Chapter 4

Understanding molecular mechanisms in peritoneal dissemination of colorectal cancer; Future possibilities for personalised treatment by use of biomarkers

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Abstract

Objective: When colorectal cancer (CRC) metastasizes, this is mostly to the liver via the portal circulation. In addition, 10-25% of CRC patients eventually show metastases in the peritoneum. A selection of these patients is treated with Cytoreductive Surgery (CRS) and Hyperthermic Intra-Peritoneal Chemotherapy (HIPEC). However, several clinical needs still exist in which biomarkers could play an important role. Relatively little is known about the biology of peritoneal spread of CRC. The development of peritoneal metastases (PM) involves several steps, including: detachment of malignant cells; anoikis evasion; attachment to and invasion of the peritoneal surface ultimately ending in a colonization phase in which the malignant cells thrive in the newly formed niche. In this paper we provide an overview of molecules associated with peritoneal dissemination and explore the clinical possibilities of these candidate biomarkers.

Methods: A literature search was conducted using the PubMed database of the U.S. National Library of Medicine and Medline to identify studies on the biological behaviour of peritoneal metastases (PM) of CRC.

Results: In a series of over 100 studies on PM published between 1990 and 2010, IGF-1, HIF1α, VEGF, EGFR and ITGB1 emerge as most interesting candidates for possible clinical application.

Conclusions: Even though these promising candidate biomarkers have been identified, all of these require extensive further validation prior to clinical application. Yet, the pace of the omics revolution makes that the question is not if, but when biomarkers will be introduced to improve diagnosis and ultimately outcome of patients with PM due to CRC.
Introduction

Colorectal carcinoma (CRC) is a major health problem, being the third most common cancer worldwide. Each year more than one million people develop CRC and over 600,000 patients die of this cancer, despite improvements in diagnostic and treatment modalities. 1 Approximately half of CRC patients develop metastases, mainly to the liver via the portal vein system. 2,3 Another category of distant metastases from CRC are those to the peritoneum, i.e. peritoneal metastases (PM). In up to 25% of patients with metastatic CRC, the peritoneum is the sole site of metastasis. 3,4 Untreated PM is associated with poor survival of about 6-12 months, while systemic chemotherapy alone does not appear to yield any clinically significant survival benefit. 5,6

In the early 1990’s, a treatment with a curative intent for patients with PM of CRC, without evidence of distant metastasis to other sites, was introduced. This consists of debulking surgery (cytoreductive surgery, CRS) combined with heated intraperitoneal chemotherapy (HIPEC). 7,8 This approach is based on the hypothesis that peritoneal dissemination is a form of local rather than systemic spread, which in theory makes PM amenable to a form of local disease control, i.e. surgery. 9 This theory is further supported by the observation that a subgroup of patients, in whom no residual macroscopic tumour is left post-resection, shows a five-year survival of 35-45%. 10 This is equal to the survival of patients undergoing resection for liver metastasis, which to date is part of the standard of care for liver metastases of colorectal origin (CRLM). 3,10,11

Thus, patients with resectable metastatic colorectal cancer, either to the liver or the peritoneum, have benefitted from the aggressive surgical management. However, because treatment with CRS and HIPEC has a morbidity and mortality rate of 15-18% and 5%, respectively, it is of utmost importance to be able to carefully select those patients who will benefit most from this treatment. 4,5,10,12 In addition, major challenges in diagnosing and treating PM remain. For example, most early peritoneal lesions are smaller than 1 cm, and as such not visible on pre-operative CT- or PET-scans, which hampers both timely diagnosis and adequate monitoring of patients with PM. 12 Furthermore, the chemotherapy that is used in HIPEC is still based on the ‘one size fits all’ principle, without any biological stratification.

Presently, the selection of patients is made roughly on clinical parameters and intra-operative findings. Based on the hypothesis that the phenotype, and thus clinical behaviour, of PM in CRC is driven by underlying biological mechanisms, read outs of disease biology, i.e. biomarkers, will be of help for more refined identification of suitable patients. Additionally, adequate targets may be of great value in prognosis assessment, imaging and guidance of therapy.
The aim of the present paper is therefore to provide an overview of promising molecular changes reported to be involved in peritoneal dissemination of CRC and to evaluate the potential for clinical application of these biomarkers.

**Materials and methods**

A literature search was conducted using the PubMed database of the U.S. National Library of Medicine and Medline using the following keywords: peritoneal metastasis, CRC, biological behaviour, detachment, motility, anoikis, attachment, invasion, angiogenesis, proliferation, epithelial to mesenchymal transition, immunohistochemistry (IHC), proteins, HIPEC and combinations of these keywords. These search terms were used in order to include as many articles as possible on the subject. Additional papers were included by cross-referencing from papers retrieved in the first search. Articles from 1990 up to 2010 on biomarkers in PM of CRC were included for individual review, and only so when fitting the scope of this paper. A second reader conducted additional review when necessary. Studies on ovarian-, gastric-, and carcinoma of the pancreas spread to the peritoneum were included for review as well. Reports on in-vivo, in-vitro as well as ex-vivo experiments were included.

**Results**

The process of metastasis in general comprises different phases: First, tumour cells detach from the primary tumour, second, they migrate to the distant site and third they adapt to the new microenvironment, i.e. niche, and grow out. 13,14

The niche in question, the peritoneum, is the surface of the abdominal wall. It is made up of a single layer of mesothelial cells on a basement membrane supported by a connective tissue or stroma compartment, also referred to as the submesothelium, which forms the niche for PM. This submesothelium consists of stromal cells and extracellular matrix (ECM), which is composed of laminin, collagen, fibronectin, proteoglycan and elastin. 15

**Cellular Mechanisms involved in peritoneal spread**

Below, candidate biomarkers are discussed in the order of the sequential steps of the metastatic process. However, as considerable interactions exist between these processes, this classification is by no means absolute.
1. Detachment and increased motility

Upon detachment from the primary CRC, in addition to anoikis evasion, tumour cells have to enable motility in order to reach the target organ. This is acquired by downregulation of cell-cell adhesion molecules (CAM’s) and changes in the cytoskeleton, such as the extension of lamellipodia and filopodia.

Cell-cell adhesion molecules include the integrin family of proteins, cadherins, selectins and members of the immunoglobulin superfamily (e.g. ICAM and VCAM), which are discussed below in section 3. Also, other adhesion molecules, like CDH2, and cell surface molecules with signal transduction function, like EGFR and c-MET play a role here. These phenotypic alterations that take place during invasion, with tumour cells exhibiting reduced adhesion and increased motility, are also referred to as epithelial-mesenchymal transition (EMT). In the context of PM, the molecule TWIST is reported to be involved in this process.

**Twist homolog 1 (TWIST)**

TWIST is a transcription factor that is most abundantly expressed in tissues of mesodermal origin. TWIST is known as an EMT trigger. Studies aiming to block the function of TWIST by siRNA in ovarian adenocarcinoma cells have shown suppression of adhesion molecules and matrix metalloproteinases (MMPs). Levels of migration, invasion and adhesion to peritoneal mesothelium were significantly lower in siTWIST cells, supporting that downregulation of TWIST could, in theory, ultimately inhibit peritoneal dissemination.

**Met proto-oncogene (c-MET)**

c-MET is a proto-oncogene that encodes for the hepatocyte growth factor receptor (HGFR). It has tyrosine kinase activity and is essential in embryonic development and wound healing. Activation of MET by hepatocyte growth factor (HGF) initiates an invasive growth program, which physiologically occurs during embryonic development and organ regeneration, but that is hijacked by cancer cells to promote invasion. Abnormal MET expression in cancer has been linked to poor survival in several malignancies, including CRC. c-MET was found to play an important role in peritoneal dissemination of ovarian carcinoma (OvCa) cells in culture, most probably through an α5β1 integrin-dependent mechanism. Using tissue microarrays (TMAs), cell lines and animal models, the role of c-MET in PM of OvCa was investigated. The disease specific survival of patients with high c-MET protein expression was significantly lower than those with low expression (N=138). In experiments using ECM components, primary mesothelial cells, full-thickness human peritoneum and in-vivo mouse peritoneum, marked inhibition of dissemination was noted by siRNA targeting c-MET. Furthermore, a reduction in α5β1 protein and
mRNA, accompanied by a reduction in MMP2 and MMP9 activity, was noted. 25 As such, theoretically c-MET represents a very attractive candidate biomarker of PM formation risk as well as a possible therapeutic target in prevention of peritoneal dissemination.

**Epidermal growth factor receptor (EGFR)**

EGFR is a cell surface protein and when its ligand binds, a signalling cascade starts that leads to cell proliferation. 3,10,11,26 Activation of EGFR is thought to promote the downregulation of E-cadherin, causing disruption of adherens junctions, through induction of MMP9. This interaction has been investigated in OvCa by blocking with an siRNA directed against MMP9. 27 This study involved two cell lines and tissue samples from 146 primary OvCas, including 17 paired samples of primary tumour and PM. Treatment with recombinant MMP9 or transient expression of MMP9 was sufficient to regain function and reduce E-cadherin levels in the cell lines, which subsequently caused a migratory and invasive response. These findings were validated by IHC on TMAs containing patient material, which exhibited co-localization of activated EGFR and MMP9 accompanied by E-cadherin loss. 27 Clinically elevated levels of both EGFR and MMP9 have been associated with worse survival. 28-30

Other markers associated with detachment and increased motility in in-vitro and in-vivo studies in the context of PM are HGF, RHOA and CDH2. 24,27,31-34 However, the clinical significance of up or down regulation of these specific genes in PM of CRC remains to be verified.

**2. Anoikis evasion**

Anoikis is a form of programmed cell death that takes place when cells detach from the surrounding extracellular matrix (ECM), thereby loosing integrin-ligand interactions, as occurs during tumour cell invasion. 13,35 In normal tissue anoikis is critical in maintaining tissue homeostasis by preventing tissue proliferation in unwanted sites. Evading anoikis is a condition for cancer cells to survive and is believed to be an essential step in metastasis. 36,37 This also is the case during the process of PM formation where detached cells exhibit anchorage independent growth, meaning that they are able to survive without intact cell-cell signalling, a requisite for cell survival in normal cells. 38 In PM, this is most likely achieved by forming tumour cell clusters and retaining the ability to proliferate in cell clusters, as opposed to the adherence to platelets as described in haematogenous spread. 35,39 In peritoneal spread, two molecules are reported to play a role in anoikis evasion.
Kallikrein-related peptidases

The process of evading anoikis in PM involves the activation of the kallikrein-related peptidases (KLKs), a group of serine proteases. There are 15 proteins belonging to the kallikrein family. Known functions of the KLKs include degradation of ECM molecules, contributing to activation of growth factors and proteases. One of the most well known KLKs, utilized in diagnosis and follow-up of prostate cancer, is KLK3 or prostate specific antigen (PSA). Interestingly, there is a link between cell clustering and KLK expression. Cell clustering is a frequent event in OvCa ascites, which may contribute to anoikis evasion. The aggregation of these malignant cells was associated with a higher protein expression of KLK7. Hence, it is assumed that treatment response is, at least partly, governed by anoikis resistance under the influence of KLK7. Further investigation revealed that α5β1 integrins and KLK7 co-express in OvCa. Based on these findings an association between KLK7 levels and PM via cell aggregates and α5β1 integrin mediated cell-adhesion was assumed. Several integrin inhibitors, such as Volociximab are currently under development and are of interest as targeted therapy in PM, based not only on the abovementioned results but also on a proof of principle study with blocking of β1 integrin (ITGB1 ) in a murine and ex-vivo peritoneal model, described under “Adhesion to peritoneal surface”. KLKs have also been investigated as prognostic markers in CRC in primary tumours. A higher KLK 7 protein expression was found to be associated with a shorter overall and disease free survival in a study containing 105 patients. In addition, analysis of a panel of KLKs shows overexpression of KLK5, KLK7 and KLK 14 to be prognostically relevant in patients with primary CRC in a multivariate analysis.

V-Src sarcoma viral oncogene homolog (Src)

Src is a tyrosine kinase that is described as a key player in cell-matrix contact-mediating focal adhesions, in cell-cell contact-mediating adherens junctions and in cell-anchorage dependent signalling. A Src-dependent mechanism has been reported that provides transient protection of colonic epithelial cells from anoikis. Using a Src-specific inhibitor, loss of protection from anoikis was shown in various experiments. CRC cell line experiments have shown that enforced Src expression increases resistance to anoikis. As well as having a role in anoikis evasion, upregulation of c-SRC causes adherent cells to scatter and to extend their lamellipodia, thus making tumour cells highly motile. In a murine model, blocking of Src showed reduced peritoneal dissemination compared to control transfectants. These results support the hypothesis that c-SRC may play a role in detachment and motility of CRC cells.
3. Adherence to peritoneal surface

After evasion of anoikis, detached malignant cells will have to adhere to the peritoneal surface to be able to ultimately invade and colonize the peritoneum. Adherence to the peritoneal surface depends largely on the interaction between malignant cells and ECM components through integrins, cadherins, and cell-adhesion molecules.

Integrins

Integrins are a family of receptors that play a major role in cell-ECM attachment and signalling. Integrins may also play a role in attachment of malignant cells to the peritoneum. The integrin family consists of 24 members, and function in different combinations of α and β subunits. Integrins are expressed on epithelial as well as endothelial cells, leukocytes and platelets. Altered expression patterns of integrins are frequently associated with a malignant phenotype, supporting invasive and metastatic behaviour. One of the integrins involved is the ITGB1. In a surgical trauma animal model, blocking ITGB1 caused less tumour cells to adhere to the peritoneum, followed by impaired outgrowth of tumours. These results were confirmed in an ex-vivo peritoneal and in-vivo mesothelial model. This suggests a role for integrins in the adherence and growth of tumour cells on (surgically traumatized) peritoneum. Another integrin, α5β3, has been identified in PM of pancreatic cancer. It is the primary receptor for Cyr61, one of the molecules described under “Inducing Angiogenesis”. The experiments involving this receptor-ligand couple were carried out on a murine pancreatic cancer model, using microarray analysis, RT-PCR and IHC to confirm the results. With IHC, co-localization of Cyr61 and α5 was observed, suggesting that the interaction between Cyr61 and α5β3 promotes the formation of PM.

Cadherin 1 (CDH1 or E-cadherin)

This protein is part of the cadherin family of cell adhesion molecules, consisting of glycoproteins that mediate cell-cell adhesion in epithelial cells. Reduced cell surface expression of this protein has been observed in many cancers, including oesophageal, colon, breast and OvCa. As a consequence these cells show easy disaggregation, facilitating invasion and metastasis. In PM of CRC one in-vivo study has been described with a LoVo clone showing E-cadherin downregulation to be associated with metastasis. These cells formed a primary colon carcinoma in the host model, which was then resected. Seven out of ten mice showed isolated mesenteric lymph node metastases, not peritoneal carcinomatosis, post-resection, compared to 10 disease free mice in the control group.
In an OvCa model however, downregulation of E-cadherin by siRNA was followed by upregulation of α5β1 integrin. 34 In this study, intra-peritoneal administration of an α5β1 integrin antibody significantly reduced ascites and the number of metastases compared to the control group. 34 In a cohort of 107 OvCa patients the overexpression of α5-integrin correlated with worse survival. 34 Altogether, these findings imply an effect of downregulation of E-cadherin through an integrin-mediated pathway.

InterCellular Adhesion Molecule 1 (ICAM-1)

The ICAMs are a subfamily of the immunoglobulin superfamily of cell adhesion molecules. ICAM 1 is one of five ICAMs. 18 The role of ICAM 1 has been investigated in a pneumoperitoneum-simulated environment using a CRC cell line and a mesothelial cell monolayer. 56 In this study increased adhesion of CRC cells to the stimulated mesothelium was observed. 56 In an attachment assay to detect MMP’s activity in naive (unstimulated) mesothelium, it was confirmed that increased expression of ICAM-1 led to increased adhesion of malignant cells. 57 Moreover, the link between ICAM1-CD43 interaction and MMP production was analysed on invasion of attached cells. An increase in adhesion and invasion was noted, which was manifested by an increase in MMP expression. 57

Epithelial cell adhesion Molecule (Ep-CAM)

Ep-CAM is a type 1 transmembrane glycoprotein and not structurally related to the four major families of cell adhesion molecules. 58 Ep-CAM is frequently highly expressed in malignancies of epithelial origin, including CRC. 58,59

In peritoneal dissemination of OvCa the expression of Ep-CAM in primary, metastatic and recurrent epithelial OvCa was studied. 60 Using real-time polymerase chain reaction (qRT-PCR) and IHC in patient material and cell lines, high expression of Ep-CAM was detected in all three disease stages, with a significant difference in Ep-CAM expression levels between metastatic, recurrent and chemotherapy-resistant OvCa compared to normal ovarian epithelium. 60

CD 44 molecule (CD44)

The protein encoded by the CD44 gene is a cell surface glycoprotein that plays a role in cell-adhesion, cell-cell interaction and migration. It is a receptor for hyaluronic acid and can interact with MMPs and collagens as well. It participates in several cellular functions, one of which is metastasis. 61

Alternative splicing of CD44 pre-mRNA results in different isoforms of the protein. In CRC CD44 exon v6 splice variants are present in advanced stages of disease. 62,63
In PM, in-vitro adhesion assays have been performed with the use of mesothelial cell cultures and OvCa cells. A CD44 monoclonal antibody partially inhibited adhesion of tumour cells to the mesothelium. In nude mice, treatment with CD44 monoclonal antibody led to significantly less tumour implants on the peritoneal surface. Although some adhesion of OvCa cells to the mesothelial layer still occurred, albeit to a lesser extent, it ultimately lead to less PM.

Interactions have also been reported between chemokine receptors present on malignant cells and targets in the mesothelium in OvCa. Examples of this mechanism are the binding of CXCR4 to CXCL12 and the binding of MUC16 to Mesothelin.

Altogether these data suggest that the blocking of these attachment-mediating molecules could be of clinical importance. For example, in the peri-operative window, blocking of these candidates could lead to less implantation and subsequently less tumour outgrowth.

4. Invasion of the peritoneum

Proteolytic enzymes to degrade the ECM are necessary for tumour cells to be able to invade beyond the basement membrane. These enzymes are secreted by tumour cells themselves or by the stromal cells surrounding them. ECM degrading metallopeptidases (MMPs) have been extensively linked to this step in peritoneal dissemination.

The matrix metallopeptidases (MMPs)

Proteins of the MMP family break down the ECM in normal physiological processes as well as in disease, including cancer. MMPs facilitate invasion of the tumour cells into the nearby stroma by degrading multiple types of ECM proteins. They play a role in metastasis of CRC, including in liver- and lung metastases.

In gastric carcinoma overexpression of MMP7 in primary tumours, as seen by IHC and RT-PCR, was an independent risk factor for formation of PM. In OvCa overexpression of MMP9 was reported to promote a migratory and invasive phenotype in functional experiments performed in two OvCa cell lines. Altogether, the invasion of the peritoneal surface by cancer cells has been linked to altered MMP2, MMP7 & MMP9 expression in several gastro-intestinal tract malignancies. For this reason, attempts have been made to treat PM with MMP inhibitors. Indeed, inhibition of MMPs with the drug Batimastat in rats inhibited development of CRC metastasis. However, intra-peritoneal administration of this drug resulted in marked peritoneal inflammation and ascites, reason why no clinical studies with Batimastat in humans have been initiated to date.

In addition, the Wilms Tumour gene protein 1 (WT1) and TIMP2 are also reported to promote invasion. Contact between malignant cells and the submesothelial collagen results in WT1 overexpression and subsequent invasion in OvCa. Furthermore, decreased
invasive capacity is observed after blocking WT1 by siRNA. TIMP-2 is a tissue inhibitor of metalloproteinases, for which a twofold upregulation of TIMP-2 mRNA was observed in the PM of CRC patients, next to upregulation of HIF-1α and IGF-1 that are described below. TIMP-2 is part of the TIMP gene family and proteins encoded by this gene are inhibitors of the matrix metallopeptidases (MMPs). In contrast to other TIMP family members, TIMP-2 also has the ability to directly suppress the proliferation of endothelial cells. Several other genes have also been implicated in invasion of the peritoneum. These include: c-MET, ITGB1 and CDH1, which have been discussed previously under “Adhesion to the peritoneal surface”.

5. The colonization phase - Survival in the new niche

Sustaining proliferative signalling, enabling replicative immortality and evading growth suppressors

One of the most fundamental characteristics of malignant cells is their ability to sustain proliferation. Cancer cells can achieve this by various methods, such as producing growth factors and receptors for autocrine stimulation and by stimulating the tumour-associated stroma to deliver various growth factors. Additionally, changing receptor and downstream signalling facilitates ligand-independent signalling, also leading to proliferation induction. In this category, IGF-1, VEGF and HIF1α have been reported in PM.

Insulin like growth factor 1

Insulin like growth factor 1 (IGF-1) is part of a family of peptides that play important roles in growth and development in mammals. In a microarray study on PM and liver metastasis from CRC patients, IGF-1 mRNA was exclusively and fairly consistently overexpressed in PM when compared to liver metastasis. Conversely, circulating levels of insulin-like growth factor binding protein 3 (IGFBP3), an endogenous antagonist of IGF-1, correlates with slower progression in patients with metastatic CRC. Both these findings imply a role for IGF-1 in the formation of PM and underline that gene expression profiles differ per site of metastasis.

Inducing angiogenesis

As all cells, cancer cells are in need of nutrients for survival and 150-180µm is the maximum distance at which these nutrients can diffuse from blood vessels. Beyond this distance the tumour cannot proliferate without active angiogenesis, since the resulting hypoxia induces apoptosis. One of the mechanisms used by cancer cells to overcome this limitation is the induction of angiogenesis, a process in which genes like Hypoxia Inducible Factor 1 (HIF1) and Vascular Endothelial Growth Factor (VEGF) play a role.
Hypoxia inducible factor (HIF) 1

Hypoxia within the tumour microenvironment can induce angiogenesis by release of HIF1, which regulates production of VEGF. In the same study discussed above, 20 colorectal adenocarcinomas and eight high-grade appendiceal adenocarcinomas were analysed for genome wide mRNA expression using oligonucleotide microarrays. Site-specific gene expression profiles were observed. A twofold upregulation of HIF1α specific for PM was noted in this cohort, next to TIMP-2 and IGF1, which were discussed above. HIF1α has also been described as being upregulated in primary CRC when compared to normal mucosa.

Vascular endothelial growth factor (VEGF) and Vascular endothelial growth factor receptor (VEGFR)

VEGF has been extensively described as one of the most important angiogenic factors in solid tumours. An IHC study of PM tissue samples from patients with adenocarcinoma of the appendix or colon demonstrated that higher VEGF expression was associated with a worse clinical prognosis. However, no significant correlation was noted between microvessel density and survival. Mutations in VEGF, as well as the tumour suppressor gene p53 have been shown to play a role in metastasis of CRC. About 50% of patients with metastatic disease are known to be positive for both mutations in p53 and VEGF. VEGF targeted therapies are clinically widely used in combination with chemotherapeutic agents in patients with histologically confirmed CRC metastasis and measurable disease in the absence of absolute contraindications. In a PM of CRC murine model, blocking of VEGFR with DC101, a VEGFR-2 specific antibody, has been shown to have a negative effect not only on angiogenesis but also on the proliferation of both tumour cells and endothelial cells, effectively showing significantly reduced intra-abdominal tumour burden and ascites formation.

Immune aggregates & VEGF

As an alternative mechanism for the pathogenesis of PM, one paper described the preferential attachment of malignant cells to immune aggregates in the omentum. In a murine model using several cell lines, including one OvCa cell line, these immune aggregates in the omentum were noted to have a complex network of capillaries with a high vascular density. The mesothelial cells overlying these immune aggregates were observed to be hypoxic and VEGF-A production co-localized with these hypoxic regions. This mechanism was hypothesised to contribute to the survival of these tumour cells on the peritoneum, due to apparent constitutive readiness of the vasculature in the immune aggregates to expand. Interestingly, these immune aggregates have also been observed
in humans. Theoretically, their presence and reported make-up could form a plausible explanation for the attachment of free cancer cells to these sites in the peritoneal surface.

**Cysteine-rich protein 61 (Cyr61)**

Cyr61, already referred to above, is a secreted matricellular protein that binds to integrins. The Connective tissue growth factor, Cysteine-rich protein, and Nephroblastoma overexpressed gene (CCN) family consists of six members that play a role in processes such as fibrosis, angiogenesis and wound healing. Overexpression of Cyr61 in tumour cells has been linked to PM in pancreatic cancer. Cyr61 is the ligand for the α5β3 integrin, described under “Attachment to peritoneum”. Therefore, Cyr61 is known to activate both angiogenesis and cell-cell attachment related signalling pathways in PM. Molecules such as CD44 and α5β3 have also been linked to angiogenesis in disseminated disease in several experiments. These results are mostly based on comparative genomic hybridization and IHC (validation) studies in pancreatic, colorectal and OvCa.

Taken together, these findings support an important role of angiogenesis in the process of peritoneal dissemination. For this reason, interfering with angiogenesis has been investigated as a method for treating PM. In nude mice, inhibition of growth of PM of CRC was demonstrated when intra-peritoneally injected tumour cells were treated with the angiogenesis inhibitor TNP-470. TNP-470 inhibits methionine aminopeptidase type II, which causes proliferation of endothelial cells. The TNP-470 treated mice in this study had significantly less tumour foci intra-peritoneally, smaller tumours and a longer survival compared to the control group. These effects are similar to those that have been reported in the aforementioned VEGFR-blocking experiments, suggesting that anti-angiogenic therapy holds promise in the treatment of PM.

A summary of the process of peritoneal dissemination and the proposed effects of the identified molecules in PM of CRC is depicted in figure1. Of all the described molecules, albeit highly experimental, several show not only biological relevance, but have been shown to have either a prognostic effect in PM or hold promise as a therapeutic target in the future. (Table1)
Figure 1: The roles of the identified molecules in PM of CRC per dissemination step

Table 1: Summary of targets with possibility for clinical implementation in PM in the future

<table>
<thead>
<tr>
<th>Promising Target in PM</th>
<th>Biological relevance in PM</th>
<th>Difference with CRLM</th>
<th>Prognostic relevance</th>
<th>Possible therapeutic implications</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>Growth factor</td>
<td>Yes, &gt;2-fold change in mRNA ⁷⁴</td>
<td>Not clear</td>
<td>Monoclonal antibody Figitumumab ¹⁰²,¹⁰³</td>
</tr>
<tr>
<td>KLK7</td>
<td>Anoikis Evasion</td>
<td>Not clear</td>
<td>Yes, worse overall survival in CRC ⁴⁵,⁴⁶</td>
<td>Inhibition of KLK7 would lead to less evasion of anoikis. ⁴⁵ However, interaction with αβ integrin, thus integrin inhibitors such as Volociximab may also be of interest. ⁴²,⁴³</td>
</tr>
<tr>
<td>HIF1</td>
<td>Angiogenesis</td>
<td>Yes, &gt;2 fold change in mRNA ⁷⁴</td>
<td>Not clear, trend was observed</td>
<td>HIF1 inhibitors could potentially be of interest in PM. HIF1 upregulation seems also to be specific for PM ⁷⁴,¹⁰⁴</td>
</tr>
<tr>
<td>VEGF</td>
<td>Angiogenesis</td>
<td>No</td>
<td>Yes, high VEGF expression correlated to worse survival ⁸⁶</td>
<td>Currently anti-VEGF antibody therapies already in use in clinical setting, very promising for clinical application. ⁸⁷</td>
</tr>
<tr>
<td>Promising Target in PM</td>
<td>Biological relevance in PM</td>
<td>Difference with CRLM</td>
<td>Prognostic relevance</td>
<td>Possible therapeutic implications</td>
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</tr>
<tr>
<td>Cyr61</td>
<td>Angiogenesis</td>
<td>No</td>
<td>Not clear</td>
<td>Is the ligand for α5β3. If possibility for blocking this interaction blocking of attachment to peritoneal surface ensues and subsequent angiogenesis is inhibited. 53</td>
</tr>
<tr>
<td>TWIST</td>
<td>Detachment and motility</td>
<td>No</td>
<td>Not clear</td>
<td>In experimental model blocking of TWIST showed less migration, invasion and adhesion to peritoneal surface. In theory powerful inhibition of PM formation. 20</td>
</tr>
<tr>
<td>c-MET</td>
<td>Detachment and motility</td>
<td>No</td>
<td>Yes, higher expression of c-MET correlated to worse survival 24</td>
<td>In multiple experimental models blocking of c-MET showed marked inhibition of dissemination. Theoretically attractive therapeutic target. 25</td>
</tr>
<tr>
<td>EGFR</td>
<td>Detachment and motility</td>
<td>No</td>
<td>Not clear</td>
<td>Cetuximab is a clinical grade antibody already widely in use for metastasized CRC. No clinical data on specific EGFR inhibition in PM is available.</td>
</tr>
<tr>
<td>Integrins</td>
<td>Adhesion molecule</td>
<td>Not clear</td>
<td>Not clear</td>
<td>In multiple experimental models blocking of integrins showed marked inhibition of dissemination. Theoretically attractive therapeutic target. 44,77,78 Several integrin inhibitors currently under development. 42,43</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Adhesion molecule</td>
<td>Not clear</td>
<td>Not clear</td>
<td>Highly experimental, dubious if therapeutically significant. Theoretically blocking of ICAM-1 leads to less PM due to less attachment to peritoneum. 56</td>
</tr>
<tr>
<td>Ep-CAM</td>
<td>Adhesion molecule</td>
<td>Not clear</td>
<td>Yes, higher expression of Ep-CAM correlated to worse survival 60</td>
<td>Dubious if therapeutically significant. Theoretically blocking of Ep-CAM leads to less PM due to less attachment to peritoneum.</td>
</tr>
<tr>
<td>CD44</td>
<td>Adhesion molecule</td>
<td>Not clear</td>
<td>Yes, exon v6 variant in advanced disease 62,63</td>
<td>Highly experimental, dubious if therapeutically significant. Blocking of CD44 does not completely block attachment to peritoneum in experimental model. 61</td>
</tr>
<tr>
<td>MMPs</td>
<td>Proteolytic enzyme</td>
<td>No</td>
<td>Yes, higher expression of MMP7 in primary tumour independent risk factor for PM. 16</td>
<td>In experimental model, treatment with Batimastat showed inhibition of PM. However serious adverse events reported, thus no clinical studies to date. 72</td>
</tr>
</tbody>
</table>
### Table 2: Targets extracted from PM of CRC studies and possible clinical implications

<table>
<thead>
<tr>
<th>Target expressed in HIPEC patient material from PM of CRC</th>
<th>Possible clinical implementation</th>
<th>Correlation with outcome of HIPEC patients after treatment with CRS and HIPEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1 (^{74,102,103})</td>
<td>Therapeutic</td>
<td>Unknown</td>
</tr>
<tr>
<td>TIMP2 (^{16,74})</td>
<td>Possible stratification tool</td>
<td>Unknown</td>
</tr>
<tr>
<td>HIF1 (^{74,84})</td>
<td>Possible stratification tool</td>
<td>Unknown</td>
</tr>
<tr>
<td>VEGF (^{86,87})</td>
<td>Possible stratification tool</td>
<td>Yes (^{86})</td>
</tr>
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### Discussion

PM of CRC, but also other malignancies, form a serious threat for the patient’s survival. While systemic drug therapy has only limited effect on the patient’s outcome, the best option for changing the clinical course is aggressive surgery in combination with HIPEC. At least a subset of patients has been demonstrated to benefit from this approach. Yet despite the reported survival benefit reported after CRS & HIPEC in patients with PM of CRC, several unmet clinical needs still exist, mainly in the field of diagnostics. Basically, these fall apart into three topics; A first major issue is the challenge to be able to diagnose the presence of PM earlier, at a stage when the disease is less widespread. Secondly, there is a need for diagnostic tools to discriminate between patients who are likely to benefit from this intensive treatment, and those that will not, and thus would be exposed to the risk of morbidity and mortality of this treatment in vain. A third, but perhaps more remote goal, is to arrive, even in this disease state, to a more personalised approach. In an ideal situation the cytoreductive drugs used during HIPEC would be more in tune with the biological characteristics of the individual tumour, rather than the current ‘one size fits all’ approach.

A common denominator in trying to achieve these goals is making use of relevant information on the biology of the disease process, because these biological mechanisms ultimately dictate the clinical phenotype. This biological information can be read out from patient samples at the DNA, RNA or protein level, but in principle also by molecular imaging modalities. These reporter molecules are then referred to as biomarkers. As the present review shows, the field of biomarkers for these three indications is still rather in its infancy. Most observations concern initial proof of concept studies, there is hardly any validation in independent series, and no prospective stratification studies yet. Most work has been done on retrospectively collected tissue samples, and limited efforts have been conducted to develop assays that would work in clinical diagnostic settings. Also, the field of molecular imaging of PM is still in its early days.

More specifically, with respect to biomarkers for early detection of PMs, several clinical studies have shown that a lower peritoneal cancer index (PCI), i.e. the amount of PM
present at the start of treatment, as well as the extent to which the surgeon has succeeded in removing all PM, are both strongly associated with disease outcome after CRS and HIPEC for PM of CRC. Yet, detection of small lesions remains a challenge, making pre- and intra-operative assessment of the extent of PM difficult. Recently, targeted imaging strategies have been applied in PM of OvCa by means of intra-operative tumour specific fluorescent imaging using a Folate Receptor-α-targeted fluorescent agent. This allows for more precise resection of small lesions otherwise overlooked. Theoretically, similar approaches could work in PM of CRC, e.g. targeting molecules like EGFR and VEGFR. As for pre-operative imaging, alternative PET-tracers to 18-FDG could provide the additional accuracy needed for timely diagnosis of PM. However, challenges here also exist in the fact that the currently available pre-operative imaging modalities such as CT, PET and MRI come with technical limitations, such as a maximum resolution of the obtained image. After meticulous analysis of the available literature we have to conclude that currently the only targets that have the potential to be assessed in a clinical setting in the near future are IGF1, TIMP2, HIF1 and VEGF. These are the only known targets to have been solely extracted by analysis of PM of CRC in two separate studies. It is also known that HIF1 regulates production of VEGF, making VEGF, an even more interesting target. As to biomarkers for the prediction of response to CRS & HIPEC for PM of CRC, i.e. discriminating between patients that may and patients that probably will not benefit from the treatment, only VEGF has been reported to be associated with outcome after CRS and HIPEC. Yet all four markers currently lack enough promise for translation on short notice into a diagnostic test of use in a clinico-pathological setting in the assessment of material acquired from PM of CRC.

With respect to personalised therapy approaches, currently little data exist on candidate biomarkers for prediction of response to Mitomycin C (MMC) despite the fact that this drug has been around for quite some time. Interestingly, MMC based functional assays are used for the diagnosis of Fanconi anaemia, a rare hereditary disorder in which the function of one of several genes in the Fanconi pathway is disrupted. So far, however, no clear role for the Fanconi pathway has been established in CRC. Also other biomarkers that may be associated with response to cross linking agents have not yet been evaluated in CRC PM. To date, established targeted therapies in CRC include drugs against EGFR, like Cetuximab, or anti-angiogenic drugs like Bevacuzimab. For the former drug, a number of biomarkers exist, of which KRAS mutation status is most established, while for the latter so far no good biomarkers have been established. Remarkably, most of the molecules involved have not yet been reported as having an effect on survival in PM of CRC, except for VEGF.
Areas for further investigation

The present review of literature suggests that there is opportunity for both discovery studies and further validation of the identified candidates for implementation in treatment stratification in patients with PM of CRC. Mainly the lack of genome-wide studies on well-defined patient cohorts poses an interesting avenue of research to identify other molecules possibly omitted by the thus far implemented candidate based approach. Several other aspects of metastasis also require further elucidation. One of which is acquiring insight into the differences or similarities between the biology of primary colorectal tumours versus their corresponding metastases. Secondly, a better understanding of metastasis promoting features of the peritoneal environment itself, including tumour-stroma interaction.

Conclusions

Currently, several promising candidates have been identified that may have potential for use as a biomarker in PM of CRC, but all of these require extensive further validation. Therefore, rapid changes in clinical decision-making and therapy design based on current data would be premature. Yet, the pace of the omics revolution makes that the question is not if, but when biomarkers will be introduced to improve diagnosis and ultimately outcome of patients with PM due to CRC. This will certainly have the potential for a significant reduction in treatment related morbidity and mortality in this population by bringing the right treatment to the right patient.
References


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