of TGF-β1 from intestinal epithelial cells significantly contributes to restoration of the immune balance in the intestinal tract, and the alleviation of IBD.[78] EVs secreted by epithelial cells of the large intestine inhibited CD4+ T cell proliferation *in vitro*, with a noticeable stronger inhibitory effect if these vesicles were isolated from IBD mice compared to from healthy mice. These EVs were found to contain high levels of TGFβ1 and required EpCAM (epithelial cell adhesion molecule) to bind in the gastrointestinal tract. Intravenous transfer of these TGF-β1-rich EVs to DSS-induced colitis mice led to decreased colon shortening, reduced body weight loss, and less colonic inflammation.[78]

Besides the above examples of proteins present in EVs that influence barrier restitution, several miRNAs transported by EVs derived from immune-, epithelial- and endothelial cells have been implicated in intestinal homeostasis, each with different target genes and effects.[38] A few examples are miR-29a explained above, and miR-21. miR-21 influences epithelial permeability by activation of the PTEN/PI3K/Akt signaling pathway, and targeting Ras-related small GTP-binding protein B (RhoB) and cell division control protein 42 (CDC42), and is found to be increased in the serum of IBD patients.[38,79,80]

It should be noted that besides cell-derived EVs, microbiota-derived EVs also can have significant impact on immunomodulation and intestinal barrier integrity.[81] Examples of EVs derived from Gram-negative bacteria in the context of IBD have been previously reviewed.[81] While at first the presumption was made that EVs could not cross the thick cell wall of Gram-positive bacteria, more recently vesicle formation from Gram-positive bacteria was shown.[82,83] A decreased abundance of Gram-positives is frequently reported in IBD patients[84], and it was shown that Gram-positive commensal bacteria can induce colitis by recruiting monocytes and macrophages.[85] Whereas the general role of Gram-positive bacteria EVs in health and disease is being unraveled, their specific implications in IBD need more extensive research.[82]

6. Clinical potential of EVs in IBD

A general overview of different categories for clinical potential of EVs in IBD is given in **Figure 1**.

- <u>EVs as biomarkers</u>

Clinical practice today has many challenges, including reducing the diagnostic delay and the possibility to stratify patients to guide follow-up and treatment. Because of their currently limited sensitivity, molecular (genetic or serological) markers are at present not recommended to use in routine diagnosis by recent European guidelines.[6] Yet, given the limitations of clinical factors in identifying patients at risk of a complicated disease course, molecular markers have been explored as alternative or additional predictors, with varying success as reviewed in detail elsewhere.[86] EVs and their content could be promising candidates to further fill this gap. The main advantages of using EVs as disease biomarkers is their presence in many biological fluids (**Figure 1A**), and the more stable content of EVs as opposed to free circulating RNAs or proteins as the content is protected by the EV membrane. Also, with EV content being – at least in part – specific for the parental cell, isolation of these EVs from biofluids of patients, and characterization of their cargo, could be an interesting method for disease detection and monitoring.

A potentially interesting set of biomarkers in IBD are miRNAs. Research on this has shown promising results, with for example the identification of an increased expression of 46 circulating miRNAs isolated from serum, plasma, or peripheral blood among IBD patients.[87] While these all could be potential blood-based biomarkers, miRNAs residing in EVs are protected by the EV membrane and thus more stable and potentially even more promising. While there are no specific examples yet known for IBD, in the context of colorectal cancer, a combination of two EV mRNA markers showed high sensitivity, specificity and accuracy in diagnosing colorectal cancer.[88]

With respect to proteins, a potential EV protein biomarker for IBD is ANXA1. As discussed above, serum EVs isolated from IBD patients with active mucosal inflammation have higher ANXA1 levels than healthy controls, and even more so in patients with higher levels of mucosal inflammation.[77] This suggests that EV ANXA1 levels could serve as biomarker for active mucosal inflammation, and are even correlating with the severity of inflammation.

Another promising biomarker is the proteasome subunit alpha type 7 (PSMA7). Research comparing protein profiles of EVs isolated from the saliva of UC and CD patients, and healthy controls, showed eight proteins that are only present in the EVs of IBD patients.[89] One of these is PSMA7 which in addition also correlated with disease activity. Significantly lower levels of EV PSMA7 were detected in IBD patients in remission than in patients with active disease, also highlighting the potential of EV-based biomarkers for monitoring disease. EV PSMA7, and the other seven IBD-specific proteins (TKT, TLN1, WDR1, NUCB2, BASP1, PSMB7, IGHV4OR), could thus be potential biomarkers for IBD.

Despite their promise, currently there are no examples available where EVs (or their content) are used in a clinical setting as biomarkers for diagnosis or prognosis of a chronic (auto)immune disease. Several steps are needed before this will even be possible. These include increasing fundamental knowledge; standardization of sample collection and efficient isolation methods for EVs[90], and further processing pipelines for application in clinical settings[91]; development of high-quality ISO-norm compliant assays; and clinical trials.[92]

- <u>EVs as therapeutic targets</u>

Even with the rapidly evolving therapeutic options in IBD, many patients do not respond to current treatment modalities or lose response over time.[10,12] This underscores the need for continued efforts towards exploiting novel therapeutic approaches in IBD.

It is clear that EVs are implicated in many pathological functions. Once they are taken up by their target cells, EV mRNA can be translated, EV proteins can directly activate or inhibit biological pathways, and EV miRNAs can regulate mRNA silencing and post-translational regulation of gene expression (**Figure 1B**). The involvement of EVs in intercellular communication and in IBD pathophysiology suggests that targeting EV biogenesis or EV content could be a promising therapeutic option. This section will give an overview of different strategies that involve the therapeutic targeting of EVs to alleviate disease progression, and how they could be employed in IBD (**Table 2**).

i. Inhibition of EV biogenesis

As described above, the main players involved in EV biogenesis are the ESCRT-machinery and their associated proteins. Multiple studies have shown the effect of inhibition of these proteins and the

subsequent inhibition of EV production and secretion. One of these target proteins is VSP4 which associates with ESCRTIII to function in membrane budding and scission (**Figure 1B**).[93] Inhibiting VSP4 in HEK293 cells resulted in reduced release of EV proteins and EV RNA, corresponding with an overall decrease in the number of EVs.[94] Further therapeutic possibilities include inhibition of ceramide formation by inhibiting n-SMases, blocking of tetraspanins, or interfering with the syndecan/syntenin/ALIX complex.[95] The consequences of their inhibition should however be carefully studied, as for example inhibition of n-SMases has been shown to not only result in inhibition of exosome formation, but also in an increased microvesicle formation.[96]

Instead of targeting the production of EVs itself, an alternative strategy is aimed at inhibiting the release of EVs from their parental cell. Specifically, this would involve suppressing proteins involved in the docking and fusion of the EVs with the plasma membrane. It was shown that knockdown of different Rab GTPases could prevent exosome secretion by inhibition of multivesicular body docking (**Figure 1B**).[31] Furthermore, manumycin A, a potent inhibitor of the Ras signaling pathway – to which Rab GTPases belong – also suppresses exosome biogenesis and secretion.[97]

For more information on inhibiting EV biogenesis and release, we kindly refer to a more extensive review.[98] Although inhibition of the discussed target proteins might be an option for therapy, to date no studies have been performed assessing the potential of this targeted inhibition in relation to IBD.

ii. Inhibition of EV uptake

EV uptake is established by several different energy-dependent mechanisms like phagocytosis, macropinocytosis, and clathrin-dependent/-independent endocytosis.[99] These EV uptake mechanisms can be blocked in multiple ways such as by inhibition of phosphatidylserine on the EV membrane; siRNA-mediated knockdown of proteins important in EV uptake; and blocking EV internalization proteins with cell-permeable inhibitors (e.g. dynasore) (**Figure 1B**).[100-102] An extensive summary of numerous compounds, chemicals, and peptides that could block EV uptake in different cells is provided in the review by Mulcahy et al.[99] The existence of different parallel processes that regulate EV uptake might make it difficult to completely abrogate the process, but still it should be considered as a viable therapeutic option.

iii. Other therapeutic options

Since the composition of EVs change in different conditions, targeting the cargo of EVs might also be a feasible option.[103] An example is miR-21. miR-21 is overexpressed in UC patients and can impair intestinal epithelial barrier function.[104] Another study showed that by activating a substance P related pathway, human colonic epithelial cells can create miR-21 enriched exosomes and hereby transfer miR-21 to neighboring naïve colonic epithelial cells.[105] Together these studies suggest that the

possibility of inhibiting or silencing miR-21 in EVs might be a possible therapeutic approach for the treatment of IBD.

- EVs as therapeutic agents

Apart from targeting EV biogenesis or uptake as a therapeutic option, using EVs as direct therapeutic agents holds great promise (**Figure 1C**). This therapeutic approach uses the beneficial properties of EVs secreted *in vitro* from different cells, or even autologous EV transfer (**Table 2**). A few examples that showed beneficial effects of EVs as therapeutic nanomedicine for immunomodulation and recovery of IBD in animal models are given here.

A first possibility is to simulate the pro-repair effect of endogenous ANXA1. A proof of principle was shown by Leoni et al. who did an intramucosal injection of nanoparticles containing the ANXA1 mimetic peptide Ac2-26.[77] By engineering these nanoparticles to have collagen IV at their surface, they are directed to vessels and injured mucosa. This approach has shown to induce recovery in DSS colitis mouse models and colonic biopsy-induced wounds.[77] As EVs would be even better vehicles than nanoparticles (seen below in the part on EVs as delivery vehicles), this option should certainly be considered.

An emerging aspect in the field of EVs involves the therapeutic potential of parasites and their secreted EVs. EVs derived from *Nippostrongylus Brasiliensis*, a gastrointestinal nematode, protected against intestinal inflammation in a TNBS-induced mouse model of colitis. This was indicated by a reduced pro-inflammatory cytokine profile (IL-1β, IL-6, IL-17a, and IFNγ), weight recovery, reduced tissue inflammation, and the absence of ulcerations or mucosal edema.[106] Another application of parasites in IBD EV therapy is the use of the modulatory properties of *Schistosoma japonicum*. Wang et al. treated dendritic cells with soluble egg antigen (SEA) of *S. japonicum*, and EVs derived from treated dendritic cells were then isolated for treatment of DSS-induced colitis. This resulted in reduced cytokine expression (TNF-α, IFN-γ, IL-17A, IL-12, IL-22), ameliorated colon length, decreased severity of diarrhea/bleeding, and improved macroscopic and histologic scores of the colon.[107]

The use of exosomes from different subtypes of M2 macrophages has been analyzed as an IBD treatment in the DSS-induced mouse model of colitis.[108] Administration of exosomes from M2b regulatory macrophages, that play a role in Th2 activation and immunoregulation, significantly alleviated colon damage and improved colon length as well as macroscopic and clinical scores.

For medicinal use, EVs from mesenchymal stem cells and antigen-presenting cells seem to exhibit the most promising properties.[109] Numerous pioneering studies have been published assessing the potential of mesenchymal stem cell derived EVs for IBD therapy.[110-114] They all showed improved

disease activity index (DAI) scores and positive effects on mitigating DSS-induced colitis after treatment with these EVs. Besides direct IBD-related improvement, introducing these EVs also had effects on the modulation of immunity, suppression of oxidative stress, and alleviation of apoptosis. Both immature and mature dendritic cells can produce and secrete EVs with certain immunomodulatory properties. A study assessing these beneficial properties for IBD therapy administered EVs derived from dendritic cells treated with IL-10 to a rat TNBS-induced colitis model.[115] These EVs were able to reduce all analyzed clinical, macroscopic, and histopathologic colitis parameters.

Lastly, a very recent study assessed the possibility of autologous EV transfer in DSS-induced colitis, showing very promising results.[116] Intestinal exosomes obtained from mice in the healing phase after colitis induction showed high *in vitro* and *in vivo* anti-inflammatory activity. These exosomes were orally administered to the same mice after re-inducing colitis, and were able to specifically target the colon and reduce intestinal inflammation without inducing any immune response in the host. This example shows that this type of approach could be safe and effective for treating IBD, and contribute to the possibility of personalized medicine.

Similar as with biomarkers though, certain aspects need to be taken into consideration before EVs will be able to be used as therapeutic agents in a clinical setting. Examples include: exploring all risks related to the administration of EVs, determining long-term effects, and establishing the adequate dosage of EVs for therapy.[117]

- <u>EVs as delivery vehicles</u>

Finally, EVs could be used as vehicles to deliver drugs and/or nucleic acids to specific tissues or cells (**Figure 1D**), and thus provide a targeted approach.

To date, mostly liposomes or nanoparticles have been used to introduce drugs. With the reduced toxicity of EVs and their great stability in biological fluids and circulation, EVs however may pose as the better alternative. Moreover, the addition of cell- or tissue specific targeting ligands to the surface of EVs provides the possibility to target specific tissues and minimize potential cytotoxicity and side effects. In comparison with regular delivery of therapeutic compounds to patients, loading them into EVs will also protect them from degradation. Results of a recent phase 2 clinical trial applying drug-loaded EVs for chemotherapy in lung cancer patients highlighted the safety and feasibility of using EVs as therapy, and suggested potential clinical activity.[118] Although research surrounding EV delivery has not yet come that far in the IBD field, several options for loading components into EVs and subsequently delivering these EVs into living organisms exist.

Antimisiaris et al. distinguishes two methodologies for loading drugs into EVs: before and after their isolation.[119] Firstly, the parental cells could be modified, e.g. by transfection, to produce certain proteins or drugs which could then be released in EVs by traditional biogenesis. Besides this endogenous loading of EVs, drugs can also be loaded into the EVs exogenously by means of incubation, electroporation, or sonication. These methods have been extensively reviewed in Tang et al., together with several advantages and disadvantages.[120]

Several small molecule drugs (e.g. doxorubicin, which is used for tumor treatment) have already been successfully loaded into EVs.[21] These EVs were modified with ligands such that they could be specifically targeted to the tumor cells, and showed large *in vitro* and *in vivo* anti-tumor effects after intravenous delivery. Compared to intravenous delivery, intraperitoneal and subcutaneous injections resulted in significantly lower EV accumulation in the liver, but an increased accumulation in the pancreas and gastrointestinal tract.[121] This suggest that for targeted EV therapy in IBD, an intraperitoneal or subcutaneous injection approach would probably be favored.

Besides delivery of drugs, EVs could also be used for gene therapy by transporting and delivering nucleic acids to target cells. The possibility of isolating EVs from a patient, loading them with RNA, and introducing them back into the same patient,[122] highlights the low immunogenicity of EV therapy. Loading siRNA and miRNA into other carriers than EVs as RNA-interference therapy for IBD has been considered before.[123] The option of using EVs instead should thus surely also be considered.

Despite the increased attention to bio-inspired drug delivery systems, the potential of EVs has been relatively unexplored in the field of gastrointestinal diseases. However, with the extending research regarding the modification of EVs, and the potential to load them with numerous compounds, a few proposals could be made. For example, studies could be performed inserting therapeutic agents into EVs which have proven to be effective in treating IBD in animal models, such as the ANXA1 mimetic peptide. Another option is uncovering the potential of autologous EV transfer in IBD patients. Several steps still need to be taken to enable EVs to be used in IBD therapy, but the discussed research shows the great potential these small vesicles hold.

It should however be noted that there are also some potential downsides to working with EVs. These include the non-specific isolation of EVs (i.e. the co-isolation of several other components), and the low-yield production of EVs which makes large scale production difficult. For a more extensive elaboration on the advantages and challenges of designing EVs for drug delivery, as well as further directions for clinical application, we kindly refer to other reviews (e.g. Armstrong et al. and Fais et al.).[124,125]

7. Conclusion

In this review we have discussed the current knowledge about the biogenesis, cargo, and function of extracellular vesicles; and how they could be involved in IBD pathogenesis, or exploited as biomarkers or treatment for IBD. Although the exact details of their role in IBD remain to be fully elucidated, it is clear that their capacity to transport cell-derived cargo and their immunomodulatory properties make them potential key players in modulating intestinal inflammation and maintaining or restoring intestinal barrier integrity. Elevated levels of EVs and/or EV content have been identified in IBD patients, suggesting EVs should be considered potential candidates when looking for biomarkers for disease diagnosis, prognosis, and therapeutic response, and for potential therapeutic strategies in the context of IBD. To exploit EVs as a biomarker tool, establishing a consensus surrounding the isolation methods and the standardization of EV analysis will however be necessary.

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Conflict of Interest

None of the authors declare any conflict of interest related to this work.

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Author Contributions

M.V. and S.V. conceptually designed the review and figures. M.V. searched the literature, prepared the tables and wrote the manuscript. S.V. and J.A.F.F. provided intellectual input and edited the manuscript. S.V. and J.A.F.F. prepared the figures. I.C. provided intellectual input, participated in the conceptual design of the review and assisted in writing the manuscript.

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Figure Legends

Figure 1: The potential of EVs in IBD. EVs are important mediators of intercellular communication, enabling the functional transfer of molecules from one cell to another. Although studies focusing on EVs in the context of IBD are limited, they are supportive for important roles of EVs in this disorder, demonstrating their clinical potential. (a) EVs have biomarker potential for many diseases, including IBD, as they are present in several body fluids, and can provide unique molecular signatures for early detection, disease progression and prediction of therapy response in IBD; (b) EVs are involved in many pathological functions, suggesting that disease progression could be alleviated by inhibiting the biogenesis or release of EVs, or by targeting EV components and inhibiting their uptake in the target cell; (c) Regulatory EVs can be exploited as therapeutic agents to improve intestinal barrier repair and to restore the dysregulated immune system in IBD; (d) EVs can also be involved in drug delivery systems that use EVs as vectors to deliver therapeutic entities to the site of repair. EVs can for example be pre-loaded with therapeutic cargo – either directly or via modified parental cells, including small molecules, nanoparticles, proteins and oligonucleotides. EE, early endosome; LE, late endosome; EV, extracellular vesicle; MVB, multivesicular body

Figure 2: Schematical overview of the membrane composition and cargo of EVs. The membrane consists of a lipid bilayer, and also includes different types of transmembrane proteins that can either be specific for the originating cell (f.e. MHC-I and MHC-II), or more general (f.e. tetraspanins). Examples of possible intravesicular proteins, and lipids and metabolites are given. Nucleic acids like DNA, mRNAs of short length, and several non-coding RNAs (e.g. miRNA, tRNA, rRNA) can also be loaded into EVs.

Tables

Table 1: Examples of EV secreting cell types and EV containing biological fluids

		References
Secreted by	Mesenchymal stem cells	Zhang et al., 2014
	Intestinal epithelial cells	Van Niel et al., 2002
	Platelets	Heijnen et al., 1999
	Tumor cells	André et al., 2002
	Lymphocytes (B & T)	Raposo et al., 1996; Blanchard et al., 2002
	Dendritic cells	Théry et al., 1999
	Mast cells	Skokos et al., 2001
	Neutrophils	Majumdar et al., 2016
Present in	Blood	Caby et al., 2005
	Saliva	Michael et al., 2010
	Urine	Gonzales et al., 2010
	Breast milk	Wang et al., 2017
	Nasal secretions	Lässer et al., 2010
	Cerebrospinal fluid	Street et al., 2012
	Intestinal fluids	Wong et al., 2016
	Faeces	Park et al., 2018

Table 2: Some examples of how EVs could be used as therapeutic targets or agents.

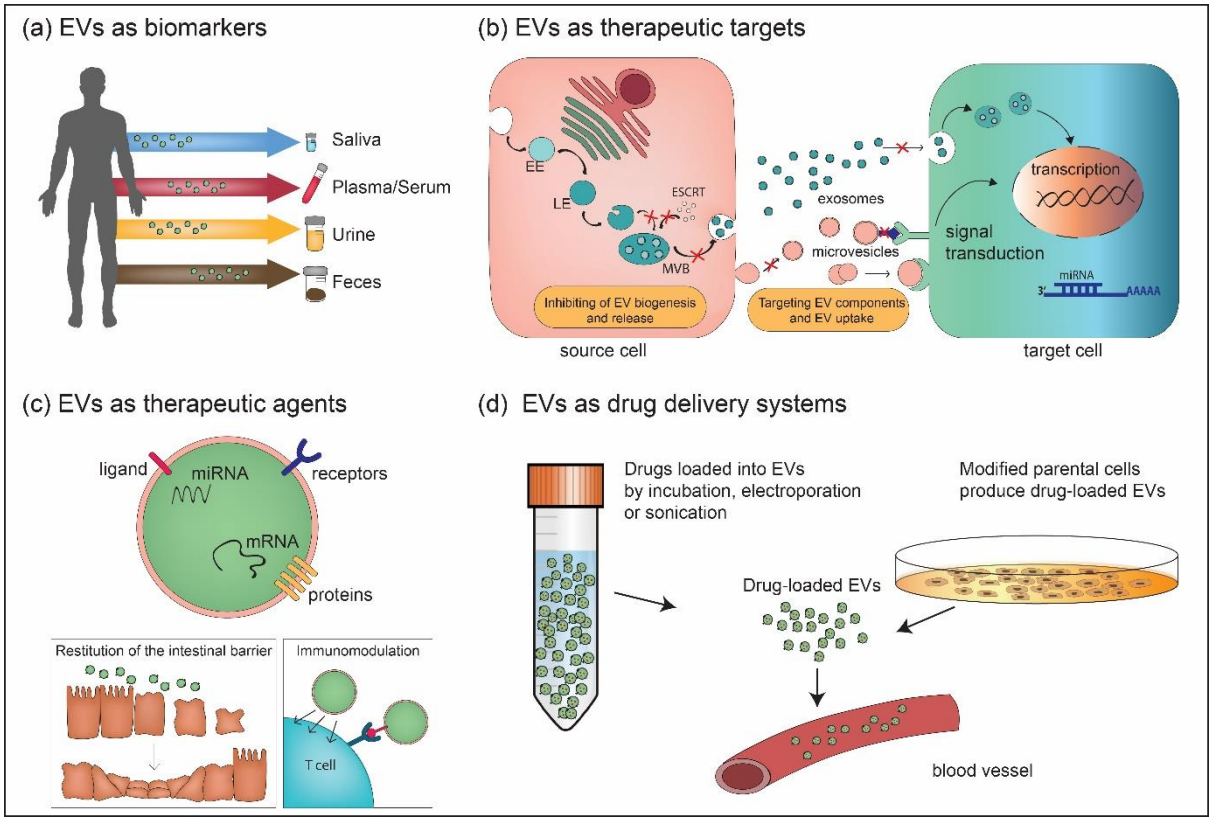
EVs AS THERAPEUTIC TARGETS		
	Inhibition of	**Reference**
Inhibition of EV biogenesis[1]	ESCRT machinery, f.e. VSP4	82
	ceramide formation, f.e. by targeting n-SMases	83
	tetraspanins	83
	syndecan/syntenin/ALIX complex	83
Inhibition of EV release[1]	multivesicular body docking, f.e. by targeting Rab GTPases	29
	Ras signaling pathway (f.e. by manumycin A)	85
Inhibition of EV uptake[2]	phosphatidylserine	88
	dynamin (f.e. by dynasore)	89
	siRNA-mediated knockdown	90
EVs AS THERAPEUTIC AGENTS		
	EVs originating from	**Reference**
External EVs	Nippostrongylus Brasiliensis	94
	dendritic cells treated with S. Japonicum soluble egg antigen	95
	M2b regulatory macrophages	96
	bone marrow mesenchymal stem cells	98, 102
	umbilical cord mesenchymal stem cells	99, 100, 101
	dendritic cells treated with IL-10	103
Autologous EV transfer	IBD healing/recovery phase	104

[1]Note that more targets are possible. We kindly refer to for example the review by Catalone et al.[86]

[2]An extensive summary of compounds that could block EV uptake is for example provided in the review by Mulcahy et al.[99]

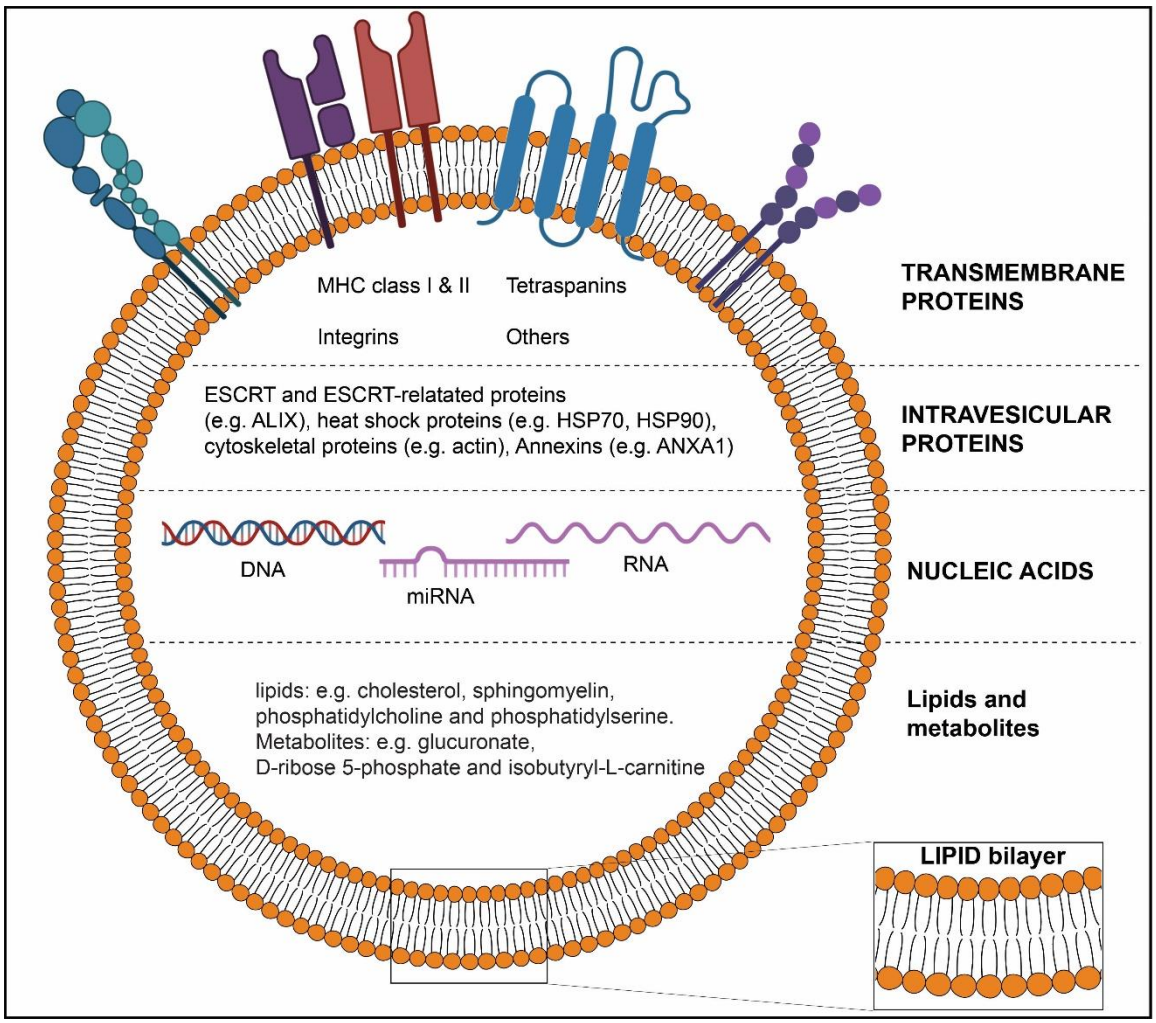
802 **Figures**

803 Figure 1



804

805 Figure 2



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