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 (bMVs - 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 though 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 interspecies 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 conserved protein sequences. Using this approach we identified Bacteroides spp. pseudokinase with activation loop and homology to PDGFRα, providing a proof-of-concept strategy. We speculate that existence of microbial pseudokinase that ‘mimics’ PDGFRα may be related to PDGFRα and Bacteroides spp. roles in colorectal carcinogenesis that require further investigation.

**Abbreviations:** AChE, acetylcholinesterase esterase; Ago, Argonaute protein family, essential catalytic components of RISC; AnV, annexin V; APC, antigen-presenting cells; ARF6, ADP-ribosylation factor 6; bMVs, bacterial microvesicles; BPD, benign pancreatic disease; CEA, carcinoembryonic antigen; CEC, colonic epithelial cells; CEA, carcinoembryonic antigen; 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, monosialoganglioside; GPCR, G protein-coupled receptor; GTPase, Rho family small GTPases; HIF, hypoxia-inducible factor; IEM, immunoelectron microscopy; IL, interleukin; IHC, immunohistochemistry; IHC, immunohistochemistry; IL, interleukin; LSPR, localized surface plasmon resonance; MHC-I, MHC class I; PKC, protein kinase C; RAS, rat sarcoma; RISC, RNA-induced silencing complex; SCID, severe combined immunodeficiency (non-human); SELN, silencing enhancer of longevity; siRNA, small interfering RNA; TEV, tumor-originating extracellular vesicles; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; 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, IFNγ 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' PDGFRα.

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 [25-28]. 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 Tsc6 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>
<thead>
<tr>
<th>Cancer type</th>
<th>Cancer subtype</th>
<th>EVs source</th>
<th>EVs size</th>
<th>Detection and purification methods</th>
<th>Subpopulations of EVs</th>
<th>Potential biomarkers</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal cancers</td>
<td>Gastric cancer</td>
<td>Serum samples</td>
<td>App. 100 nm (TEM)</td>
<td>TEM, western blotting</td>
<td>FCM, RT-PCR</td>
<td>CEACAM5, CD97, tetraspanins, plexin, TACSTD1, trophoblast glycoprotein, CEACAM5, CD97, tetraspanins, plexin, TACSTD1, trophoblast glycoprotein</td>
<td>[50]</td>
</tr>
<tr>
<td>Intestinal cancer</td>
<td>Gastric cancer</td>
<td>Human serum</td>
<td>App. 100 nm (TEM)</td>
<td>TEM, western blotting</td>
<td>FCM, RT-PCR</td>
<td>CEACAM5, CD97, tetraspanins, plexin, TACSTD1, trophoblast glycoprotein, CEACAM5, CD97, tetraspanins, plexin, TACSTD1, trophoblast glycoprotein</td>
<td>[50]</td>
</tr>
<tr>
<td>Gastrointestinal stromal cancer</td>
<td>Gastric cancer</td>
<td>Human serum</td>
<td>&lt; 100 nm</td>
<td>ExoQuick™; qRT-PCR</td>
<td>TEM, Western blotting</td>
<td>CEACAM5, CD97, tetraspanins, plexin, TACSTD1, trophoblast glycoprotein</td>
<td>[83]</td>
</tr>
<tr>
<td>Pancreatic cancers</td>
<td>Pancreatic ductal adenocarcinoma</td>
<td>Human serum</td>
<td>&lt; 100 nm</td>
<td>ExoQuick™; qRT-PCR; TEM, Western blotting</td>
<td>CEACAM5, CD97, tetraspanins, plexin, TACSTD1, trophoblast glycoprotein</td>
<td>N/A</td>
<td>[83]</td>
</tr>
<tr>
<td>Pancreaticobiliary cancer and PDAC</td>
<td></td>
<td></td>
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<td>[87]</td>
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</tbody>
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Table 1 (continued)

<table>
<thead>
<tr>
<th>EVs source</th>
<th>Subpopulations of EVs</th>
<th>Detection and purification methods</th>
<th>Potential biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasma-derived Robertson et al. [59]</td>
<td>Through differential ultracentrifugation</td>
<td>Proteins and RNA</td>
<td>MIF + [88]</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Human serum</td>
<td>NTA, EM, CM, SEM</td>
<td>CA-199, TSG101 [89]</td>
</tr>
<tr>
<td>PDAC</td>
<td>SoM tissue and plasma samples</td>
<td>TEM, LSPR, EM, RT-PCR</td>
<td>miR-10b [90]</td>
</tr>
<tr>
<td>PDAC</td>
<td>Human blood samples</td>
<td>ULPS-MS, RT-PCR</td>
<td>GPC1 + [91]</td>
</tr>
<tr>
<td>PDAC</td>
<td>Serum samples</td>
<td>RT-PCR, qRT-PCR</td>
<td>PC-derived EVs S-Exo PaCIC markers [92]</td>
</tr>
<tr>
<td>PDAC</td>
<td>Human serum</td>
<td>RT-PCR, qRT-PCR</td>
<td>miR-21, miR-17-5p [93]</td>
</tr>
</tbody>
</table>

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 on how specific cargoes are selectively absorbed and accumulated by different classes of EVs. It has been shown that posttranslational modifications of proteins with acetyl, myristoyl, palmitoyl or glycosphatidylinositol anchors may facilitate recruitment of specific proteins to EVs [11,63-64]. However, since recruiting of proteins happens 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 understood.
<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Cancer subtype</th>
<th>EVs source</th>
<th>EVs size</th>
<th>Detection and purification methods</th>
<th>Subpopulations of EVs</th>
<th>Potential biomarkers</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal cancers</td>
<td>CRC and PC</td>
<td>Human serum</td>
<td>ND</td>
<td>FCM</td>
<td>EpCAM$^+$, EpCAM$^+$ CD147$^-$, CD147$^-$</td>
<td>Increased EpCAM$^+$</td>
<td>[95]</td>
</tr>
<tr>
<td></td>
<td>CRC</td>
<td>Platelet-poor plasma</td>
<td>ND</td>
<td>FCM</td>
<td>EpCAM-MVs</td>
<td>ND</td>
<td>[96]</td>
</tr>
<tr>
<td></td>
<td>CRC</td>
<td>Solid tissue (surgically or endoscopically resected)</td>
<td>&lt; 1000 nm</td>
<td>RT-PCR, NTA</td>
<td>CRC-derived EVs</td>
<td>miR-1246</td>
<td>[97]</td>
</tr>
<tr>
<td></td>
<td>CRC</td>
<td>Human plasma</td>
<td>&lt; 1000 nm</td>
<td>FCM</td>
<td>CRC-derived EVs</td>
<td>ND</td>
<td>[98]</td>
</tr>
<tr>
<td></td>
<td>CRC</td>
<td>Bone marrow</td>
<td>20–2000 nm</td>
<td>SEM</td>
<td>TEVs</td>
<td>ND</td>
<td>[99]</td>
</tr>
<tr>
<td></td>
<td>CRC</td>
<td>Human body fluids (pleural liquid)</td>
<td>&lt; 1000 nm</td>
<td>FCM</td>
<td>TF$^+$, PS$^-$ EVs</td>
<td>D-dimer to detect VYE in CRC patients</td>
<td>[100]</td>
</tr>
<tr>
<td>Gastrointestinal cancers</td>
<td>Gastric cancer, III-IV stage</td>
<td>Human plasma</td>
<td>ND</td>
<td>Centrifugation, FCM, procoagulant activity</td>
<td>Leukocyte-derived EVs, Er-derived EVs, Endo-derived EVs, PS$^+$ EVs</td>
<td>Leukocyte EVs, Er EVs, Endo EVs</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td>Gastric cancer</td>
<td>Human plasma</td>
<td>10–800 nm</td>
<td>TEM, FCM, AFM</td>
<td>TEVs, PEVs</td>
<td>GC36 and HER2/neu</td>
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<tr>
<td></td>
<td>Gastric cancer</td>
<td>Human platelet</td>
<td>ND</td>
<td>FCM</td>
<td>Circulating tumor-derived EVs</td>
<td>PEVs</td>
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<tr>
<td></td>
<td>Gastric cancer</td>
<td>Human pancreatic adenocarcinoma cell lines BxPc3 and Low.6pl</td>
<td>ND</td>
<td>FCM</td>
<td>TF$^+$ EVs</td>
<td>TF$^+$ EVs for PC-associated thrombosis</td>
<td>[105]</td>
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<tr>
<td></td>
<td>Pancreatic cancers</td>
<td>TF$^+$ EVs from PC injected in Par-4-deficient mice</td>
<td>ND</td>
<td>FCM</td>
<td>PMPs, EMPs, Er-derived EVs, LeuEVs, TF$^+$, Fibrin$^+$, CD11$^+$, CA19-9$^+$ EVs</td>
<td>Specific signature</td>
<td>[96]</td>
</tr>
<tr>
<td></td>
<td>Pancreatic cancer with associated thrombosis (IVC-stenosis mice model)</td>
<td>Human blood</td>
<td>200–2000 nm</td>
<td>SEM</td>
<td>TF$^+$</td>
<td>ND</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td>Pancreatic carcinoma</td>
<td></td>
<td></td>
<td></td>
<td>MVs, PEVs</td>
<td>ND</td>
<td>[100]</td>
</tr>
</tbody>
</table>

$^{*}$ ND $>$ 300 nm in the studies where conventional flow cytometry (FCM) was used for characterization of EVs (standard limit of sensitivity for conventional flow cytometer).
<table>
<thead>
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<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal cancers</td>
<td>Colorectal carcinoma</td>
<td>HCT-8</td>
<td>ND</td>
<td>TEM</td>
<td>Fraction obtained with exosome-precipitation solution</td>
<td>miR-210</td>
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<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>SW480</td>
<td>ND</td>
<td>0.22 mkm filtration and differential ultracentrifugation; western blotting, microarray, functional assays, qRT-PCR</td>
<td>Fraction obtained from 48 h cultural supernatant</td>
<td>Erk1/2 kinase dependent internalization</td>
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</tr>
<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>SW480</td>
<td>ND</td>
<td>Differential ultracentrifugation; single molecule-localization microscopy (SMLM), real-time PCR, FCM, western blotting</td>
<td>48–72 h exosomal (EX) fractions</td>
<td>miR31</td>
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<tr>
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<td>Colorectal carcinoma</td>
<td>SW480, A181.4, Capan1</td>
<td>ND</td>
<td>Differential ultracentrifugation; FCM, zymography, RT-PCR</td>
<td>EX fraction obtained from 48 h cultural supernatant</td>
<td>CXCR4-axis</td>
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<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>HT-29, Caco-2 (in vivo mouse model)</td>
<td>30–150 nm (TEM)</td>
<td>Differential ultracentrifugation and EX purification kit; TEM, FCM, western blotting (HSP70), qRT-PCR, functional assays</td>
<td>IX fraction from 24 and 48 h supernatant</td>
<td>CD44v6</td>
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<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>LIM1863</td>
<td>Different 30–100 nm, 30–1000 nm fractions</td>
<td>Differential ultracentrifugation, ExoQuick&lt;sup&gt;™&lt;/sup&gt;; TEM, NTa, western blotting, mass-spectrometry, qRT-PCR</td>
<td>IX fraction obtained from cultural supernatant of MF associated with gastric cancer cells</td>
<td>Different proteomic signatures for EX and MVs</td>
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<td></td>
<td>Murine colorectal carcinoma</td>
<td>CT26 murine colorectal carcinoma associated with mouse macrophage Ana-1 cell line</td>
<td>ND</td>
<td>Differential ultracentrifugation; FCM, western blotting, mass-spectrometry, CD9, functional assays</td>
<td>IX fraction obtained from murine colorectal carcinoma-associated with mouse macrophage Ana-1 cell line</td>
<td>Proteomic signatures</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>DLD-1 and DLD-1/5FU</td>
<td>~100 nm</td>
<td>Ultrafiltration and column separation; qRT-PCR, western blotting (CD9), deep RNA sequencing</td>
<td>DLD-1 and DLD-1/5FU-derived MVs</td>
<td>miR-34a, miR-145</td>
<td>[115]</td>
</tr>
<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>LIM1863</td>
<td>50–400 nm (EX) and 100–1500 nm (MVs)</td>
<td>NTa, RT-PCR</td>
<td>D33-EX and EpCam EX and MVs</td>
<td>32-mRNA signature</td>
<td>[116]</td>
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<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>HCT-116</td>
<td>~100 nm</td>
<td>Differential ultracentrifugation; TEM, NTa, Ache assay, western blotting</td>
<td>CD9&lt;sup&gt;+&lt;/sup&gt; and CD63&lt;sup&gt;+&lt;/sup&gt; exosomal fraction</td>
<td>CD147/C9D, CA119-9</td>
<td>[97]</td>
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<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>DLD-1, WiDr, SW480, and COLO201</td>
<td>~100 nm</td>
<td>NTa, western blotting</td>
<td>DLD-1, WiDr, SW480, and COLO201 exosomes</td>
<td>miR-1246</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>HCT116, HCT15, HT29, COLO201, COLO205, WiDr, SW1116</td>
<td>~100 nm</td>
<td>ExoScreen, immunoblot, NTa</td>
<td>HCT116, HCT15, HT29, COLO201, COLO205, WiDr, SW1116 exosomes</td>
<td>CD147/C9D, CA119-9</td>
<td>[69]</td>
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<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>LIM1215</td>
<td>&lt; 100 nm</td>
<td>Ultrafiltration and 0.1 mkm filtration; immunoprecipitation, western blotting, LC-MS/MS</td>
<td>LIM1215-derived fraction</td>
<td>Cadherin17</td>
<td>[118]</td>
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<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>SW480 and SW620</td>
<td>40–130 nm</td>
<td>EM, Cryo-EM, GelLC-MS/MS</td>
<td>SW480, SW620 secretome and exosomes</td>
<td>TSG101, Axl, CD63, MET, S100A8, S100A9, TNC, FN2, EGFR, JAGI, SRC, TNK, CAV1, FLOT1, FLOT2, PROM1</td>
<td>[119]</td>
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<tr>
<td></td>
<td>Colorectal carcinoma</td>
<td>HT29</td>
<td>90 nm to 812 nm</td>
<td>Ultrafiltration, combined with filtration; hA33/EpCam immunosaffinity purification, western blotting (TS4011, Axl, EpCam, A33), TEM, gel-C-MS/MS</td>
<td>HT29 cell line-derived EVs</td>
<td>CD63, CD9, and CD81, miR-21</td>
<td>[120]</td>
</tr>
<tr>
<td></td>
<td>Murine LIM1863</td>
<td>90 nm to 812 nm</td>
<td>~100 nm (TEM)</td>
<td>NTA, TEM, FCM, western blotting</td>
<td>HT15 cell line-derived EVs</td>
<td>Tetraspanin and their associated proteins, FSCN1, STRAP, S100A4, S100A14 expression</td>
<td>[121]</td>
</tr>
<tr>
<td></td>
<td>HCT15, SW480 and WiDr</td>
<td>CRC</td>
<td>~220 nm</td>
<td>SDS-PAGE, western blotting, RT-PCR</td>
<td>HT15, SW480 and WiDr-derived EVs</td>
<td>CD63, CD9, and CD81, miR-21</td>
<td>[122]</td>
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<tr>
<td></td>
<td>SW480 and SW620</td>
<td>23 to 636 nm and 26–576 nm (as defined by NTA)</td>
<td>TEM, Nano-LC-ESI-MS/MS, NTa, peptide OPGGIL fractionation</td>
<td>SW480 and SW620 derived EVs</td>
<td>Tetraspanin and their associated proteins, FSCN1, STRAP, S100A4, S100A14 expression</td>
<td>[123]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCT116, SW480, SW620, HT29, CaCo2</td>
<td>LIM1215</td>
<td>&lt; 200 nm</td>
<td>TEM, Nano-LC-ESI-MS/MS, NTa, peptide OPGGIL fractionation</td>
<td>HCT116 EX fraction SW498, SW620, SW980, HT29, CaCo2 secretomes and EX fraction A33 EX-fraction</td>
<td>TSPN6</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td>HCT116, SW480, SW620, HT29, CaCo2</td>
<td>LIM1215</td>
<td>&lt; 200 nm</td>
<td>TEM, Nano-LC-ESI-MS/MS, NTa, peptide OPGGIL fractionation</td>
<td>HCT116 EX fraction SW498, SW620, SW980, HT29, CaCo2 secretomes and EX fraction A33 EX-fraction</td>
<td>Glod4, Rad23b-Cterminal fragment of agrin, Proteome analysis</td>
<td>[124]</td>
</tr>
<tr>
<td></td>
<td>CX2 and Colo357</td>
<td>&lt; 200 nm</td>
<td>TEM, Nano-LC-ESI-MS/MS, NTa, peptide OPGGIL fractionation</td>
<td>HCT116 EX fraction SW498, SW620, SW980, HT29, CaCo2 secretomes and EX fraction A33 EX-fraction</td>
<td>HCT116 EX fraction SW498, SW620, SW980, HT29, CaCo2 secretomes and EX fraction A33 EX-fraction</td>
<td>Proteome analysis</td>
<td>[125]</td>
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<tr>
<td>Cancer type</td>
<td>Cancer subtype</td>
<td>EVs source</td>
<td>EVs size</td>
<td>Detection and purification methods</td>
<td>Subpopulations of EVs</td>
<td>Potential biomarkers</td>
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<tr>
<td>Pancreatic cancer</td>
<td>Pancreatic cancer cell line and pancreatic cancer cell lines</td>
<td>Immortalized pancreatic cancer cell lines</td>
<td>30–150 nm</td>
<td>Defined by EM, Mallory staining, FCM, functional assays</td>
<td>CD63, TSG101</td>
<td>Increased miR-451</td>
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</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Pancreatic cancer cell lines</td>
<td>Primary A2.521, M2.3-D3</td>
<td>ND</td>
<td>Most of experiments with pancreatic cancer A2.521 cell line</td>
<td>ND</td>
<td>Increased miR-451</td>
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<td>Most of experiments with pancreatic cancer A2.521 cell line</td>
<td>ND</td>
<td>Increased miR-451</td>
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<td>Pancreatic cancer</td>
<td>Pancreatic cancer cell lines</td>
<td>Defined by EM, Mallory staining, FCM, functional assays</td>
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Table 3 (continued)
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<th>EVs source</th>
<th>EVs size</th>
<th>Detection and purification methods</th>
<th>Subpopulations of EVs</th>
<th>Potential biomarkers</th>
<th>Ref.</th>
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<tr>
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<td>PDAC</td>
<td>BxPC-3 and HPAF-II</td>
<td>100 nm</td>
<td>EM, FCM, NTA</td>
<td>markers confirmed</td>
<td>PDAC-derived EX fraction</td>
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<td>PDAC</td>
<td>PANC-1, SW1990, BxPC-3</td>
<td>30–110 nm</td>
<td>NTA, RT-PCR, FISH</td>
<td>PANC-1, SW1990, BxPC-3 derived EX fraction</td>
<td>RFXAP, miR-212-3p</td>
<td>[143]</td>
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<td>epithelioid carcinoma PDAC</td>
<td>PDAC</td>
<td>AsPC-1, BxPC-3, PANC-1</td>
<td>50–120 nm</td>
<td>SEM, LSPR-based sensing platform</td>
<td>Cell lines-derived EX fraction</td>
<td>miR-10b</td>
<td>[90]</td>
</tr>
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<td>PDAC</td>
<td>Panc1, MIA Paca2, T3M4</td>
<td>&lt; 100 nm</td>
<td>EM, FCM, UPLC-MS, RT-PCR</td>
<td>Panc-1, MIA Paca2, T3M4 EX fraction</td>
<td>GPC1</td>
<td>[91]</td>
</tr>
<tr>
<td>Pancreatic carcinoma</td>
<td>PDAC</td>
<td>bXPC-3 SW</td>
<td>40–150 nm</td>
<td>Ultrafiltration, AcH activity, NTA, DIS</td>
<td>Panc-1-derived EX fraction</td>
<td>miR155</td>
<td>[145]</td>
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<td>Pancreatic adenocarcinoma</td>
<td>INS-1, HPDE PANC-1</td>
<td>Panc-1</td>
<td>~100 nm</td>
<td>TEM, LC-MS</td>
<td>Panc-1-derived EX fraction</td>
<td>GA19-9 and AM</td>
<td>[89]</td>
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<td>Pancreatic adenocarcinoma</td>
<td>Panc-1</td>
<td>PANC-1, MIA Paca2, HPSC and HPDE</td>
<td>&lt; 100 nm</td>
<td>TEM, EM, SEM</td>
<td>Panc-1, MIA Paca2, HPSC and HPDE EX fraction</td>
<td>MET, CIT, MAP4K4, Yes1, DDR1, WEE1, SRC, CDCP1, TCSF</td>
<td>[146]</td>
</tr>
<tr>
<td>Pancreatic carcinoma</td>
<td>Panc02 cells, cultivated and injected in mice</td>
<td>Panc-1</td>
<td>30–100 nm</td>
<td>RT-PCR, EM, western blotting</td>
<td>Panc-1-derived EX fraction</td>
<td>miR-203</td>
<td>[147]</td>
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<td>Pancreatic cancer</td>
<td>AsPC1, BxPC3, Capan1, Capan2, MiaPaca1, Panc1, Pt45P1, 8.18, HD3522, HD3542, HD3577</td>
<td></td>
<td>60–100 nm</td>
<td>Magnetic bead-based exosome extraction; AchE assay, TEM, mRNA detection/measurement</td>
<td>IX fraction purified with magnetic beads coated with biotinylated lectin specific to 2,6-linked sialic acid sandwich</td>
<td>ASPN, Foxp1, Apbb1lp, BC031781, Da2f, Gag2, Incomp</td>
<td>[148]</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>AsPC1, BxPC3, Capan1, Capan2, MiaPaca1, Panc1, Pt45P1, 8.18, HD3522, HD3542, HD3577</td>
<td></td>
<td>60–100 nm</td>
<td>Magnetic bead-based exosome extraction; AchE assay, TEM, mRNA detection/measurement</td>
<td>IX fraction purified with magnetic beads coated with biotinylated lectin specific to 2,6-linked sialic acid sandwich</td>
<td>Tspan8, CD44v6, alpha6beta4</td>
<td>[60]</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>BxPC3, MiaPaca2, Panc1</td>
<td>ND</td>
<td>34–45 nm</td>
<td>Ultrafiltration and ultracentrifugation, western blotting, ESI-MS/MS</td>
<td>IX fraction was enriched for IX marker proteins Alix, C99, Lamp3, syntenin</td>
<td>EGFR</td>
<td>[149]</td>
</tr>
<tr>
<td>Rat pancreatic adenocarcinoma</td>
<td>RSp73ASML</td>
<td>ND</td>
<td>34–45 nm</td>
<td>Ultrafiltration, SDS-PAGE, western blotting, MALDI-TOF</td>
<td>IX fraction</td>
<td>CD44v6-dependent exosomes</td>
<td>[55]</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>SOU-6, BxPC3, MiaPaCa-2, and Panc-1</td>
<td>34–45 nm</td>
<td></td>
<td>EM, SDS-PAGE, MALDI-TOF</td>
<td>SOU-6, BxPC3, MiaPaCa-2, and Panc-1 nanoparticles</td>
<td>ND</td>
<td>[150]</td>
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</table>
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 vehicles 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′ uridylation of 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 [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...
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 envi-
ronment facilitated angiogenesis.

It has recently been established that gut microbiota has a significant
influence on the expression pattern of miRNA of intestine cells in-
cluding 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 re-
constitution 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 bor-
ders 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 in-
terrelationships 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 gas-
trointestinal 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 inter-
connected 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 rib-
osylation factors (ARF), such as ARF1 and SRC kinases have the es-
tential 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 me-
tastasis 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 diag-
nostic 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 mole-
cules (JASG1, SRC, TNIK) 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 mod-
ulation 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,
phosphatidylethanolamine (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-en-
riched membrane domains (TEMs) associated with gangliosides and cho-
lesterol. 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 and important
components of lipid metabolism such as phospholipases, hydroxycholesterol, 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, Ris-
torcelli 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 exo-
somes 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-spec-
trometry, they demonstrated that secreted exosome glycerophos-
pholipid compositions are clearly distinct from parental cells in-
dicating 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, plasmalogens- 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 frac-
tions 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 com-
parison 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

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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 mRNA may 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 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,
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-tumor 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 (POD), 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 clinicopathological 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) and required functional ATG16L1 to deliver 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* secreted 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**

<table>
<thead>
<tr>
<th>Protein family</th>
<th>Protein found in human colorectal EVs proteome</th>
<th>Microorganism containing matched protein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrins</td>
<td>Integrin-α2 precursor; Integrin-α5-precursor; Integrins α1, 2, 3, 6; β1, 4</td>
<td><em>Helicobacter pylori</em> (CagL) interacts with α5β1 and α5β1 integrins</td>
</tr>
<tr>
<td>Regulatory proteins (regulation of actin skeleton)</td>
<td>Tuba-protein</td>
<td><em>Candida albicans</em> (SAP4, SAP5) [285]</td>
</tr>
<tr>
<td>Keratins</td>
<td>2a, 10, 13, 19, 24, type I, cytoskeletal 9, 14, 18, 19, 20, type II, cytoskeletal 1, 2, 5, 8</td>
<td><em>Listeria monocytogenes</em> (InC binds with high affinity to SH3 Tuba domain) [286]</td>
</tr>
</tbody>
</table>

**References**

Microbial commensals can induce cross-reactive lymphocytes (rev. [279]) and antibody responses in the gut can be directed against commensal and self-antigens cases being polyreactive in up to 25% [280].

We hypothesized that colorectal cancer proteomes derived from EVs released by GI tract cancer cells may have protein sequences matching gut microbiome organisms. To investigate this, we re-analyzed the colorectal EV proteome studied by the Choi group [123,281,282] and compared it with protein sequences from different commensal bacteria and viruses. As a result, a number of matching microbial sequences was identified (please, see Table 4 for detailed analysis results).

![Multiple sequence alignment for three kinase domains from Human, Mycobacterium tuberculosis, and Bacteroides fragilis, respectively. Left part shows multiple sequence alignment of three kinase domains P16234 (591–983), P9WI79 (13–295), and A0A017PL20 (1–295) from Human, Mycobacterium tuberculosis, and Bacteroides fragilis, respectively. Here P16234 is Platelet-derived growth factor receptor alpha (PDGFR-α), whereas P9WI79 and A0A017PL20 are Serine/threonine-protein kinase (PknD); (B) Superposition of the kinase domains from Human (represented in thin ribbon) and B. fragilis (represented in thick cartoon). Residues that are critical for kinase activity are shown red in the multiple sequence alignment. For Human and Mycobacterium tuberculosis, we show G-rich loop in cyan and activation loop in purple; whereas for Bacteroides fragilis, G-rich loop is shown in green and activation loop in yellow (detailed description in Suppl. File 1). From the Fig. 4, the PknD from B. fragilis lost the most part of G-rich loop, as well as sequences differ largely at activation loop. Since G-rich loop is critical for ATP-binding, PknD from B. fragilis could lose the kinase activity and become a pseudo-kinase. (Methods are provided in Suppl. File 1).

Furthermore, as shown in Fig. 5, we identified the pseudokinase domain in the B. fragilis genome with sequence matching platelet-derived growth factor receptor alpha (PDGFR-α). Mutations of PDGF/PDGFRs genes, particularly in kinase and juxtamembrane domain [295-297] can lead to an increased PDGFR signaling and is notably associated with the stage, grade and poor outcomes of various cancers including colorectal cancers [298,299]. Interestingly, while orthologs in Mycobacterium tuberculosis possess the characteristic catalytic residue (aspartate), this residue and segment of 16 amino acids is absent in Bacteroides spp., so this protein is predicted to be catalytically inactive, but functional pseudokinase [300]. The loss of catalytic activity is mirrored by the acquisition of a potential activation loop (Fig. 5). We hypothesize that the presence of this pseudokinase with activation loop...
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 bio signatures 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.jbcan.2017.06.005 associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jbcan.2017.06.005.

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vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin, Blood 99 (2002) 2569–2577.


PLASMA LONG NONCODING RNA PROTECTED BY EXOSOMES AS A POTENTIAL STABLE BIO-MARKER FOR CANCER DETECTION, USING EXOSOME-BOUND MICRO-RNA-21 AS A CLINICAL BLOOD BIOMARKER FOR CANCER DIAGNOSIS AND prognoses.


