Extracellular vesicles derived from gut microbiota in inflammatory bowel disease and colorectal cancer

Giusi Alberti[a#], Margherita Mazzola[a#], Carola Gagliardo[b], Alessandro Pitruzzella[a], Alberto Fucarini[a], Marco Giammanco[c##], Giovanni Tomasello[a##], Francesco Carini[a##]

The human gut microbiome encompasses inter alia, the myriad bacterial species that create the optimal host-microorganism balance essential for normal metabolic and immune function. Various lines of evidence suggest that dysregulation of the microbiota-host interaction is linked to pathologies such as inflammatory bowel disease (IBD) and colorectal cancer (CRC). Extracellular vesicles (EVs), found in virtually all body fluids and produced by both eukaryotic cells and bacteria are involved in cell-cell communication and crosstalk mechanisms, such as the immune response, barrier function and intestinal flora. This review highlights advancements in knowledge of the functional role that EVs may have in IBD and CRC, and discusses the possible use of EVs derived from intestinal microbiota in therapeutic strategies for treating these conditions.

Key words: extracellular vesicles, gut microbiota, inflammatory bowel disease, colorectal cancer

Received: February 25, 2021; Revised: May 10, 2021; Accepted: June 16, 2021; Available online: July 2, 2021
https://doi.org/10.5507/bp.2021.042

[a]Institute of Human Anatomy and Histology, Department of Biomedicine, Neurosciences and Advanced Diagnostics, (BIND), University Hospital Policlinico Paolo Giaccone of Palermo, Palermo, Italy
[b]Department of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties, Palermo, Italy
[c]Department of Surgery, Oncologicical and Stomatological Sciences (Di.Chir.On.S), University Hospital Policlinico Paolo Giaccone of Palermo, Palermo, Italy
[#]These authors contributed equally
[##]These authors contributed equally
Corresponding author: Margherita Mazzola, e-mail: margheritamazzola@hotmail.it

INTRODUCTION

In the last few years, the intestinal microbiota has become a focus of prolific research for its pivotal role in human health and disease. It is understood that our gut hosts more than 1000 bacterial species, defined as our hidden metabolic "organ" for its immense impact on human wellbeing. The gut microbiota consists of a multispecies microbial community composed of bacteria, viruses, yeast, fungi, and others capable of establishing symbiosis with the host organism[1,2]. In particular, about 90% of the total microbiome of the mammalian gut is represented by two main commensal bacteria groups: Firmicutes (gram-positive bacteria) and Bacteroidetes (gram-negative bacteria) (ref.[3]). The microbial community has been shown to be implicated in several processes ranging from energy harvest and storage[4], to normal intestinal development through several mechanism, among which the processes that lead to the formation of short chain fatty acids (SCFA), such as butyrate involved in intestinal homeostasis [5]. Furthermore, some commensal bacteria (e.g. Bacteroides fragilis) participate at the synthesis of vitamins, such as B vitamins, with an important role in the maintenance of immune homeostasis[6]. It has been demonstrated that commensal bacteria shape oth innate and adaptive immunity[7]. In particular, the host-commensal microbiota communication is triggered from soluble mediators and extracellular vesicles (EVs) that can diffuse through the mucin layer, influencing the maturation and development of the digestive and immune system[8,9]. From a physical point of view, this is guaranteed by the separation of commensal bacteria from the epithelial layer by of a highly compact mucus layer which prevents the entry of bacteria[10]. A mechanism that favors the host-commensal bacteria interaction involves EVs derived from bacteria that carry molecules with signaling properties, in combination with other mechanisms. The EVs released by commensal bacteria can be taken up by eukaryotic host cells modulating changes, as well as alteration of the gene expression[11]. For example, EVs derived from B. fragilis are enriched in immunomodulatory molecules (i.e. capsular lipolysaccharide A, PSA) which induces protection against colitis by an autophagy pathway that involves genes associated with IBD, Nucleotide Binding Oligomerization Domain Containing 2 (NOD2) and Autophagy related 16 like 1 (ATG16L1) (ref.[12]). Likewise, bacterial EVs when absorbed by macrophages can induce a massive release of pro-inflammatory cytokines, such as Tumor Necrosis Factor alpha (TNF-α) and interleukin -6 (IL-6). By contrast, EVs released from the bacterium Campylobacter jejuni possess cytotoxic

activity able to induce an immune response in the host[13], and EVs-derived from Bacteroides thetaiotaomicron have the ability to cross the intestinal mucosal barrier reaching the intestinal cells and initiating a localized inflammation process[14]. Despite the important functions of bacterial EVs, to date, the mechanism of biognesis remains poorly known vis a vis that of eukaryotic cells. Consequently, it is plausible to think of an involvement of EVs derived from intestinal microbiota in the systemic inflammatory responses with an important role in the development of colorectal cancer (CRC). Here, we review the different mechanisms of inter-cellular communication between bacterial and host cells in CRC and their potential effect on inflammatory bowel disease (IBD) (ref.[15-19]).

EXTRACELLULAR VESICLES DERIVED FROM GUT BACTERIAL

Currently, the ubiquity of EVs in all the kingdoms of life, iks well-known, underlining their high evolutionary importance[20]. All gram-negative and some gram-positive bacteria constitutively generate EVs from the outer membrane and accordingly, namely outer membrane vesicles (OMVs) [(Ref. 20,21)]. Briefly, OMVs are characterized by an external lipopolysaccharide sheet and an internal phospholipid sheet, enriched in membrane and surface proteins given their origin. Unlike mammalian EVs, OMVs are particularly enriched in cell wall components, peptidoglycans, lipopolysaccharides (LPS), phospholipids, as well as soluble proteins, and others[22]. Although OMVs are passively produced under natural conditions, they may be released in greater numbers in response to stress, either via explosive cell lysis or actively[23]. However, the exact mechanism that results in outer membrane budding remains unknown making it challenging to unravel the basic mechanisms of vesicle transport. In consideration of this, several models for OMV biogenesis are currently being discussed, as well as autolysin[24], DNA fragments[25,26], biofilm formation[27,28], toxin delivery[29], antibiotic resistance[30], and the transfer of nucleic acids[31-33]. Generally, they have ranges from less than 100 nm to a few hundred nanometers, and are considered to be distinct communication system between cells and cooperation including multicellular development, quorum sensing, and virulence factors[34-36]. Also, OMVs surrounding bacteria can protect from viral attack for example by absorbing viruses[37], or to excrete misfolded proteins following to stress response[38]. In addition to this knowledge, it should be added that, OMVs have the immunogenic capacity to carry a wide spectrum of endogenous antigens, and the natural self-adjuvanticity exerted by toll-like receptor (TLR) agonists, such as LPS (ref.[39]). Considering this, we will briefly discuss the process of Gram negative and Gram positive EVs biogenesis.

Gram-negative bacteria

The first identified of the presence of OMVs in gram-negative bacteria dates back to 1960s by observing bacterial structure by electron microscopy[40]. Gram-negative bacteria have two main pathways for vesicle biogenesis. Brefly, the first pathway of formation involves the blebbing of the outer membrane of the bacterial envelope with generation the OMV, while the second involves the explosive cell lysis with formation of vesicles of the outer-inner membrane (OIMV) and explosive vesicles of the outer membrane (EOMV) (ref.[41]). EVs produced by Gram-negative bacteria secreted EVs with sizes ranging from 10 to 400 nm by breaking the connection directly in specific areas[42-43]. This process can lead to the incorporation of peptidoglycan and fragment of OM-peptidoglycan bridging proteins in the OMV[44], since they have a cell wall consisting of a thin layer of peptidoglycan in the periplasmic space between two double layers of membrane, the inner (or cytoplasmic) membrane and the outer one[41]. The outer membrane contains LPS and many membrane-bound proteins and channels, such as porins, that intervene in the non-vesicle mediated transport[41].Given this architecture, the outer membrane of Gram-negative is known to engage Toll-like receptor 4 (TLR4)[(Ref.45)]. Gram-negative EVs carry bacterial components including proteins, peptiglycan, LPS, O-antigen, nucleic acid, enzymes and other moleculs[44-49], and probably the different biogenesis route is responsible of a different composition. Gram-negative EVs are involved in intra-and-intercellular communication and mediating a plethora of biological processes. Accordingly, they can also deliver their cargoes (e.g toxins and virulence factor) to prokaryotic kingdoms and eukaryotic cells regulating many processes including host-pathogen interaction, regulation of the host immune response, and others[50,51]. Of note, intraluminal DNA from gram-negative EVs has been observed to be enriched in specific regions of the bacterial chromosome and to be involved in virulence, stress response, antibiotic resistance and metabolism[52].

Gram-positive bacteria

In the 1990s, it was discovered that Gram-positive bacteria (*Bacillus subtilis* and *Bacillus cereus*) were also able to release membrane vesicles[53]. Unlike gram-negative bacteria, gram-positive bacteria lack an outer membrane and present a thick peptidoglycan layer composing their cell walls[41]. To date, the specific mechanisms behind the release of EVs in Gram-positive bacteria are not fully understood, but support for the hypothesis that describes the origin of the EVs from the cytoplasmic membrane named as cytoplasmic membrane vesicles (CMVs) (ref.[41,54]). Consequently, EVs-secreted from Gram-positive bacteria do not show the characteristic toxicity of the LPS, the main component of the bilayered lipids of OMVs, but are particularly enriched in lipoteichoic acid (LTA) that might engage the Toll-like receptor 2 (TLR2) (ref.[55]). This difference in composition makes it possible to distinguish EVs from gram positive bacteria[56,57]. EVs secreted by gram-positive bacteria intervene in the pathogen-host interaction and in the regulation of inflammatory processes by their molecules cargo[58]. For example, it has been demonstrated that the pneumococcal surface protein A (PspA), expressed by all strains of *Streptococcus pneumoniae*, when introducted into a gram-negative *Salmonella enterica* strain and, in turn, released into EVs, is capable

of conferring immunity to mice treated with the latter [59]. For their safety features and others, EVs released from gram-positive bacteria are currently under investigation as an antigen adjuvant for vaccine candidate[60].

POTENTIAL OF BACTERIAL EVS IN IBD AND CRC

EVs play an important role in carcinogenesis, particularly in cancer progression and growth. They interacting with a variety of cells within the tumour microenvironment favoring cell proliferation, angiogenesis (e.g. thourgh the induction of vascular endothelial growth factor (VEGF) expression and the autocrine activation of its receptor), promoting metastases, and transmitting chemoresistence abilities to nearby cells. Also, EVs might play a role in the induction of immune tolerance in cancer cells, for example, by modulating the activity of T cells[61,62]. EVs have been studied in many pathological and non- pathological conditions, including CRC and IBD. IBD forms a group that encompasses chronic autoimmune diseases that affect the gastrointestinal tract. They are emerging as an inflammatory component coupled with immune dysregulation responsible for the damages to the gastrointestinal tract[63]. Regardless of source of the EVs, they can be released by immune cells (i.e. macrpghages, monocytes and dendritic cells), intestinal epithelial cells (IECs), stem cells, tumor cells, and others. Besides, EVs from some nutritional sources have attracted interest due to the fact that they are ingested daily and therefore are generally considered safe. For example, breast milk contains a diversity of biologically active components like EVs that, together with the microbiota, assist in mucosal tissue, immune system and microbiome development and maintenance[64]. This highlights that EVs do not just regulate gut microbiome communities, but actively participate in the disharmony between bacteria and their hosts. Here the focus on associations that are the focus of greatest attention, that is, the possibility of a link between the gut microbiota and a chronic gastrointestinal disease, i.e IBD, as well as the onset of CRC (Table1).

IBD

IBD is the consequence of a dysregulated mucosal immune system and has been extensively studied. IBD patients have a higher concentration of EVs than the healthy subject, thus representing potential biomarkers. The expression of such biomarkers within the serum and tissues of IBD patients would indicate the onset of a molecular and genetic imbalance originating probably from pro-inflammatory conditions. EVs contain molecules from their parental cells, including proteins, lipids, and miRNAs, through which they can intervene in different processes, including promoting pro-inflammatory conditions[71]. EVs derived by intestinal luminal aspirate from IBD subjects contain markedly higher mRNA and protein levels of IL-6, interleukin – 8 (IL-8), and TNF-α than those of healthy controls. In detail, EVs are absorbed by colonic epithelial cells, resulting in an increase in the level of IL-8 expression and subsequent induction of macrophage migration by epithelial cells[72]. IECs are among the first to perceive luminal stimuli related to the entry of food, pathogens and more capable of triggering the encounter with IBD. It has been reported that EVs released from IECs play important roles in immune tolerance, and can function critically in immune responses in the pathogenesis of IBD[73]. EVs secreted by the IECs interact preferentially with dendritic cells (DCs), thus intervening in the presentation of exogenous antigens through their major histocompatibility complex of class II (MHC-II) (ref.[73]). In this manner, these EVs link local immune cells and luminal antigens in a powerful way through mediated transfer of luminal antigenic information and to facilitate immune surveillance on mucosal surfaces[73]. EVs released by IECs are able to limit expansion of CD4+ T cells, and of T helper 1 (Th1) and T helper 2 (Th2). In turn, IECs intervene in the interaction between gut microbiome and immune cells[74]. EVs luminal play important role in the IECs-microbiome-immune system interaction to maintain mucosal homeostasis[74]. Other studies have demonstrated that the involvement of EVs in the activation of macrophages having a key role in the pathogenesis of IBD, because they are involved in the maintenance of homeostasis and regulation of the intestine[75]. In the IBD microenvironment, EVs inducing Treg, regulatory DCs and M2 phenotype macrophages, resulting in immunosuppressive action on the host immune system. Major histocompatibility complex of class I (MHC-I) on the surface of EVs can mediate apoptosis of CD8+T cells through regulating the activity of natural killer cells and DCs mediating inflammation tolerance[76]. Besides, in the pathogenesis of IBD, have been investigated he heat shock protein (Hsp), as Hsp70 particularly enriched in EVs (ref.[77]). Exosomal HSP70 interact with gram-negative

Table 1. Correlation between gut microbiota diversity and pathological condition.

Pathological condition	Increase of some microbes in relation to the disease	Decrease of some microbes in relation to the disease	Ref.
IBD (incl. CD and UC)	Gamma–proteobacteria, Enterobacteraceae, Escherichia coli, Clostridium spp.	Firmicutes Bacteroidetes Lachnospiracheae Clostridium leptum Coccoides group	65-67
CRC	Fusobacterium spp. E. coli	Not dectable	68-70

bacteria receptors, i.e. TLR4 and gram-positive bacteria receptors (TLR2), to stimulate proinflammatory responses and, also, exosomal heat shock protein 72 (HSP72) in the functions of IECs (ref.[78]). The upregulation of heat shock protein 60 (Hsp60) in cells typical of inflammation of the lamina propria (eg CD68 cells), following alterations in the homeostasis of the MuMi layer, suggests that this chaperonin may be involved in the activation of the immune system and therefore in evolution of the inflammatory process[79]. Accordingly, EVs might play a pro-inflammatory role during active IBD, by inducing, maintaining and regulating the required functions of intestinal tissues. These modulatory properties exhibited by EVs make them ideal candidates for the treatment and prevention of IBD relapses[79]. Currently, hyperactivation of pro-inflammatory pathways is blocked i.e by inhibition of tumor necrosis factor TNF-α, gut-homing α4β7 integrin, interleukin 12 (IL-12) (ref.[80]), given their capacity to induce serious negative effects, including infections and malignancies[81]. EVs-derived from enteropathogenic bacteria induce the secretion of intestinal mucosa-derived EVs carrying an elevated level of C-C motif chemokine 20 (CCL20) and prostaglandin E2 (PGE2) causing inflammation[82]. Oxidative antimicrobial activity induces an increase in proteins in EVs at the interface between the intestinal mucosa and the intestinal lumen in IBD patients compared to control subjects[83]. Intestinal microbiota plays a major role in the development of IBD (ref.[84]). In Figure 1, we show the involvement of gut microbiota in maintaining intestinal homeostasis following activation of inflammatory pathways underlies IBD pathogenesis[85].

Fig. 1. Schematic illustration of involvement of gut microbiota in maintaining intestinal homeostasis following to activation of inflammatory pathways underlies IBD pathogenesis. Both macrophages and DCs actively promote the transition from inflammation to the return to homeostasis after immune system activation, by internalizing EVs which mediates an immunomodulatory response through Toll-like receptors (TLR2 and TLR4) signaling. Once activated, the DCs promote the release of anti-inflammatory cytokines, such as IL-4, IL-10, and IL-22, and down regulating of pro-inflammatory cytokines. Also, EVs interacting with intestinal epithelial cells favoring the expression of tight junction proteins and, in turn, modulating cytokine secretion, with consequent reinforcementof the intestinal barrier. Both mechanisms contribute, modulation of the immune response and improvement of the gut barrier[85].

Microbial dysbiosis is an important contributing factor to oncogenesis and tumor progression including colorectal cancer, which may also adversely affect treatment response to chemotherapy and immunotherapy[86].

CRC

Colorectal cancer (CRC) is one of the most common malignant tumors, ranking in the top 3 causes of cancer-related death worldwide. High-throughput microbiome sequencing has shown that patients with CRC have reduced bacterial diversity and richness compared with those of healthy individuals[87]. However, conflicting results make the precise community dynamics between the gut microbiota and CRC unclear. It has been shown that one of the most consistent bacterial groups associated with CRC carcinogenesis is Bacteroides spp., in particular Bacteroides fragilis implicated in an increase in inflammation[88,89]. Furthermore, the intestinal microbiota CRC

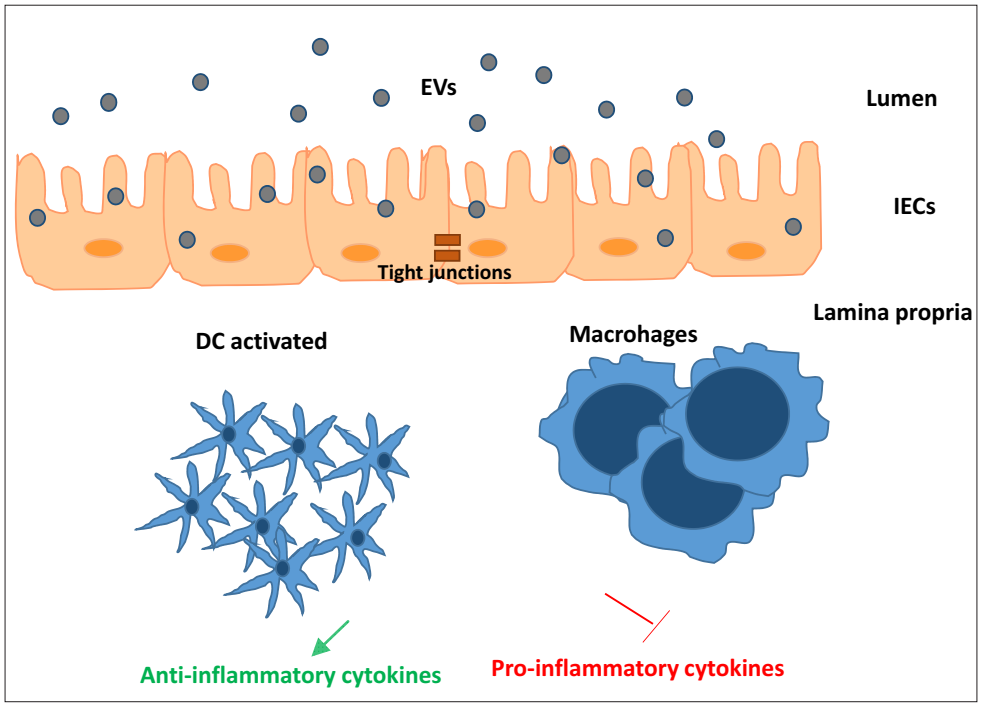

Fig. 1. Gut microbiota and intestinal homeostasis in pathogenesis of IBD.

is characterized by a lower presence of lactic bacteria, a greater presence of Fusobacterium and altered levels of Bacteroides/Prevotella[90]. Jung and coworkers demonstrated the correlation between microbial changes and metabolic alterations within EV samples from patients with CRC. There was a strong association between the abundance of gut flora (*Firmicutes* and *Proteobacteria*) and relevant candidate metabolites (predominantly amino acids) (ref.[90]). This suggests that altered composition of macronutrient-fermenting and degrading bacteria in CRC might result in the accumulation of amino acids and the depletion of energy sources. Moreover, our findings indicate that EVs secreted by gut microbes carry a dynamic range of metabolic information reflecting the host's nutritional state, metabolism, and immune responses in the presence of disease[91]. The mechanism by which bacteria affect carcinogenesis and tumor progression are different, for example they could act as tumor promoting entities by invoking tolerogenic immune reprogramming of the micro environment (TME). It has been proposed that EVs secreted by the gut microbiota could drive suppressive cellular differentiation in a TLR-dependent manner, to indirectly elicit T-lymphocyte anergy[92]. Also, it has been observed that EVs infected by toxins can be released by some intestinal bacteria promoting the development of CRC by exacerbating an inflammatory condition. For example, fragilisin toxin secreted into EVs by Bacteriodes fragilis is able to promote tumor growth of the colon mediating both splitting of E-cadherin[86] and production of IL-8 (ref.[79]). CRC patients have an increase in Bacteroidetes which can control inflammation by regulating the differentiation of Tregs. Capsular polysaccharide A released by B. fragilis has immunoregulatory properties by which it mediates the conversion of CD4 + T cells to Foxp3 + Treg via TLR2-mediated signaling. These cells have a greater suppressive capacity through increased production of the anti-inflammatory cytokine interleukine 10 (IL-10) (ref.[93]). Popēna et all have reported that primary CRC-derived EVs modulate the immunophenotype and secretory profile of monocytes and inactive macrophages towards M1 type of response whereas metastatic CRC-derived EVs induce a mixed M1 and M2 cytokine response in inactive macrophages in the THP-1 monocyte differentiation model. Furthermore, although CRC EVs decrease HLA-DR expression in M1 and M2 polarized macrophages, their effect on the secretory profile of these cells is negligible[94]. Together these studies provide evidence to support the notion that there may be two-way IV-mediated communication between bacteria and human host cells. However, to date, there is no mechanistic study investigating how bacterial EVs can impact oncogenesis and tumor progression, and their role is likely to be context-dependent.

CONCLUSION

Various lines of evidence suggest that dysregulation of microbiota-host interaction is linked to various pathologies, such as IBD and CRC. An ever-growing number of studies have highlighted the key role of EVs as a source of diagnostic and prognostic markers or as promising pharmaceutical vehicles. Scientific interest in EVs has been stimulated due to their key role in cell-cell and cell-organism communication. Many studies have shown that circulating EVs increase in patients with gastrointestinal tumors, compared to patients with inflammatory gastrointestinal diseases such as IBD[95-97]. Nevertheless, it is likely that the amount of circulating EVs is high in the active phase of inflammatory diseases in comparison to healthy people[98].A higher number of EVs was found in IBD patients in remission than in healthy donors supporting the inflammatory cell recruitment hypothesis[99]. Recently, one study has reported that intestinal luminal fluid is enriched in proinflammatory EVs of various origins (e.g secreted by cells that structure the intestinal wall or vesicles of the bacterial outer membrane) carrying markers IL8, IL6, IL10 and TNF (ref.[100,101]). Apropos CRC, a number of publications describe the microRNA profile in supernatants of epithelial cell line biomarker cultures as good biomarker candidates for CRC[102]. However, there is always a tendency to consider the functions of EVs on inflammatory gastrointestinal diseases and gastrointestinal tumors from the point where EVs originate only from eukaryotic cells, neglecting EVs originating from the human microbiota. This suggests a possible therapeutic role of AmEV in the treatment of chronic intestinal inflammation. Consequently, the administration of EVs derived from specific bacterial strains can modulate the immune signaling pathways and other related processes. Finally, the use of EVs could allow the construction of a network of ecological units organized within the intestinal microbiota capable of bringing about improvements in a number of pathological conditions.

Search strategy and selection criteria

Our aim was to offer an overview of EVs with particular attention to their possible therapeutic role. Expanding therapeutic perspectives towards IBD and CRC could guarantee better patient outcomes in terms of disease remission and life expectancy. Scientific articles were searched using the PubMed adatabases. All searches were up to date as of 2020. The search terms used included "extracellular vesicles", "gut microbiota", "inflammatory bowel disease", "colorectal cancer".

Author contributions: GA, MM: drafting, composition and correction of the article; CG, AF, AP: litearature research; MG, GT, FC: coordination, correction and evaluation.
Conflict of Interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

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