



Review

Extracellular vesicles in gastrointestinal cancer in conjunction with microbiota: On the border of Kingdoms



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ABSTRACT

Extracellular vesicle (EV) production is a universal feature of metazoan cells as well as prokaryotes (bacterial microvesicles). They are small vesicles with phospholipid membrane carrying proteins, DNA and different classes of RNAs and are heavily involved in intercellular communication acting as vectors of information to target cells. For the last decade, the interest in EV research has exponentially increased through thorough studies of their roles in various pathologies that was not previously possible due to technical limitations. This review focuses on research evaluating the role of EV production in gastrointestinal (GI) cancer development in conjunction with GI microbiota and inflammatory diseases. We also discuss recent studies on the promising role of EVs and their content as biomarkers for early diagnosis of GI cancers.

The bMVs have also been implicated in the pathogenesis of GI chronic inflammatory diseases, however, possible role of bMVs in tumorigenesis remains underestimated. We propose that EVs from eukaryotic cells as well as from different microbial, fungi, parasitic species and edible plants in GI tract act as mediators of intracellular and inter-species communication, particularly facilitating tumor cell survival and multi-drug resistance.

In conclusion, we suggest that matching sequences from EV proteomes (available from public databases) with known protein sequences of microbiome gut bacteria will be useful in identification of antigen mimicry between evolutionary conservative protein sequences. Using this approach we identified *Bacteroides* spp. pseudokinase with activation loop and homology to PDGFR α , providing a proof-of-concept strategy. We speculate that existence of microbial pseudokinase that 'mimics' PDGFR α may be related to PDGFR α and *Bacteroides* spp. roles in colorectal carcinogenesis that require further investigation.

Abbreviations: AchE, acetylcholinesterase; Ago, Argonaute protein family, essential catalytic components of RISC; AnV, annexin V; APC, antigen-presenting cells; ARF6, ADP-ribosylation factor 6; bft, *Bacteroides fragilis* toxin; bMVs, bacterial microvesicles; BPD, benign pancreatic disease; CEA, carcinoembryonic antigen; CEC, colonic epithelial cells; CrD, Crohn's disease; CRC, colorectal cancer; DAPI, (4',6-diamidino-2-phenylindole); DHR, dihydrorhodamine; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; EpCAM, epithelial cell adhesion molecule; EVs, extracellular vesicles; FFPE, formalin-fixed paraffin embedded; FP, fluorescent protein; GI, gastrointestinal; GM1, monosialotetrahexosylganglioside; GPC1, membrane-anchored proteoglycan molecule glypican-1; IBD, inflammatory bowel disease; IEM, immunoelectron microscopy; IFC, imaging flow cytometry; IL, interleukin; KRAS, GTPase, that in human is encoded by the *KRAS* gene; lncRNAs, long non-coding RNAs; LSPR, localized surface plasmon resonance; MEK-ERK, mitogen-activated protein kinase kinase/extracellular matrix-anchored proteoglycan molecule glypican-1 cell-signal regulated kinase; MPs, microparticles; MUC1, mucin1; MVs, microvesicles; MVBs, multivesicular bodies; OD, optical density; OMPs, outer membrane vesicles; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PCA, capsular polysaccharide A; PCD, programmed cell death; PDAC, pancreatic ductal adenocarcinoma; PDGFR, platelet-derived growth factor receptor alpha; PE, phycoerythrin; PI, propidium iodide; PODO, podoplanin; qRT-PCR, quantitative RT-PCR; RFP, red fluorescent protein; RISC, RNA-induced silencing complex; SCID, severe combined immunodeficiency (non-human); SELN, exosome-like synthesized nanoparticles; SMLM, single-molecule localization microscopy; TEV, tumor-originating extracellular vesicles; TNF-alpha, tumor necrotic factor alpha; TF, tissue factor; YSPAN8, tetraspanin 8; VEGF, vascular endothelial growth factor; VTEs, venous thromboembolic events

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1. Introduction

Chronic inflammation pathologies of GI such as inflammatory bowel disease (IBD), Crohn's disease (CrD), *Helicobacter pylori*-associated inflammation and chronic pancreatitis have been identified as strong risk factors for cancer development [1–6]. The initial hypothesis that the emergence of the tumor is associated with chronic inflammation was postulated by Rudolf Virchow in 1863 [7]. Today, the causative link between cancer and chronic inflammation is widely accepted, though molecular and cellular mechanisms of this association have not been resolved [8]. About 15% of the global cancer burden can be attributed to infectious agents [9], of which chronic inflammation is a major component [1]. Acute inflammation is self-limiting, since the production of anti-inflammatory cytokines (IL-1, IL-10, IL-13 etc.) follows the production of pro-inflammatory cytokines (IL-1, TNF-alpha, IFN γ etc.) [8]. The strongest association of chronic inflammation and underlying infection with cancerogenesis is found between inflammatory bowel diseases and colon cancer, *Helicobacter pylori* and gastric cancer, hepatitis C and liver carcinoma, schistosomiasis and bladder and colon carcinomas [10]. The broader implication of these observations was that chemokines and other cytokines are widely involved in cancer development, however, the detailed mechanisms linked to infection and inflammation in relation to cancer are not well understood.

It is well known that cell activation and pathogenesis of a variety of diseases are often associated with increased levels of EVs released in body fluids including plasma, liquor, urine, bile, saliva, semen, vitreous and synovial fluids, atherosclerotic plaques, mucus and intestinal fluids, ascitic and pleural fluids [11–14]. EVs production by eukaryotic cells is upregulated during cell activation and growth, thus playing an essential role in cellular communication during cancer development. However, until recently it was not clear how commensal and pathogenic bacteria, and members of gut microbiota, communicate with other microbial and eukaryotic cells and the immune system. Studies during the last decade that are focusing on the association between gastrointestinal cancer and inflammatory diseases with certain types of bacterial infections often are overlooking the intensive production of outer membrane vesicles (OMVs) by different types of gastrointestinal bacterial commensals and pathogens as well as fungi, parasites invading the GI tract, and nematodes.

This review focuses on research assessing EVs originating from

different types of eukaryotic and prokaryotic cells inside the GI tract which separates symbionts and commensals in our bodies. We also discuss the role of EVs originated from GI cancers as potential biomarker tools. Early diagnosis of GI cancers continues to be a major challenge with a miss rate of up to 6.7% - for the upper GI tract with endoscopy and colonoscopy [15] and up to 6% miss rate for colorectal cancers [16]. Due to the invasiveness of these procedures and the possibility of complications [17], there is a high need for robust circulating biomarkers of GI cancers and effective non-invasive monitoring. In conclusion, we suggest that matching sequences from EV proteomes (available from public databases) with known protein sequences of microbiome gut bacteria will be useful in identification new molecular mimicry target protein sequences and provide a proof-of-concept strategy identifying *B. fragilis* pseudokinase that 'mimic' PDGFR α .

2. Extracellular vesicles (EVs) structure and biogenesis

Generally, three different classes of EVs (Fig. 1) are produced by metazoan cells, namely, exosomes, microparticles (MPs) or microvesicles, sometimes also named ectosomes, and apoptotic bodies. These EVs are distinguished by size, their content, morphology and by different mechanisms of their biogenesis [18–19]. Moreover, it is well accepted that production of EVs represents a universal feature of life, since gram-negative and gram-positive bacteria as well as *Archaea* generate outer membrane vesicles [20–24]. It is believed that the formation of microparticles (MPs) usually happens via plasma membrane budding and shedding associated with membrane sites enriched with lipid rafts [25–26,18]. Unlike MPs, exosome formation occurs via re-routing of multivesicular bodies (MVBs) to the cell surface [27–28], where they fuse with the cell membrane and exit the cell through exocytosis (detailed rev. [29]). As a result, exosomes are enriched with endosome- and MVBs-associated proteins, such as tetraspanins (CD9, CD63, CD81 and CD82 and CD151; whereas CD37, CD53 and Tssc6 are restricted by hematopoietic cells) [30–31]. CD63 is important for exosomal secretion, as the reduction of exosomal production was observed in CD63-knockout HEK-293 cells. However, at the same time no reduction in the secretion of microparticles > 150 nm size was noted, supporting the observation that CD63 is important for exosomal

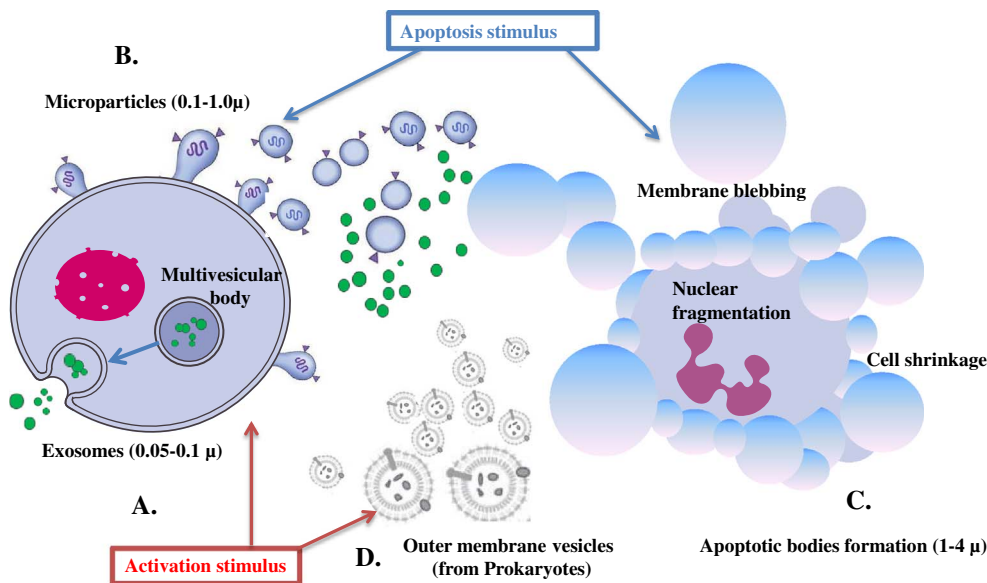


Fig. 1. Production of different classes of EVs. (A) Microparticles; (B) exosomes; (C) apoptotic bodies; (D) outer membrane vesicles.

Table 1
Circulating exosomes and microvesicles (below 200 nm) in patients with GI cancers.

Cancer type	Cancer subtype	EVs source	EVs size	Detection and purification methods	Subpopulations of EVs	Potential biomarkers	Ref.
Colorectal cancers	Colorectal carcinoma—adenoma—NC	Human serum	ND	ExoQuick™	Exosomal fraction from ExoQuick™	miR-21, miR-291a, miR-92a	[66]
	CRC	Human serum	App. 50 nm (TEM)	Ultracentrifugation and Exosome Isolation Kit (Invitrogen), TEM, microRNA microarray, qRT-PCR	Exosomal fraction	miR-17-92a (recurrence biomarker)	[67]
CRC	Human serum and cell lines FHC, HCT116, HT-29, RKO, SW48, SW480	Human serum and cell lines FHC, HCT116, HT-29, RKO, SW48, SW480	App. 100 nm < 220 nm	Ultracentrifugation, Western blotting (CD81), miRNA microarray, qRT-PCR	EX fraction	7 miRNA (let7a, miR-1220, miR-1246, miR-150, miR-21, miR-223, miR-23a)	[68]
CRC	Serum samples	Human plasma	App. 100 nm	ExoScreen, NTA, ELISA	CRC-derived EVs	CD147, CD9	[69]
CRC	Human plasma and HCT116 cell line	Human plasma and HCT116 cell line	< 220 nm	0.22 µm filtration, Ultracentrifugation and CD63+ beads isolation, FCM, RT-PCR, western blotting	CD63+, EpCAM fractions of CRC-derived EVs	TSAP6 and p21 mRNA	[70]
CRC	Human ascites	Human ascites	30–150 nm	Western blot, nano-LC-MS	Ascite-derived EVs	CEACAM5, CD97, tetraspanins, plexin, TAGSD1, trophoblast glycoprotein	[50]
CRC	Human plasma	Human plasma	50–100 nm	FCM, Western blot, IEM	FasL+, TRAIL+, CD63+ and CEA+ CRC EVs	CEA and CD63	[71]
Gastric cancer	Gastric cancer, TNM stages I-IV	Human serum	ND	ExoQuick™	Exosomal fraction	miR-10b-5p; miR-132-3p; miR-185-5p; miR-195-5p; miR-20a-3p; miR-296-5p	[72]
	Gastric adenocarcinoma	Peritoneum lavage fluid	ND	Microarray, qPCR	Exosomal fraction	miR-21; miR-1225-5p	[73]
Gastric cancer (pre- and post-operative)	Gastric cancer (pre- and post-operative)	Paired plasma samples	100 nm (TEM)	TEM, qRT-PCR	Exosomal fraction from "Total Exosome Isolation kit" (Invitrogen)	Long intergenic non-protein-coding RNA 152 (LINC00152)	[74]
Gastric cancer	Gastric cancer	Human plasma	ND	FISH, qRT-PCR, ExoQuick™	Exosomal fraction (ExoQuick™)	Five-miRNA signature; miR-185 associated with TNM stage	[75]
Gastrointestinal stromal cancer	Gastrointestinal stromal tumors (GIST)	Ascites	30–200 nm	STEM	Exosomal fraction	ND	[76]
	GIST solid tissues	GIST solid tissues	30–200 nm	STEM	Exosomes, microvesicles, spherosomes (multivesicular spherules)	ND	[77]
Esophageal cancers	Esophageal squamous cell carcinoma	Human serum	ND	ExoQuick™; Exiqon miRNA panel; qRT-PCR	Exosomal fraction (ExoQuick™)	Five-miRNA signature (miR-20b-5p; miR-28-3p; miR-192-5p; miR-223-3p; miR-296-5p)	[78]
	Esophageal squamous cell carcinoma	Human serum	ND	Differential centrifugation; TaqMan OpenArray miRNA Profiling; immunoblotting; Exosome isolation kit (Invitrogen); AChE activity; NTA	Exosomal fraction	miRNA-21	[79]
Esophageal squamous cell carcinoma: control esophagitis	Esophageal squamous cell carcinoma: control esophagitis	Human plasma	ND	ExoQuick™; miRNA profiling	Exosomal fraction	Exosomal (or EVs) number in peripheral blood as prognostic biomarker	[80]
	Advanced esophageal carcinoma (HC and Barrett's esophagus)	Human plasma	ND	ExoQuick™; qRT-PCR; microRNA array, ELISA	Exosomal fraction	Multi-miRNA panel (RNU6-1/miR-16-5p; miR-25-3p/miR-320a; let-7e-5p/miR-15b-5p; miR-30a-5p/miR-324-5p; miR-17-5p/miR-194-5p)	[81]
Advanced Esophageal adenocarcinoma vs T2-T3 adenocarcinoma and squamous carcinoma	Advanced Esophageal adenocarcinoma vs T2-T3 adenocarcinoma and squamous carcinoma	Human serum	ND	ExoQuick™; qRT-PCR; microRNA array, ELISA	Exosomal fraction (ExoQuick™)	miR-223-5p, miR-483-5p	[82]
	Esophageal adenocarcinoma	Human serum	ND	ExoQuick™; qRT-PCR; TEM, Western blotting	Exosomal fraction (ExoQuick™)	miR-21	[83]
Barrett's esophagus metaplasia-dysplasia-adenocarcinoma	Barrett's esophagus metaplasia-dysplasia-adenocarcinoma	Esophageal solid tissue samples	< 100 nm	ExoQuick™; qRT-PCR; TEM, Western blotting	Exosomal fraction (ExoQuick™)	N/A	[84]
	Large bowel cancer	Human serum	90–200 nm	TEM	Matrix EVs	N/A	[85]
Intestinal cancer	Large bowel cancer	Human serum	App. 100 nm	IEM, TEM, AChE assay, qRT-PCR; Western blotting, ELISA	Exosomal fraction from ultracentrifugation	HSP60	[86]
	Pancreatic ductal adenocarcinoma (PDAC)	Human plasma before/after surgery	60–120 nm	IEM, LS-MS/MS, RT-PCR, ELISA	Exosomal fraction from ultracentrifugation	miRNA signature (high miR-10b, miR-21, miR-30c, miR-181, low miR-let7a; glypican-1 (GPC1); CA-19-9)	[86]
Pancreatic cancers	Pancreaticobiliary cancer and PDAC	Pleural effusion and	Peak at 128 nm	TEM and SEM, NTA, FCM, western	Exosomal fraction from	N/A	[87]
	Pancreaticobiliary cancer and PDAC	Pleural effusion and	Peak at 128 nm	TEM and SEM, NTA, FCM, western	Exosomal fraction from	N/A	[87]

(continued on next page)

Table 1 (continued)

Cancer type	Cancer subtype	EVs source	EVs size	Detection and purification methods	Subpopulations of EVs	Potential biomarkers	Ref.
PDAC	PDAC	plasma-derived Solid tissue and plasma	App. 100 nm	blotting, NGS EM, FCM, NTA	ultracentrifugation PDAC-derived EVs	MIF ⁺	[88]
PDAC	Pancreatic cancer	Human serum	App. 100 nm	NTA, EM, CM, SEM		CA-19-9, TSG101	[89]
PDAC	PDAC	Human plasma	N/A	LSPR, TEM		miR-10b	[90]
Pancreatic cancer, PDAC	Pancreatic cancer, PDAC	Solid tissue and plasma	< 100 nm	EM, FCM, ULPS-MS, RT-PCR	PDAC-derived EVs	GPCI ⁺	[91]
Pancreatic cancer	Pancreatic cancer	Human blood samples	30–100 nm	EM, ExoChip	Pancreatic cancer (PC)-derived EVs	CD63 and Rab5	[92]
Pancreatic cancer	Pancreatic cancer	Serum samples	N/A	RT-PCR, qRT-PCR	PC-derived EVs	S-Exo PaCIC markers	[93]
PDAC	PDAC	Human serum	< 220 nm	RT-PCR	PC-derived EVs	miR-21; miR-17-5p	[94]

secretion only [32]. Heat shock proteins HSP 70/90 are also enriched in exosomes, but not in shedded MPs [33]. The exosomal membrane is rigid with rigidity increasing from pH 5 to 7 as demonstrated by membrane fluidity probes (diphenylhexatriene), which is a result of increased amounts of desaturated phosphatidylcholines (PC), phosphatidylethanolamine, sphingomyelin, cholesterol [34] and ceramides [35]. Circulating exosomes are resistant to lipolytic enzymes and can stay for up to two weeks in the lymph nodes [36]. MPs are much more heterogeneous in size and structure, spanning from 0.1 to 1.0 μm, whereas exosomes are typically homogenous in size (0.04–0.1 μm) and shape [37,12]. Cells secrete exosomes and MPs simultaneously, but their ratios differ depending on the cell type and cell status [38–39].

Though their biogenesis is different and sizes are overlapping, separation and purification of these two major classes of EVs has been hampered by the limitations of existing methods [40–42]. Currently, various strategies including differential ultracentrifugation, filtration, density gradient separation, immunoaffinity, bead purification, and size exclusion chromatography have been used for isolation of different EV fractions, however, no universal approach exists. Limitation factors for ultracentrifugation include co-sedimentation of protein aggregates and co-purifying of non-specifically bound proteins [40]. The most straightforward approach for separating exosomes and MPs is FACS-based sorting accomplished by sorting similarly sized viral particles [43]. However, FACS-based cell sorting of EVs normally leads to size-based selection, could also be biased by the choice of antigens selected for FACS-sorting.

Both major EV classes contain different protein, RNA and DNA cargo [44–47], and also significantly differ by their lipid content. EVs may transfer information from the host cell to various target cells by direct cell-to-cell contact or alternatively, through secretion of soluble mediators and effectors [48–49]. Moreover, EVs may interact with target cells via surface-bound ligands, transferred surface receptors and membrane-associated enzymes, or deliver cytoplasmic or membrane-associated constituents, such as cytosolic proteins, different classes of RNA molecules, DNA, bioactive lipids and even cellular organelles such as mitochondria [11,14]. Several studies indicate that tumor-derived extracellular vesicles (TEVs) harbor signaling proteins that affect cell metabolism, mRNA processing, angiogenesis, and cell growth and motility, in addition to molecules that are likely required for TEV biogenesis [50,28]. It has been demonstrated that at least in some cases cellular proteins are selectively integrated into TEVs via ARF6-regulated endosome recycling, where the expression and activation of ARF6 is associated with an increase of tumor-invasive potential [51,52]. Plasma membrane-derived EVs, secreted from the parent cell, strengthens some, but not all plasma membrane proteins and those that integrated inside EVs conserve the topology of the parent cell plasma membrane [12].

The function of EVs appears to be dependent on their cargo. EVs shed from various tumor-cell lines have been thought to facilitate extracellular matrix (ECM) invasion, evasion of the immune response [53–54,37] and potentiate formation of metastasis in PDAC and other cancers [55–57]. Shedding of tumor-derived EVs can stimulate metastasis spreading, tumor-stroma interactions and angiogenesis [55,58–62]. EVs contain complex sets of cargo, depending on the physiological conditions in which they are generated and released, and once shed may impact a variety of cellular processes and modulate inflammatory response. The major question that remains to be elucidated is how specific cargoes are selectively absorbed and accumulated by different classes of EVs. It has been shown that posttranslational modifications of proteins with acyl, myristoyl, palmitoyl or glycoposphatidylinositol anchors may facilitate recruitment of specific proteins to EVs [11,63–64]. However, since recruiting of proteins happens also in the absence of these post-translational modifications, there are other yet unknown mechanisms that participate in protein cargo selection by EVs. The mechanisms of RNA and miRNA selection by EVs are poorly

Table 2
Circulating microvesicles (between 200 and 2000 nm) in gastrointestinal cancers.

Cancer type	Cancer subtype	EVs source	Detection and purification methods	EVs size	Subpopulations of EVs	Potential biomarkers	Ref.
Colorectal cancers	CRC and PC	Human serum	FCM	ND*	EpCAM ⁺ , EpCAM ⁺ CD147 ⁺ , CD147 ⁺ EpCAM-MVs	Increased EpCAM ⁺ CD147 ⁺	[95]
	CRC	Platelet-poor plasma	FCM	ND	CRC-derived MVs	ND	[96]
	CRC	Solid tissue (surgically or endoscopically resected)	RT-PCR, NTA	< 1000 nm	CRC-derived MVs	miR-1246	[97]
	CRC	Human plasma	FCM	< 1000 nm	TEVs	ND	[98]
	Colon cancer	Bone marrow	FCM	ND	CD34 ⁺ /HER2 ⁺ /neu ⁺ stem cell EVs	ND	[99]
	Colon cancer	Human body fluids (pleural liquid)	SEM	20–2000 nm	MVs, PEVs	ND	[100]
Gastrointestinal cancers	CRC	Human plasma	FCM	< 1000 nm	TF ⁺ , PS ⁺ EVs	D-dimer to detect VYE in CRC patients	[101]
	Gastric cancer, III-IV stage	Human plasma	Centrifugation, FCM, procoagulant activity	ND	Leukocyte-derived MVs, Er-derived MVs, Endo-derived MVs, PS ⁺ MVs	Leukocyte MVs, Er MVs, Endo MVs > > In GC III-IV patients, PS ⁺ MVs	[102]
	Gastric cancer	Human plasma	TEM, FCM, AFM	10–800 nm	TEVs, PEVs	CCR6 and HER2/neu	[103]
	Gastric cancer	Human plasma	FCM	ND	Circulating tumor-derived EVs	ND	[104]
	Human pancreatic adenocarcinoma cell lines BxPc-3 and Low.6p]	Human platelet	FCM	ND	PEVs	ND	[105]
	CRC, PC, IBD	TF ⁺ MVs from PC injected in Par4-deficient mice	FCM	ND	TF ⁺ MVs	TF ⁺ MVs for PC-associated thrombosis	[106]
Pancreatic cancers	Pancreatic cancer with associated thrombosis	Platelet-poor plasma	FCM	ND	PMPs, EMPs, Er-derived MVs, LeuMVs, TF ⁺ , Fibrin ⁺ , CEA ⁺ , CA19-9 ⁺ MVs	Specific signature	[96]
	Pancreatic cancer with associated thrombosis (IVC-stenosis mice model)	Panc02 pancreatic ductal cell line induced in C57Bl/6 mice	FCM	ND	CD31, CD68, AnnV ⁺ TF ⁺ , total AnnV ⁺ , AnnV ⁺ Muc1 ⁺	TF ⁺ MVs for PC-associated thrombosis	[107]
	Pancreatic carcinoma	Human blood	Centrifugation, FCM	200–2000 nm	SEMI	ND	[108]

* ND – > 300 nm in the studies where conventional flow cytometry (FCM) was used for characterization of MVs (standard limit of sensitivity for conventional flow cytometer).

Table 3
Characterization of EVs derived from human cell lines.

Cancer type	Cancer subtype	EVs source	EVs size	Detection and purification methods	Subpopulations of EVs	Potential biomarkers	Ref.
Colorectal cancers	Colorectal carcinoma	HCT-8	ND	TEM	Fraction obtained with exosome-precipitation solution	miR-210	[109]
	Colorectal carcinoma	SW480	ND	0.22 mkm filtration and differential ultracentrifugation; western blotting, microarray, functional assays, qRT-PCR	Fraction obtained from 48 h cultural supernatant	Erk1/2 kinase dependent internalization	[110]
	Colorectal carcinoma	SW480	ND	Differential ultracentrifugation; single molecule-localization microscopy (SMLM), real-time PCR, FCM, western blotting	48–72 h exosomal (EX) fractions	miR31	[111]
	Colorectal carcinoma and pancreatic carcinoma	SW480, A818.4, Capan1	ND	Differential ultracentrifugation; FCM, zymography, RT-PCR	EX fraction from 24 and 48 h supernatant	CD44v6	[112]
	Colorectal carcinoma	HT-29, Caco-2 (in vivo mouse model)	30–150 nm (TEM)	Differential ultracentrifugation and EX purification kit; TEM, FCM, western blotting (HSP70), qRT-PCR, functional assays	EX and MVs fractions	CXCR4-axis	[61]
	Colorectal carcinoma	LIM1863	Different 30–100 nm, 30–1000 nm fractions	Sequential ultrafiltration (0.1, 0.22, 0.45, 0.65 mkm), cryo-EM	EX fraction obtained from cultural supernatant of MF associated with gastric cancer cells	Different proteomic signatures for EX and MVs Proteomic signatures	[113] [114]
	Murine colorectal carcinoma	GT26 murine colorectal carcinoma associated with mouse macrophage Ana-1 cell line	~100 nm	Differential ultracentrifugation, ExoQuick™, TEM, NTA, western blotting, mass-spectrometry, qRT-PCR			
	Colorectal carcinoma	DLD-1 and DLD-1/5FU LIM1863	50–450 nm 50–150 nm (EX) and 100–1500 nm (MV's)	NTA, RT-PCR Ultracentrifugation and column separation; qRT-PCR, western blotting (CD9), deep RNA sequencing	DLD-1 and DLD-1/5FU-derived MVs A33-EX and EpCam EX and MVs	miR-34a, miR-145 32-miRNA signature	[115] [116]
	Colorectal carcinoma	HCT-116	~100 nm	Differential ultracentrifugation; TEM, NTA, Ache assay, western blotting	CD9 ⁺ and CD63 ⁺ exosomal fraction		[117]
	Colorectal carcinoma	DLD-1, WiDr, SW480, and COLO201	30–200 nm	NTA, western blotting	DLD-1, WiDr, SW480, and COLO201 exosomes	miR-1246, TGF-β	[97]
	Colorectal carcinoma	HCT116, HCT15, HT29, COLO201, COLO205, WiDr, SW1116	~100 nm	ExoScreen, immunoblot, NTA	HCT116, HCT15, HT29, COLO201, COLO205, WiDr, SW1116 exosomes	CD147/CD9, CA19-9	[69]
	Colorectal carcinoma	LIM1215	< 100 nm	Ultracentrifugation and 0.1 mkm filtration; immunoprecipitation, western blotting, LC-MS/MS	LIM1215-derived fraction	Cadherin17	[118]
	Colorectal carcinoma	Sw480 and SW620	40–130 nm	EM, Cryo-EM, GeLC-MS/MS	Sw480, SW620 secretome and exosomes	TSG101, Alix, CD63, MET, S100A8, S100A9, TNC, FNB2, EGFR, JAG1, SRC, TNK1, CAV1, FLOT1, FLOT2, PROM1 CEACAMs	[119] [120] [121]
	Colorectal carcinoma	HT29 Murine LIM1863	90 nm to 812 nm ~100 nm (TEM)	NTA, TEM, FCM, western blotting Ultracentrifugation, combined with filtration; hA33/EpCam immunoaffinity purification, western blotting (TSG101, Alix, EpCam, A33), TEM, Gel C-MS/MS)	HT29 cell line-derived EVs EX immunoaffinity purified fraction		[122]
Colorectal carcinoma	HCT15, SW480 and WiDr CRC	< 220 nm	SDS-PAGE, western blotting, RT-PCR	HT15, SW480 and WiDr-derived EVs	CD63, CD9, and CD81, miR-21	[123]	
Colorectal carcinoma	SW480 and SW620	23 to 636 nm and 26–574 nm defined by NTA)	TEM, Nano-LC-ESI-MS/MS, NTA, peptide OFFGEL fractionation	SW480 and SW620-derived EVs	Tetraspanin and their associated proteins, FSCN1, STRAP, S100A4, S100A14 expression TSAP6	[124] [125]	
Colorectal carcinoma	HCT116 SW948, SW620, SW480, HT29, CaCo2	ND	FCM, RT-PCR, western blotting	HCT116 EX fraction		[70]	
Colorectal carcinoma	HT29, CaCo2 LIM1215	< 200 nm	MALDI-TOF/TOF-MS, nano-HPLC/ESI-MS/MS, 2DGE Filtration and differential centrifugation, and immunocapture purification (A33); TEM, IEM (GD63), LS-MS/MS	SW948, SW620, SW480, HT29, CaCo2 secretomes and EX fraction A33 EX-fraction	Glod4, Rad23bC-terminal fragment of agrin, Proteome analysis	[124] [125]	
Colorectal carcinoma and	CX2 and Colo357	< 200 nm	Filtration and Differential centrifugation; IEM	EX fraction	HSP70 ⁺ Bag4 ⁺ active fraction	[126] (continued on next page)	

Table 3 (continued)

Cancer type	Cancer subtype	EVs source	EVs size	Detection and purification methods	Subpopulations of EVs	Potential biomarkers	Ref.
Esophageal cancers	pancreas carcinoma			(HSP70), western blotting, FCM, functional assays			
	Colorectal carcinoma	SW480	40–150 nm	Microarray, TEM, IHC, RT-PCR	SW480 CRC cell line-derived EX fraction	CENPE, KIF15, CEP55, CCNA2, NEK2, PBK, and CDK8	[119]
	Colorectal carcinoma	SW403 and CRC28462	50–100 nm	FCM, western blotting, IEM	FasL ⁺ , TRAIL ⁺ , CD63 ⁺ and CEA ⁺ CRC MVs	CEA and CD63	[71]
	Colorectal carcinoma	HT29-19A and T84-DRBI*0401/CIITA	30–90 nm	IEM, FCM, MALDI-TOF-MS	Apical and basolateral EX fraction	CD63 and CD26	[127]
Esophageal squamous cell carcinoma		EC9706	ND	Differential centrifugation	EX fraction from 48-h cell supernatant		[79]
		TE2; Murine SCCVII	ND	Exosome-isolation kit (Invitrogen); qRT-PCR; AChE quantitation	CD63-GFP-tagged exosomes from TE2	CD63	[80]
		EC9706	30–60 nm	TEM, Solexa, RT-PCR	EC9706 EX fraction	let-7 family, miR-21-5p, miR-26a-5p	[128]
Gastric cancer	Co-culture esophageal carcinoma and fibroblasts	KYSE-30	ND	Differential centrifugation	EX fraction from conditioned medium	Upregulation of 18 miRNA	[129]
	Co-culture esophageal carcinoma and fibroblasts	TE1, TE2, TE4, TE6, TE11	ND	Differential ultracentrifugation	EX fraction from 48-h cell supernatant	miR-1246	[130]
	Esophageal adeno-carcinoma	Het-1A	ND	LSCM, FCM	Het-1A EX fraction	CD95L	[131]
	Gastric cancer	GES-1, MGS-803, SGC-7901	24–340 nm	NTA, TEM, IFC	48 h supernatants fraction	ND	[132]
	Gastric carcinoma	SGC-7901 (also co-cultured with HUVEC)	~100 nm (TEM)	Differential centrifugation; TEM, ELISA, qRT-PCR, functional assays, immunohistochemistry (CD31, anti-VEGF), western blotting (anti-Bim, anti-GAPDH)	Cultural supernatant EX fraction	miR-29a/c	[133]
Gastric cancer		MKN-1,-7,-45,-74	ND	Differential ultracentrifugation; qRT-PCR, western blotting (tetraspanin-8), RNAi, functional methods	48 h supernatants MVs fraction	TSPAN8 prognostic marker	[134]
		SCG-901	~50 nm (TEM)	Differential ultracentrifugation and filtration (0.20 μm); TEM, FCM, functional assays, microRNA microarray, western blotting (CD9, HSP70)	EX fraction from 48 h cultural supernatants	CD97 ⁺	[135]
		GC415	Range 60–900 nm (highest conc. 80–120 nm)	Differential ultracentrifugation; FCM, DLS, NTA, AFM, functional assays	Fractions from supernatants (confluent cell cultures)	CD44 ⁺ , CD44v6 ⁺ , CCR6 ⁺ , HER-2 ⁺	[99]
Pancreatic cancers	Scirrhous gastric carcinoma cell line and gastric cancer peritoneal lavages	OCUM-2 M, 2 M-D3		0.22 μm filtration and differential ultracentrifugation; miRNA microarray, qRT-PCR	EX fraction from supernatant	miR-320c, miR-1202, miR-1225-5p, miR-1207-5p, miR-7270 signature (validated in peritoneal lavages)	[73]
	Duodenal cancer	Primary AZ-521 (HuTu-80), metastatic AZ-P7a	ND	LC-MS/MS, RT-PCR	AZ-521 (HuTu80), AZ-P7a EX fraction	PABP1	[136]
	Gastric cancer	GC-MSC, GCN-MSC, BM-MSC from tissue samples	ND	0.22 μm filtration and differential ultracentrifugation and ExoQuick [™] , miRNA microarray analysis, qRT-PCR, functional assays	MSC-derived EX fraction	miR-214, miR-221, miR-222	[137]
	Gastric cancer	SGC7901, HGC27 and gastric epithelial cells	40–100 nm (TEM)	Differential ultracentrifugation; TEM, western blotting (CD9, CD81, TGFβ, VEGF, N-cadherin, E-cadherin, GAPDH), qRT-PCR, functional assays	EX fraction	ND	[138]
Pancreatic cancers	8 gastric cancer, 5 colon cancer, 9 pancreas cell lines	Most of experiments with gastric cancer AZ-P7a cell line	Defined by IEM	Centrifugation and filtration; immunoelectron microscopy; western blotting (CD29, Alip1/Alix, Tsg101), miRNA profiling and microarray analysis	CD63 ⁺ EX fraction after combination of centrifugation and filtration	Let-7 miRNA family	[139]
	Gastric cancer	SGC7901, BGC823	30–100 nm	EM, western blotting	SGC7901, BGC823 exosomes	Cbl-b, Cbl-c	[140]
	Pancreatic stellate cells	Immortalized pancreatic cancer cells	30–110 nm	Differential ultracentrifugation; TEM, western blotting (CD9, 63, 81, GMI130). Real-time PCR, functional assays	CD9 ⁺ , CD63 ⁺ , CD81 ⁺ , GMI130 ^{EX} fraction from conditioned media	Increased miR451	[141]
Pancreatic cancer	Pancreatic cancer	PC patient-derived cell lines	NTA data	Ultracentrifugation, NTA, western blotting	CD63, TSG101 and ALIX exosomal	ND	[142]

(continued on next page)

Table 3 (continued)

Cancer type	Cancer subtype	EVs source	EVs size	Detection and purification methods	Subpopulations of EVs	Potential biomarkers	Ref.
Pancreatic adenocarcinoma (PDAC)	Pancreatic ductal adenocarcinoma (PDAC) and epithelioid carcinoma	BxPC-3 and HPAF-II	100 nm	EM, FCM, NTA	markers confirmed PDAC-derived EX fraction	ND	[88]
		PANC-1, SW1990, BxPC-3	30–110 nm	NTA, RT-PCR, FISH	PANC-1, SW1990, BxPC-3 derived EX fraction	RFXAP, miR-212-3p	[143]
		AsPC-1, BxPC-3, PANC-1	50–120 nm	SEM, ISPR-based sensing platform	Cell lines-derived EX fraction	miR-10b	[90]
		Panc-1, MIA Paca2, T3M4	< 100 nm	EM, FCM, UPLC-MS, RT-PCR	Panc-1, MIA Paca2, T3M4 EX fraction	GPC1 ⁺	[91]
		PANC-1	40–150 nm	Ultracentrifugation, Ach activity, NTA, DLS	PANC-1-derived EX fraction	ND	[144]
		bxPC-3 SW	~ 100 nm	Ultracentrifugation		miR155	[145]
		INS-1, HPDE PANC-1	< 100 nm	NTA, EM, CM, SEM	PC-derived EX fraction	CA19-9 and AM	[89]
		Paca44, Panc1, BxPc3,	< 100 nm	TEM, LC-MS	Paca44, Panc1, BxPc3, MiaPaCa2,	MET, CIT, MAP4K4, Yes1, DDR1,	[146]
		MiaPaca2, HPSC and HPDE	30–100 nm	RT-PCR, EM, western blotting	HPSC and HPDE EX fraction	WEE1, SRC, CDCP1, TCSF	[147]
		Panc-1	60–100 nm	Magnetic bead-based exosome extraction; AchE- assay, TEM, mRNA detection/measurement	Panc-1-derived EX fraction	miR-203	[148]
Pancreatic cancer	Pancreatic cancer	Panc02 cells, cultivated and injected in mice	60–100 nm		EX fraction purified with magnetic beads coated with biotinylated lectin specific to 2,6-linked sialic acid sandwich	ASPn, Foxp1, Apbb1ip, BC031781, Daf2, Gng2, Inceup	[60]
		AsPC1, BxPC3, Capan1, Capan2, MiaPaca1, Panc1, Pt45P1, 8.18, HD3522, HD3542, HD3577	ND	FCM, functional assays	EX fraction, purified by ultracentrifugation	Tspan8, CD44v6, alpha6beta4	[60]
Pancreatic cancer	Pancreatic cancer	BxPC3, MiaPaca2, Panc1	ND	Ultrafiltration and ultracentrifugation, western blotting, ESI-MS/MS	EX fraction was enriched for EX marker proteins Alix, CD9, Lamp3, syntenin	EGFR	[149]
		Bsp73ASML	ND	Ultracentrifugation, SDS-PAGE, western blotting, MALDI-TOF	EX fraction	CD44v6-dependent exosomes	[55]
Pancreatic cancer	Pancreatic cancer	SOJ-6, BxPC-3, MiaPaCa-2, and Panc-1	34–45 nm	EM, SDS-PAGE, MALDI-TOF	SOJ-6, BxPC-3, MiaPaCa-2, and Panc-1 nanoparticles	ND	[150]

understood, however, there are indications that uncoded regions of RNA are involved in this process [65]. Protein and miRNA signatures carried by EVs may represent potential biomarkers as reported by several groups (summarized in Tables 1, 2, 3).

3. EV cargo in gastrointestinal cancer

The phenotypic and functional heterogeneity of cancer cells that arises from heritable and stochastic epigenetic and genetic changes increases risk of metastasizing or resistance to drug treatment, and therefore represents one of major challenges in cancer patients treatment [151–152]. Recent research in cancer heterogeneity is adding additional layer of complexity to systemic transfer of extracellular vesicles and their functional content between the cells, which can contribute to tumor progression and influence anti-cancer therapies [153].

3.1. EV-associated RNA in gastrointestinal cancer

All major RNA classes are present in EVs [46]. In addition to mRNA and miRNAs [48,154], vRNAs and yRNAs, the degraded products of non-coding and long-coding RNAs have been found in exosomes [155–156].

It has been shown that miRNA dysregulation is involved in the initiation and progression of human cancers although the natural mechanism of this phenomenon is still unclear [157–158]. In normal conditions, these small noncoding, 19 to 22 nucleotide length RNAs participate in regulation of cellular development, differentiation, proliferation, apoptosis, and cancer cell metabolism [158]. Goldie and coauthors [159] demonstrated that exosomes contain a higher proportion of miRNAs compared to other classes of small RNA. It has been shown that exosomes are enriched with miRNA [159], certain types of miRNA are selectively sorted to exosomes and other MPs [160].

However, the details of the mechanism of this selective sorting of miRNAs and miRNA-associated proteins in exosomes and/or MPs are not clear. In mammals, this process includes transcription of miRNA genes into primary miRNAs (pri-miRNAs), and processing by the Drosha complex to produce precursor miRNAs [161]. Pre-miRNA undergoes digestion by the Dicer complex and is sorted to exosomes after becoming mature miRNA via four potential pathways: (1) nSMase2-dependent pathway; (2) miRISC-related pathway that is co-localized

with the sites of exosome biogenesis (MTB) and their components (AGO2 protein and miRNA-targeted mRNA); (3) 3' miRNA sequence dependent pathway, and (4) miRNA motif and sumoylated hnRNPs-dependent pathway (described in detail by [161]).

Though uridylation [162] and sumoylation of miRNA [163] are implicated in preferential sorting of miRNA in exosomes, in most cases these targeting sequences are absent in secreted miRNAs [164]. McKenzie and co-authors [165] demonstrated using wild-type and KRAS-mutant colon cancer cells, that KRAS-dependent activation of the MEK-ERK pathway (mitogen-activated protein kinase kinase/extracellular-signal regulated kinase) inhibits sorting of the Argonaute (Ago) 2 dependent miRNA in exosomes. There are early reports that demonstrated the presence of Ago2, a major component of RNA-induced silencing complex (RISC) as well as other proteins responsible for RNA-processing in secreted exosomes [166–167]. Recently, another component of RISC-complex, Y-box protein-1 has been shown to be involved in sorting miRNA to CD63⁺ exosomes [168]. Surprisingly, the authors did not find evidence of Ago proteins in their isolated exosomal preparations (CD63⁺ exosomes), which led them to suggest a different route for miRNA egress via exosomes, which they named the chaperone-mediated route as opposed to the Ago2-associated route.

The physiological role of EV miRNA and other cargo has been the subject of discussion from the moment of their discovery in EVs. Though transfer of exosomal miRNA between malignant and non-malignant cells has been proved in numerous studies [48,169–170] (Fig. 2), the functional role of EV-associated miRNAs remains elusive.

Most of the GI cancer-related miRNA studies are based on analysis of tissue and stool samples [171–173] and recently on plasma-derived miRNAs [174–177].

Significant upregulation of miRNA92a, miRNA221, miRNA29a and miRNA17-3p compared to healthy control groups was revealed. Furthermore, Huang and colleagues [174] showed miR-17-3p and miR-92a to be downregulated, giving a potential biomarker for colorectal cancer (CRC) evaluation. Huang and Yu's recent work [78] elucidated the possible mechanism of circulating miRNAs and long non-coding RNAs (lncRNAs) secretion for gastric cancer diagnosis and demonstrated that circulating miRNAs and lncRNAs exhibit higher diagnostic values relative to conventional tumor markers such as CA199, CA125, CA724, CA242, CA50, CEA, and pepsinogen. Another group [177] studied circulating miRNA92a carried by EVs from CRC patient's

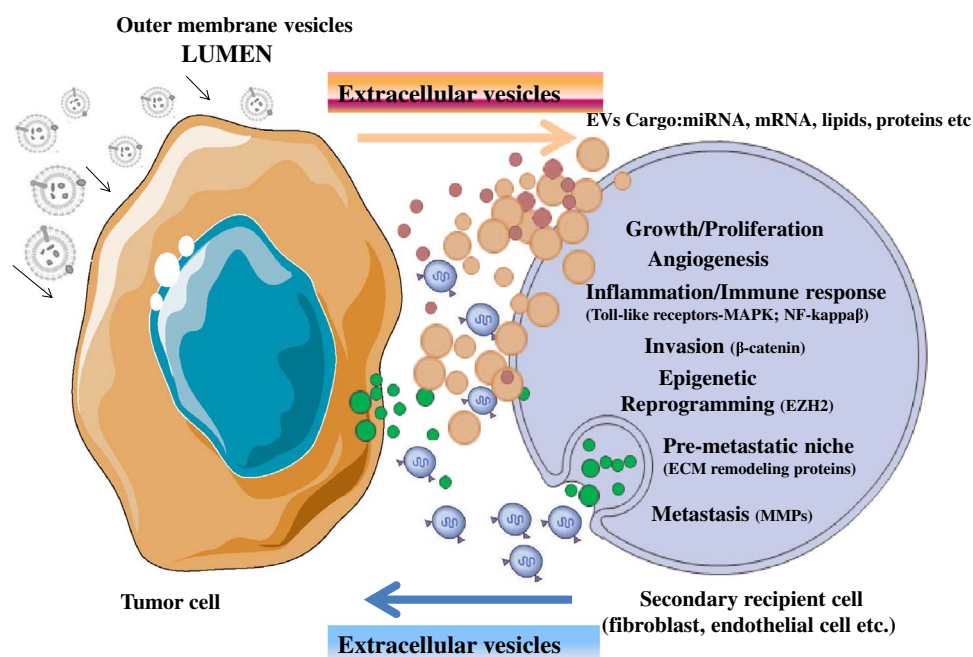


Fig. 2. Communication by EVs between lumen, tumor cells and secondary recipient cells (fibroblast, endothelial cells etc.).

plasma and demonstrated high expression levels of miR-92a leading to down regulation of the anti-oncogene Dkk-3. Moreover, secretion of miR-92a-containing EVs by cancer cells into their surrounding environment facilitated angiogenesis.

It has recently been established that gut microbiota has a significant influence on the expression pattern of miRNA of intestine cells including miRNA packed in EVs. In the Dalmasso and co-authors study [178], germ-free mice, lacking natural microbiota, were reconstituted with microbiota from normal special pathogen-free mice leading to the changes in the miRNA expression profile. It was found that after reconstitution nine miRNAs were differentially expressed with four being upregulated: miR-298, miR-200c, miR-342-p. Among these, miR-200c was known to be associated with EVs of highly invasive cancer cells [179–180]. Thus, expression profiling of miRNA in EVs may indicate changes in gut microbiota and a possible predisposition to cancer.

The most remarkable feature of miRNA is its stability in human biofluids, which allows miRNA molecules to operate outside cell borders within hostile environments such as intestinal lumen.

3.2. Proteomic studies on gastrointestinal cancer-derived EVs

The underlying mechanisms of protein interactions in EVs and interrelationships between vesicular transport proteins remain to be studied in GI cancers. To date, some authors have evaluated the role and function of the proteomic composition of EVs derived from primary human gastrointestinal cancers and EVs produced by cell lines of gastrointestinal origin [139]. Choi and co-authors [181] described how EVs derived from the HT29 cell line are organized by protein-protein interactions (PPIs) showing that vesicular transport proteins are interconnected via physical interactions and assembled into functional modules. As a result of the study, 957 of the 1261 vesicular proteins were mapped onto the extracellular vesicle PPI network, where 304 of them do not have any PPI in the Human Proteome Reference Database. Also, it has been shown that actin and actin-binding protein ADP ribosylation factors (ARF), such as ARF1 and SRC kinases have the essential signaling role in EV biogenesis [181]. Later studies confirmed the role of ARFs, such as ARF6 in exosomal biogenesis [182]. Another study [183] researching serum biomarkers for colorectal cancer metastasis using the secretome approach identified a total of 910 proteins from the conditioned media and 145 differential proteins in SW480 and SW620 cell lines. The differential expression pattern of 6 candidate proteins was validated by Western blot analysis and receiver operating characteristic curve analysis confirmed that serum trefoil factor 3 and growth/differentiation factor 15 could provide a discriminatory diagnostic test for predicting colorectal cancer metastasis. Ji and coauthors [184] found selective enrichment of metastatic factors (MET, S100A8, S100A9, TNC), lipid raft and lipid-raft associated membrane proteins (CAV1, FLOT1, FLOT2, PROM1) and some signal transduction molecules (JASG1, SRC, TNK1) in exosomes derived from metastatic SW60 cells. Enrichment of components of Src-signaling pathway in cancer cell line exosomes identified by proteomic research [181,183] has recently been shown by DeRita and colleagues [185].

3.3. Lipids in gastrointestinal cancer-derived EVs

There are a few publications describing a lipid composition of EVs, perhaps because a methodological approach for studying EV lipids is complicated and more difficult than this for EV proteomic analysis. EV membrane lipids have been shown to play an important role in the modulation of a functional response of target cells, formation, secretion and internalization of EVs [35,186–189]. Standard approaches include labeling exosomal and/or other EV subpopulations with fluorescent lipids [34,190], mass-spectrometry based lipidomics [189,191], and recently developed lipid-targeting fluorescent peptide probes [192].

Formation of EVs involves enrichment in certain classes of lipids. Several authors described the enrichment of exosomal fractions

with multiple lipid classes including cholesterol (Ch), sphingolipids, phosphatidylserine (PS), gangliosides, free fatty acids [35,188,191,193–194], whereas MPs are enriched in ceramides and sphingomyelins [191]. Lipid changes happen during different steps of EV biogenesis and affect sorting of EV cargo. For example, some proteins are sorted by lipid affinity, such as clustering tetraspanins in tetraspanin-enriched membrane domains (TEMs) associated with gangliosides and cholesterol. TEMs act as sorting machinery for loading growth factors and MHC II molecules into exosomes [195] and cholesterol and ceramides increase exosome release and affect their cargo [186,196].

Exosomes have been shown to contain functional enzymes and important components of lipid metabolism such as phospholipases, hydroxycholesterols, prostaglandins and leukotrienes [34,rev. 35,188,197–198]. Bioactive lipids contained by EVs as a part of their cargo are able to affect the metabolism of target cells (rev. [35]).

To date, the lipidomic characterization of EVs originating from GI tract cancer is limited to only a few publications describing lipid content and/or functional activities involving lipid-mediated pathways induced by exosomes originated from cell lines [150,190,199], while detailed exploration of their functional and causative role remains poorly understood. In one of the ground-breaking publications, Ristorcelli and co-authors [150] reported that the human pancreatic cell line SOJ-6 produced lipid raft-enriched exosomes capable of activating the Notch-1 survival pathway in the cells. To demonstrate the role of exosomal lipids in promoting cellular death, Beloribi and colleagues [190] used SOJ-6 cells and exosome-like synthesized nanoparticles (SELN) composed of lipids typical for lipid rafts. SELN were co-localized with a marker for lipid rafts ganglioside GM1 and Notch-1 on plasma membrane or Notch-1 and Rab5a on early endosomes. Furthermore, Beloribi and co-authors [190] demonstrated a fusion and exchange of SELN lipids with lipid rafts of plasma membrane and endosomal membrane and showed that SELN mimicked detrimental effects of exosomes on the survival of SOJ-6 cells, but not on exosome-insensitive pancreatic MiaPaCa-2 cells. Exploration of lipids as potential cancer biomarkers has only recently begun and depends on technological progress in lipidomic mass-spectrometry. One notable example is the work of Lydic and co-authors [189], who performed a comprehensive, in-depth characterization of a “shotgun” lipidomic profiling of exosomes secreted by LIM 1215 colorectal cell line in comparison with parental. Using novel sample derivatization techniques, coupled with high-resolution “shotgun” mass-spectrometry and targeted mass-spectrometry, they demonstrated that secreted exosome glycerophospholipid compositions are clearly distinct from parental cells indicating that exosome formation/secretion requires unique partitioning of particular lipid classes and subclasses. Comparison of exosome versus cellular lipid profiles reveal > 520 individual lipids in 36 lipid classes and subclasses, as well as substantial lipid remodeling including an increase of sphingolipids, plasmalogen- and alkyl ether-containing glycerophospholipids in exosomes. Importantly, obtained lipidomic results are in broad agreement with publications on exosome content from other cancer types. A deeper analysis may provide insights on lipid exosome role in cancer progression. The potential implication of these studies for diagnostics is yet to be revealed.

4. Circulating EVs in gastrointestinal inflammatory disorders and cancer-derived EVs

A number of studies demonstrated that certain circulating EV fractions are increased in patients with GI cancers, compared to patients with inflammatory gastrointestinal diseases such as Crohn's disease (CrD) and inflammatory bowel disease (IBD) [12,13,104,200–204]. However, the amount of circulating microparticles may be also elevated in the active phase of gastrointestinal inflammatory diseases in comparison with healthy controls or cancer patients in remission. Thus, conducted studies on CrD revealed that the total number of circulating MPs was significantly elevated in CrD patients with active processes

compared to healthy controls and CrD patients in remission [201,205]. CrD patients in active phases of disease demonstrated an increased total amount of circulating MPs, particularly pro-coagulant MPs, and MP fractions originating from platelets, erythrocytes, leukocytes and endothelial cells compared to the patients in remission [201].

These elevated populations of MPs may cooperate in the inflammatory process as they induce neutrophil and endothelial activation, monocyte adhesion and recruitment of various inflammatory cells [201,206–207]. Another study in IBD patients found their levels of circulating TF⁺MP to be significantly higher than in healthy donors [202]. Interestingly, higher numbers of circulating total, platelet- and endothelial-derived MPs were also observed in patients with IBD in remission as compared to healthy donors supporting the hypothesis of inflammatory cell recruitment [201]. Similar high levels of platelet-derived MPs were demonstrated in earlier studies on IBD patients with increased thromboembolism risk [208–210]. Moreover, recently, Mitsuhashi and co-authors [203] found that intestinal luminal fluid is also a rich source of proinflammatory EVs carrying IL8, IL6, IL10 and TNF markers. The luminal liquid contains high quantities of EVs of different origin (EVs produced by cells structuring intestinal wall, bacterial outer membrane vesicles, EVs coming from parasites and food) (Fig. 3).

EV fraction (< 500 nm size) from colonic luminal fluid aspirates from patients with IBD contained CD63⁺/CD66b⁺ (originated from neutrophils) and CD63⁺/MUC-1⁺ (originated from epithelium) subpopulations, though no statistical significance with healthy controls was found (8.5% vs 10.8% and 39.5% vs 47.4%). EVs of different origin are partly internalized by cell components of intestinal wall, including cancer cells (illustrated by Fig. 4).

Excess of EVs is released in the circulation. Overall, the increase in the amount of circulating EVs or circulating annexin V⁺-EVs [96,211] has been observed in many cancer types and in inflammatory diseases (rev. [13]), and may not be considered as a specific diagnostic marker without additional immunophenotyping, discovering and validating disease-specific markers.

5. Analysis of potential biomarkers of gastrointestinal cancers in EVs

For the last decade EV components have been considered and intensively investigated as potential cancer biomarkers

[66,68,75,80,91,95,169,212]. However, the majority of published studies report that the expression of biomarkers (or combinations of biomarkers) is detected only at the advanced metastatic tumor stage, i.e. when tumors are already detectable by other methods.

Recently, Melo and coauthors [91] described the first diagnostic test based on detecting of a membrane-anchored proteoglycan molecule glypican-1 (GPC1) on EVs. The authors were able to differentiate exosomes isolated from the blood of patients with benign pancreatic disease (BPD), from exosomes isolated from the patients with intraductal papillary mucinous neoplasm (pre-neoplastic lesion) based on the levels of exosomal GPC1. This non-invasive test, as authors claimed, allowed them to distinguish patients with pre-cancerous pancreatic lesions, and to identify patients with late pancreatic cancer (pancreatic ductal adenocarcinoma, PDAC) with 100% certainty. A comparison of the GPC1⁺ exosome-based test with a standard biomarker for pancreatic cancer (CA-19-9) demonstrated the advantage of using the GPC1⁺ exosome detection method. The authors analyzed blood from 190 patients with PDAC and 100 healthy controls, however, only a few patients with early pre-neoplastic disease. However, there are already new published results from other group [86] that contradict these findings.

EVs of different origins vary in their stability, but reported serum EVs and EVs associated with DNA may remain stable between 4 °C and room temperature for > 24 h [47], which is important for cancer diagnostics. Significant progress and research interest in circulating miRNAs and other non-coding RNAs could establish them robust diagnostic biomarkers of GI cancers if the feasibility is validated. In the context of cancer biology, miRNA-based signatures of circulated EVs could serve as potential biomarkers for clinical use due to their comparatively long half-life, high sensitivity and specificity, relatively easy accessibility and ability for early cancer detection [213]. A major advantage of miRNA compared to other RNA types, is in its high stability compared to longer mRNA transcripts in most biological samples including fixed and paraffin embedded (FFPE) samples [214–215]. However, during long-term storage their stability may gradually and differentially decrease depending on FFPE tissue block age [216]. miRNA stability in circulation is explained by binding to protein and lipid EV content [217–219].

A number of publications describe microRNA profiling in plasma, serum, and culture supernatants of epithelial cell lines of GI cancers [82,93,94,128,220]. Thus, four important miRNAs, namely, miR-1246,

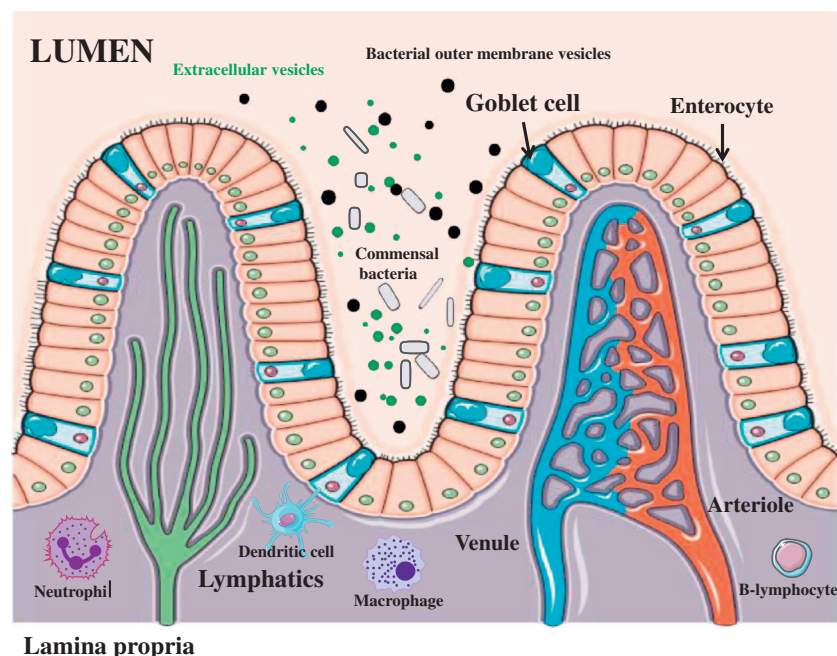


Fig. 3. Production of EVs in intestinal lumina (intraluminal EVs; tumor cell-derived-EVs; parasite-derived EVs; bacterial OMs).

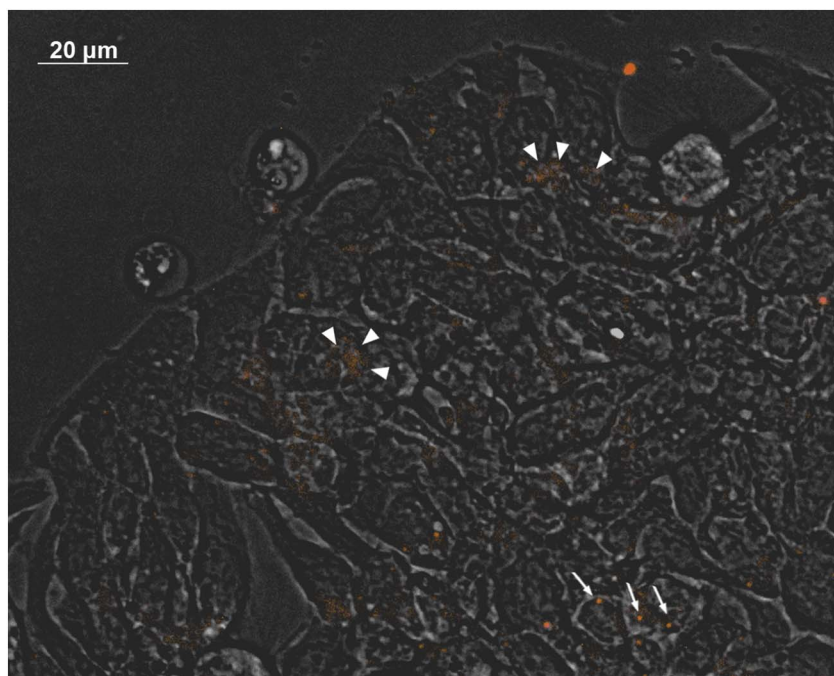


Fig. 4. Internalization of cancer-derived EVs by colorectal cancer cell line HT29. EVs from plasma of patients with colorectal cancer were purified, quantitated, labeled by PKH26 and incubated with colorectal cancer cell line HT29 for 18 h at 37 °C. Further cells were imaged with internalized individual EVs (Arrow) and cluster EVs (arrow head) using confocal laser scanning microscope ZEISS LSM780 (objective $\times 63$, 540 to 552 nm band pass excitation filters and 575 to 640 nm band pass emission filters).

miR-4644, miR-3976 and miR-4306, were upregulated in 83% of patients with pancreatic cancer and are considered by the authors [93] to be promising candidates for screening of pancreatic cancer in larger patient cohorts. It is important to note that the profile of miRNAs detected in the EVs is significantly different from that obtained from plasma samples as was demonstrated with miRNA 20a, miRNA 21, miRNA 92a, miRNA 106 and others [221]. Despite the existing optimism that miRNA/miRNA signatures may be considered as perspective biomarkers for colorectal cancer [173,222–224], and other GI tract cancers and gastrointestinal tract inflammatory diseases [225–226], other reports demonstrated that the majority of detected miRNAs are not disease specific [227–228]. Though publications suggesting EV microRNA signatures for cancer grow exponentially throughout the last decade, the suitability for clinical applications is still limited due to absence of endogenous controls to normalize circulating EVs microRNA levels, and conflicting results across the studies.

6. EVs and cancer co-evolution

It is now accepted that solid tumors are likely to derive from the co-evolution of neoplastic cells, stromal components, vasculature, and immune cells [229]. As tumors grow in size, an array of molecular modifications aggregates, giving rise to multiple cell subpopulations, each with the ability to proliferate and mutate further. Moreover, neoplastic cell populations are able to regulate the nature of other types of cells in their microenvironment, converting their intrinsic anti-tumoral response into pro-tumoral activity [230–231]. Therefore, a malignant tumor is composed not only of neoplastic cells, which are heterogeneous in terms of genetic and phenotypic features, but also of heterogeneous healthy cell populations participating in anti-tumor immune response and communicating with cells, forming a particular extracellular matrix that supports cancer evolution and progression [151]. EVs are critical mediators of intercellular communication and can orchestrate stroma, tumor microenvironments and tumor heterogeneity influencing the dynamics of disease progression and metastasis outgrowth [232–234], promoting angiogenesis via Egr-1 activation [235], down-regulation of VEGF expression [20], microRNA production [236], and implicating other mechanisms, such as epigenetic regulation of cancer progression.

Longitudinal studies of cancer patients may reveal possible

mechanisms of tumor heterogeneity/evolution [237]. Thus, Mege and co-authors [96], compared MPs from patients with CRC, pancreatic cancer, chronic pancreatitis and IBD, and characterized the distribution of different MP cellular origins (platelet-derived MPs (PMPs), endothelial MPs (EMPs), erythrocyte-derived MPs (EryMPs), leukocyte-derived MPs (LeuMPs) and MPs expressing of AnV and different MP antigens (tissue factor (TF), mucin1 (MUC1), podoplanin (PODO), etc.). Besides the differences between patient groups, authors also found microparticle signature changes before and after the “composite” complete remission (CRC), indicating that quantitative and qualitative changes happen in MP signature during cancer evolution.

Another important aspect of cancer evolution is the epigenetic regulation of gene transcription that mediates cancer cell fate, proliferation and differentiation. The initial strategy using aberrant methylation for diagnostics of tumor-specific hypermethylated genes was challenging because chronic inflammation also induced changes in methylation levels [238,239]. The search for early markers of pancreatic cancer also includes epigenetic markers [240]. Thus, DNA methylation of ADAM metalloproteinase and basonuclin in serum appears to have prognostic value for pancreatic cancer [241].

However, tumor evolution and EVs modulating effects on GI cancers are always considered from the point of EVs originating only from eukaryotic cells, and therefore completely neglects EVs originating from human microbiota.

7. EVs originated from gastrointestinal cancer and human microbiota

Gut microbiota play a crucial role in the pathogenesis of mucosal inflammation in GI tract disorders. Bacterial, mycobacterial and viral infections are found to be important in IBD pathogenesis [242]. Practically all rodent models of IBD can be mitigated by treatment with antibiotics or by transferring mice into germ-free conditions [243–244]. In mouse models, colitis-associated cancer did not develop in animals treated with antibiotics or germ-free environments [245–246].

The microorganisms that are most frequently associated with cancer development include *Mycobacterium avium* subspecies *paratuberculosis*, adherent enteroinvasive *Escherichia coli*, *Chlamydia pneumoniae* and yeasts, such as *Candida albicans* and *Saccharomyces cerevisiae* [247]. The intestine is normally colonized by approximately 100 trillion

microorganisms [248] comprising 500–1500 different species. Two major commensal groups in the mammalian intestine include *Firmicutes* (gram-positive bacteria) and *Bacteroidetes* (gram-negative bacteria) phyla [249–250] forming approximately 90% of the total microbiome. *Bacteroides* spp. dominates GI microbiota, representing approximately 30% of all bacteria in GI tract, and is most resistant to antibiotics among anaerobes [251]. Patients with GI tract cancers have specific enteric patterns of microbiome associated with an increase of certain bacterial species. Thus, stools derived from CRC patients had increased levels of *Enterococcus*, *Escherichia*, *Shigella*, *Klebsiella*, *Streptococcus*, *Peptostreptococcus*, *Firmicutes*, *Fusobacterium* and *Bacteroidetes* [245,252]. Viljoen and co-authors [253] evaluated important clinico-pathological features in *Fusobacterium* spp. and enterotoxigenic *Bacteroides fragilis* (ETBF), concluding that these bacteria were present at significantly higher levels in late-stage CRC patients in comparison with healthy individuals. *Fusobacterium* spp. is not carcinogenic, but may lead to tumorigenesis indirectly by enhancing inflammation and stimulating cancer cell proliferation [254]. *Fusobacterium* spp. acts by activating FadA adhesion, which triggers colonic epithelial cells (CEC) Wnt signaling and enhances epithelial cell proliferation. The other important bacterial species, ETBF, produces the *B. fragilis* toxin (bft), which is associated with colorectal cancer, diarrheal disease, etc. [255]. It is important, however, to emphasize that α - and ϵ - clades of *Proteobacteria* including *H.pylori* and another pathogen of digestive tract, *Campylobacter jejuni*, produce altered form of flagellin molecule that is not recognized by Toll-like receptor TLR5, but preserve bacterial motility [256].

As one of the major mechanisms, bacteria representing gut microbiota, release vesicles named outer membrane vesicles (OMVs) for Gram-negative vesicles, or blebs, for Gram-positive bacteria [257]. For simplicity we will use the term bacterial MVs or bMVs to refer to both types of these vesicles. bMVs are 40–300 nm in size, serve as part of the bacterial secretion and transport system and can deliver their cargo (DNA and RNA, protein, lipids and other biologically active molecules) [24,257] to bacterial and/or eukaryotic cells. bMVs are also a rich source of immunomodulating lipopolysaccharides, lipoproteins, peptidoglycans and other bioactive components which are described for many species of Gram-negative bacteria residing in human gastrointestinal tract including *Escherichia coli*, *Helicobacter pylori*, *Fusobacterium nucleatum*, *B. fragilis* and others [253,258–265]. Recently, Chu and co-authors [264], demonstrated that bMVs shed by *B. fragilis* deliver immunomodulatory molecules such as a capsular polysaccharide A (PSA) to intestinal dendritic cells (DC) of mice and activate a noncanonical autophagy pathway requiring IBD-associated genes, *NOD2* and *ATG16L1* for protection from colitis. Consistent with mouse research data, cells from Crohn's disease patients and healthy controls respond to purified PSA and required functional *ATG16L1* to induce CD4⁺ Foxp3⁺ IL-10⁺ T-regulatory cells in response to OMVs

from *B. fragilis*. These bMVs require human ortholog IBD associated genes *ATG16L1* and *NOD2* (the later encodes an intracellular sensor of bacterial peptidoglycan) in order to activate non-canonical autophagy protection pathway against colitis. Moreover, β -lactamases associated with bMVs that produced by *Bacteroides* sp. confer antibiotic resistance not only to the producing organism but also to other commensal bacteria and enteric pathogens (such as *Salmonella typhimurium*) against β -lactam antibiotics [266]. The bMVs have also been implicated in the pathogenesis of gastrointestinal chronic inflammatory diseases including Crohn's disease [263] and *Helicobacter pylori* associated inflammation [261].

8. Extracellular vesicles, interspecies communication and molecular mimicry

Gastrointestinal tract is a place where inter-species and even inter-kingdom communication constantly occurs. EVs derived from host eukaryotic cells and from prokaryotic symbiotic and/or pathogenic cells, edible plants [267], fungi and parasitic EVs produced by helminths [268] meet in intraluminal space and interact with intestinal cells. Recently, interspecies communication between nematodes (helminths) and host intestinal cells was reported by Buck and co-authors [269]. Remarkably, *Heligmosomoides polygyrus* secrete miRNA-loaded EVs that are internalized by host mice cells and suppress host immune response. EV secretion was shown for many human parasites, such as trematodes (*Schistosoma mansoni* and *Schistosoma japonicum*) [270,271] and nematodes (*Fasciola hepatica*, *Teladorsagia circumcincta*), and plays an important role in establishing and maintaining parasitic infection [272]. Circulating exosomal miRNAs after internalization by target cells can also act as ligands of Toll-like Receptors (TLRs) [273]. Interestingly, mice TLRs (TLR13) were reported to recognize conserved nucleic acids such as 23S ribosomal RNA molecule of bacterial pathogen *Staphylococcus aureus* [274]. Recent progress has led to identification of a large variety of cytosolic nucleic acid sensors [275]. Further research in this area offers new scenarios to understand complexity that exists in the interaction between organisms on inter-species and inter-kingdom levels.

The intestinal EVs represent a huge reservoir of microbial and parasitic antigens. New evidence demonstrates that bacterial DNA integration and related mutagenesis through lateral gene transfer may occur in cancer cells [276,277]. EVs can serve as a hypothetical vehicle for this transfer, since they carry different types of DNA and RNA. Moreover, protein epitopes can be shared between microbial molecules and self-antigens, and molecular mimicry can lead to the formation of cross-reactive antigens and/or activation T-lymphocytes. Target epitopes can have only distant homologies with initial antigenic triggers [278] and epitope spreading can lead to tissue destruction, apoptosis and concomitant presentation of self- and microbial antigens [279].

Table 4

Proteomic analysis of colorectal cancer-derived EVs [123,281–282] and matching protein sequences in commensal and pathogenic microorganisms of GI tract.

Protein family	Protein found in human colorectal EVs proteome	Microorganism containing matched protein sequence
Integrins	Integrin- α 2 precursor; Integrin- α 5-precursor; Integrins α 1, 2, 3, 6; β 1, 4	<i>Helicobacter pylori</i> (CagL) interacts with α 5 β 3 and α 5 β 1 integrins [283–284]; <i>Candida albicans</i> (SAP4, SAP5) [285]
Regulatory proteins (regulation of actin skeleton)	Tuba-protein	<i>Listeria monocytogenes</i> (InIC binds with high affinity to SH3 Tuba domain) [286]
Keratins	2a, 10, 13, 19, 24, type I, cytoskeletal 9, 14, 18, 19, 20, type II, cytoskeletal 1, 2, 5, 8	<i>Enterobacteriaceae</i> (serine protease autotransporter pet protein binding to K8) [287]; Enteropathogenic <i>Escherichia coli</i> (keratin-dependent actin reorganization) [288]; <i>Salmonella enterica</i> (interaction SspC protein with K8) [289]; <i>Salmonella enterica</i> (interaction SipC protein with K18) [290]; <i>Shigella flexneri</i> (IpaC binding to K18) [291]; <i>Streptococcus agalactiae</i> (Sar protein binding to K4) [292]; <i>Streptococcus parasanguinis</i> [293–294]

and homology to PDGFR- α in *Bacteroides* spp. may be related to PDGFR- α role in colorectal carcinogenesis.

This novel approach, (i.e. matching protein sequences from eukaryotic EV proteome with the known protein sequences of microbiome gut bacteria) (as well as the opposite – matching bMVs proteome with eukaryotic sequences) helps to identify new similarities between bacteria and eukaryotic proteins and understand their functional role.

9. Conclusions and future perspectives

Actively secreted EVs from eukaryotic cells as well as from different microbial, fungi, parasitic species and edible plants in GI tract act as mediators of intracellular and inter-species communication, promote angiogenesis, induce immune suppression, and facilitate tumor cell survival and multi-drug resistance. There is a general consensus that the specific biosignatures of multiple EV biomarkers will be required for diagnostics of GI tract cancers. However, this suggestion recently got challenged when Melo and co-authors developed a simple one-molecule EV-based test for pancreatic cancer [92]. This study was based on the search for proteomic markers highly expressed in EVs originating from patients with advanced tumors and have been identified in early stages of GI tumors. A similar approach can lead to the development of EV-based early diagnosis of different GI cancers, such as CRC (Fig. 6).

The power of EVs is related to their unique protein, RNA, miRNA and DNA profiles, ubiquitous distribution (plasma, saliva, gastric juice, intestinal luminal liquid etc.) and their efficient binding and transfer to target cells [301]. Many efforts have been devoted to EV proteome characterization and protein marker discovery [302,303]. We developed a new approach, consisting of matching protein sequences from eukaryotic EV proteome with known protein sequences to microbiome of gut bacteria as well as matching bMVs proteome with eukaryotic counterparts. We believe that using this strategy, it is possible to identify potential molecular mimicry protein sequences and these findings could be useful for further experimental research of their functional role.

The current challenges in using EV as clinical biomarkers and in researching GI tract cancers include analytical variability of different instruments used for EV detection and variability of sample preparation. The future perspective is the possibility of applying genome editing tools to engineer EVs loaded with “tailored” cargoes. Despite thorough EV research, there is much remaining to be studied to understand the contribution of EVs in GI cancers' pathogenesis and development. Overall, the development of the EV field has the potential for early-stage cancer diagnosis, tracking chemo-resistance and therapeutic efficacy, and tailor-made treatment strategies for GI cancer patients.

Conflict of interest statement

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Transparency Document

The <http://dx.doi.org/10.1016/j.bbcan.2017.06.005> associated with this article can be found, in online version.

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Appendix A. Supplementary data

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