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The evolving role of MUC16 (CA125) in the transformation of ovarian cells and the progression of neoplasia

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Abstract

MUC16 (the cancer antigen CA125) is the most commonly used serum biomarker in epithelial ovarian cancer, with increasing levels reflecting disease progression. It is a transmembrane glycoprotein with multiple isoforms, undergoing significant changes through the metastatic process. Aberrant glycosylation and cleavage with overexpression of a small membrane-bound fragment consist MUC16-related mechanisms that enhance malignant potential. Even MUC16 knockdown can induce an aggressive phenotype but can also increase susceptibility to chemotherapy. Variable MUC16 functions help ovarian cancer cells avoid immune cytotoxicity, survive inside ascites and form metastases. This review provides a comprehensive insight into MUC16 transformations and interactions, with description of activated oncogenic signalling pathways, and adds new elements on the role of its differential glycosylation. By following the journey of the molecule from pre-malignant states to advanced stages of disease it demonstrates its behaviour, in relation to the phenotypic shifts and progression of ovarian cancer. Additionally it presents proposed differences of MUC16 structure in normal/benign conditions and epithelial ovarian malignancy.

Key words: CA125 (MUC16), Mucins, Glycans, Cleavage, Ovarian cancer

Introduction

Ovarian cancer is the seventh most common cancer and the eighth most common cancer-related cause of death in women worldwide, with approximately 300.000 new cases and 185.000 deaths in 2018, according to the last report of the International Agency for Research on Cancer (1). It is predicted that, by 2035, there will be a 55% increase in incidence and a 67% increase in deaths (2). Ovarian cancer consists the most fatal gynaecological malignancy, predominantly due to its generally vague symptoms which preclude early recognition, and result in most cases (over 70%) being diagnosed when the disease has progressed to advanced stages (3). The most commonly used staging system is the one proposed by the International Federation of Gynaecology and Obstetrics (FIGO) (4). In early ovarian cancer (FIGO stages I and II) the five-year survival is high (80% - 90%) whereas in advanced disease (FIGO stages III and IV) it drops to 20% - 30% (5,6). The overall five-year survival rate generally ranges between 30% - 40% across the globe and has seen very modest increases since 1995 (7).

Ovarian cancer is a non-specific term for a variety of tumours that involve the ovary and can be classified into three large groups: epithelial, germ cell and stromal cell, depending on the different cell lineages they originate from (3). The vast majority (approximately 90%) are epithelial (EOCs), included among the most complex of all human malignancies (8,9). One of their most unusual aspects is the change in cellular differentiation that accompanies neoplastic progression (8-11). The ovarian surface epithelium (OSE) is a single cell layer primitive epithelium. During cycles of ovulation it undergoes rupture and repair with OSE cells switching between mesenchymal (migratory) and epithelial (proliferative) phenotypes to restore its integrity, whereas in resting conditions they exhibit a predominantly mesenchymal phenotype (8,10,12). With ageing, the human ovary displays increasing irregular contours and forms OSE-lined surface invaginations (clefts) and inclusion cysts. These formations have the propensity to undergo metaplastic changes and become sites of early neoplastic progression (8,13). In this sequence of events OSE acquires the characteristics of Mullerian duct-derived epithelia, i.e. the fallopian tube, endometrium, uterine cervix and vagina. This aberrant differentiation provides the basis for the classification of these cancers in several subtypes such as serous (fallopian tube-like), endometrioid (endometrium-like), mucinous (endocervical-like) and clear cell (endometrium or vagina-like) adenocarcinomas (8,10,14). Unlike carcinomas of most other organs in which epithelial cells become progressively less differentiated than the epithelium from which they arise, the differentiation of EOCs is more complex than that of OSE. Additionally they become unresponsive to signals causing mesenchymal conversion. This phenomenon called mesenchymal to epithelial transition (MET) occurs in inclusion cysts and early EOC; it is characterised by cells with cuboidal/columnar shape forming cobblestone monolayers and strong

inter-cellular contacts. In metastatic phases of EOC progression these specialised epithelial features diminish and revert to a mesenchymal (fibroblastic) phenotype, characterized by spindle-shaped cells with increased motility and invasive potential (epithelial to mesenchymal transition – EMT) (8,10-13,15,16). There is accumulating evidence that partial EMT also occurs during the metastatic process, at which an intermediate epithelial/mesenchymal phenotype is exhibited. Cancer cells in this hybrid condition demonstrate phenotypic plasticity and can shift between different morphological states, adapting to modifying conditions of their microenvironment. In this way, they can resist apoptosis more efficiently and enhance their malignant potential (15,17).

The predominant pattern of EOC metastasis is through peritoneal dissemination rather than lymphatic or haematogenous spread, distinguishing EOC from other solid tumours (11). An early event is shedding of cells from the primary ovarian tumour into the peritoneal cavity, which survive in suspension fluid (ascites) as single cells or as anchorage-independent multicellular aggregates (MCAs), also called spheroids (10). The presence of ascites is a common feature of women with disseminated ovarian cancer (18). Although most cells that detach from their tissues undergo apoptosis, EOC cells survive in ascitic fluid. This is thought to be achieved through ascites-induced apoptotic attenuation, growth factors, phenotypic shifts along the EMT spectrum and the formation of MCAs. MCAs have diverse compositions (ranging from purely epithelial to purely mesenchymal with a wide variety of mixed phenotypes) as free-floating epithelial, mesenchymal and hybrid cells join to generate them. The final steps of their metastatic journey is invasion of peritoneal surfaces, following which they undergo MET to proliferate and form firm metastases (10,11,15,17,19). The pattern of phenotypic cellular changes observed in EOC's evolution (MET → EMT → MET) is unique, as other carcinomas follow EMT → MET in their metastatic process (11).

Serous carcinomas are sub-classified into high-grade (HGSOC) and low-grade (LGSOC), the former comprising 70% - 80% of all EOCs and the latter <5%. Endometrioid, mucinous and clear cell tumours account for 10%, 3% and 10% respectively (3). These four histologic types are relatively evenly distributed among FIGO stage I cases but the vast majority of advanced stage carcinomas are high grade serous (20). Although originally OSE was considered the precursor of HGSOC tumourigenesis (through the 'incessant ovulation' hypothesis causing repetitive injuries and DNA damage), it was later identified that the fallopian tube epithelium is involved in the development of these cancers. More specifically ovarian cancers often exhibit serous tubal intraepithelial carcinomas ("STICs"), which represent the probable origin of the disease (21,22). These lesions develop at the fimbrial end of the fallopian tube and spread to the ovarian surface. During this process normal fallopian tube epithelial cells initially undergo partial EMT to form a STIC lesion, subsequently acquire a purely mesenchymal phenotype for dissemination to the ovary and eventually undergo MET

for tumour formation (15,23). It is believed that approximately 50% - 60% of HGSOCs derive from a STIC lesion (3,22).

Current treatment modalities in ovarian cancer include a combination of surgery and chemotherapy. The most commonly used chemotherapy agents are platins (either cisplatin or carboplatin) with the addition of a taxane (either paclitaxel or docetaxel) (9). Developments in the knowledge of epithelial ovarian tumours' genomic profile may lead to individualised interventions, targeting mutated genes and their oncogenic molecular pathways (22,24,25).

Despite advances in the understanding of ovarian cancer biology and application of new treatment strategies, CA125 continues to be the main clinical biomarker used for its diagnosis and surveillance (26). It was first recognized approximately forty years ago and identified as the transmembrane mucin MUC16 two decades later (27). Mucins are high molecular weight glycoproteins synthesised by specialised epithelial cells (known as goblet cells) as a major component of mucus. They are extensively glycosylated and characterised by the presence of a tandem repeat amino acid motif. The sequence and number of tandem repeats vary between different mucins. Their family can be broadly classified into two categories, based on their location relative to the cell surface: secreted mucins which are entirely extracellular (such as MUC2, MUC6 and MUC19); and mucins that are either tethered at the cell surface or secreted, which have a heavily glycosylated extracellular domain, a membrane-spanning domain and a short cytoplasmic tail (such as MUC1, MUC4 and MUC16). Mucins play a crucial role in hydration and lubrication of epithelial surfaces (such as tracheobronchial, gastrointestinal, reproductive tracts) and their protection from degrading enzymes, pathogen attacks and physical stresses. However, apart from their normal physiological roles, they also participate in various pathological states including cancer (28,29). Importantly, they possess adhesive and anti-adhesive properties, which they use in normal and malignant conditions. More specifically the expression of mucins in resting, normally polarised cells is restricted on the apical membranes of exposed epithelia. This allows for the development of cell-cell and cell-substratum contacts. Following loss of cell polarity during carcinogenesis, mucins are expressed all over their surface (30). In this way, they become available to interact with several growth factor receptors typically located to the basolateral cell surface, and modulate their downstream signalling in various malignancies (31-33). Additionally, the unrestricted expression of mucins leads to blocking of cellular junctions, which facilitates detachment of cancer cells from the tumour mass. On the other hand, tumours use the adhesive effects of mucins for metastasis, and both their adhesive and anti-adhesive properties in order to evade immune cytotoxicity (30).

The aim of this review is to investigate the role of MUC16 (CA125) in the complex milieu of ovarian cancer progression, with a demonstration of its different forms, functions and interactions.

MUC16: Functions and expression

Key advances have revealed the structure and functions of MUC16 as well as the role it plays in fundamental processes, including protection of the epithelium and human carcinogenesis (34). MUC16 is the largest mucin identified to date (35). It towers over other large mucins like MUC1 and MUC4 and, as such, it likely represents the initial point of contact with other cells and matrices (36). Its gene is present on the short arm of the human chromosome 19 and spans around 179 kb of genomic DNA (35). It encodes for a protein with a molecular mass up to 2.5 MDa, estimated to be more than 5 MDa for the predicted glycosylated mucin (37).

MUC16 forms with much lower molecular weights (ranging from 50 to 1500 kDa) have been reported by different groups and classified as splice variants (38-42). However, definitive experimental validation is lacking to support their existence and are regarded as possible degradation products from the use of experimental agents (27,43-45). On the other hand, MUC16 is known to be released from the cell following proteolytic cleavage, and the shed fragment is not significantly different in molecular weight than the intact mucin (26,27).

In foetal tissues, MUC16 is detected in the amnion and derivatives of the coelomic epithelium, *i.e.*, the Müllerian epithelium and the lining cells of the peritoneum, pleura, and pericardium (46). In adults, MUC16 is normally expressed in lacrimal, corneal, conjunctival and ocular epithelia. It is also expressed on the peritoneum, pleura and pericardium as well as respiratory and female reproductive tracts (46-50). In the female reproductive tract, it is detected in fallopian tubes, endometrium and endocervix. MUC16 is expressed by the decidua in early pregnancy, contributing to the protection of the foetus from maternal immune cells. On the other hand, loss of MUC16 from the endometrium facilitates the implantation of the trophoblast into the uterus (51,52). MUC16 is not expressed by normal OSE cells but is present on areas of metaplasia and early neoplastic progression in inclusion cysts (13,46). OSE is the only region in the ovary that expresses mucins, with MUC1 (carrier of the cancer antigen CA15-3) being the best studied one (14). MUC16 is present in STIC lesions and the majority of EOCs (53,54). Other mucins overexpressed in EOC are MUC1, MUC2 and MUC4 (14,55). The causes of mucin overexpression are not always clear but include altered gene responsiveness to factors in the tumour microenvironment (30,36). Therefore MUC16 disappears early in the course of ovarian epithelium formation and is re-expressed in certain reactive and neoplastic lesions, when transformation of OSE cells is induced (8,46). MUC16 expression has also been observed in a small percentage of pancreatic, breast and lung malignancies (56-59).

MUC16 has been extensively studied in EOC and its overexpression has been associated with attenuated cellular apoptosis, platinum chemotherapy resistance, tumour proliferation and disease progression (60-64). It also has variable representation among different histological EOC types. In the study by Hogdall *et al.*, involving 584 EOCs, MUC16 was present in 70% of cases. These included 85% of serous, 65% of endometrioid, 40% of clear cell but only 12% of mucinous adenocarcinomas (65). Similar results were reported by Rosen *et al.* who included 296 EOCs (66). A significant correlation has been found between MUC16 tissue expression and advanced FIGO stage but not with tumour grade (65,66). Additionally, data analysis from The Cancer Genome Atlas (TCGA) ovarian cancer project (including 316 FIGO stage II - IV high-grade serous ovarian cancers) showed that tumours with the highest MUC16 expression resulted in significantly worse survival outcomes. However, no correlation was observed between MUC16 expression and resistance to chemotherapy (67). In another study by de la Cuesta *et al.*, including 50 EOC patients of all histological types, high MUC16 tissue expression doubled the risk of death compared to no expression (68). Strikingly though, in the study by Hogdall *et al.*, patients with advanced disease and MUC16 tissue expression had significantly longer disease specific survival compared to those with absent MUC16 (65).

Tumour marker CA125

Shed MUC16 in the peritoneal fluid ultimately reaches the blood circulation, where it is detected as the CA125 antigen, and ascites paracentesis in ovarian cancer leads to a substantial decrease of serum CA125 levels (69,70). Hogdall *et al.* and Rosen *et al.* reported good correlation (though not always consistent) between ovarian cancer tissue and serum CA125, but used scoring patterns and not proper quantification of tissue levels (65,66). However, other studies including ovarian and endometrial carcinomas have found discrepancies, which were four-times more pronounced in the latter group (71,72). In the studies by Fleuren *et al.* and Harlozinska *et al.*, exploring differences in benign and malignant ovarian tumours, CA125 was expressed in almost all tissues; serum CA125 was elevated in the vast majority of carcinomas (with higher concentrations as the stage of disease increased) but in almost none of the benign patients. In the majority of these cases tissue CA125 concentrations were tens to thousands of times higher compared to serum ones, whereas ascites levels were up to 130 times higher (73,74). It was suggested that basement membranes surrounding the tissues in which tumours arise, as well as peritoneal barriers, hinder high molecular weight proteins such as CA125 from entering the circulation. Disruption of these barriers, either by local invasion in early or by metastatic lesions in advanced ovarian cancers, together with higher CA125 production by the latter, could explain the progressive increase in serum concentrations with disease progression (73). However, these mechanisms cannot explain the lack of elevated serum CA125 in a small (< 10%) proportion of patients with \geq FIGO stage II disease, which is even higher when all stages are included

(15% - 20%) (75). In a large study by Zorn *et al.*, including 1300 EOC patients with FIGO stage III and IV disease, 7.5% of patients had normal pre-treatment serum CA125 levels, which raised to 31% for patients with mucinous carcinomas (76). Therefore, unidentified mechanisms for CA125 tissue retention must exist, being active with variable efficiency in different gynaecological cancers and EOC subtypes (72,77).

CA125 has been extensively investigated as a serum biomarker in four separate clinical scenarios: **1.** as part of screening algorithms for the early detection of ovarian cancer (78-81); **2.** as part of diagnostic algorithms to distinguish between benign and malignant disease in women presenting with a pelvic mass (82,83); **3.** to monitor response to treatment (84); and, **4.** to detect disease recurrence (84). Setting the benchmark cut-off of 35 U/ml the antigen is elevated in more than 80% of women with non-mucinous EOC and its levels correlate with disease progression and poorer survival outcomes (69,76,85,86). However, it soon became apparent that CA125 is not a very sensitive and specific marker for screening and diagnosis of ovarian cancer, since many other non-gynaecological malignancies and non-malignant conditions lead to elevated serum levels (34). Non-gynaecological malignancies are almost invariably in advanced widely disseminated stage and include lung, liver, bladder, breast, biliary tract, stomach, colorectal, and pancreatic tumours, as well as lymphomas and leukaemia (87,88). Benign entities include ovarian cysts, endometriosis, adenomyosis, uterine fibroids, pelvic inflammatory disease, non-gynaecological inflammatory conditions, hepatic cirrhosis, heart and renal failure etc (87,89-93). In a large study by Bast *et al.* it was found that 1% of normal women, 6% of those with benign disease and 28% of those with non-gynaecological cancers have elevated serum levels (69). Furthermore, CA125 appears predominantly in the circulation of patients with advanced ovarian cancer but is raised in only 50% of those with FIGO stage I malignancy, rendering it a weak biomarker for the screening of asymptomatic women with early disease (75).

Another reason for this low sensitivity and specificity could be weaknesses in the serum CA125 assay. The two monoclonal antibodies currently used for this purpose (OC125 and M11) are known to attach in a multivalent fashion to CA125. It is therefore believed that these antibodies identify regions within the tandem repeat domains (26,94). However, our knowledge of the exact molecular characteristics and location of the epitopes they recognize on these domains remains elusive (26,95). The same applies for the role of MUC16's glycans, although it has been recently suggested that binding epitopes of monoclonal antibodies are dependent on the molecule's conformation and not on its glycosylation (95). Additionally, because these antibodies detect released CA125 in the serum, they cannot detect the proximal residual MUC16 fragment remaining on the cell surface following cleavage (34). Finally, another reason may be the rapid clearance of mucin fragments by the hepatic reticuloendothelial system, that may potentially skew the measured serum levels (96). Combined, these effects suggest that the CA125 assay significantly underestimates both the serum concentration

of this biomarker and its total quantity in the body (26). At the other end of the spectrum, a cross-reactivity of OC125 and M11 antibodies with other proteins has been observed. This could lead to falsely raised levels of measured CA125, if the serum concentration of these proteins is increased, thus also impacting the accuracy of the CA125 assay (45).

MUC16 structure

MUC16 is a transmembrane mucin composed of up to 22,152 amino acids and consists of 3 major domains: an extracellular amino-terminal (N-terminal) domain, a large tandem repeat domain interspersed with 16 sequences that are also found in Sea urchin sperm protein, Enterokinase and Agrin (called SEA domains) and a carboxy-terminal domain (37,94,97-99). MUC16 exhibits two types of glycosylation: O-linked and N-linked (94,100).

The N-terminal region is composed of 12,068 amino acids (37). This domain is dominated by its capacity for O-glycosylation, which may allow for extracellular matrix (ECM) interactions. There is one short area with little or no glycosylation, which could mediate protein-to-protein interactions in the ECM (94).

The extracellular tandem repeat domain also occupies a large part of the molecule, locating downstream from the amino-terminal domain (94). It is composed of a variable number of 45 to up to more than 60 repeats, which are arranged in tandem array (Variable Number Tandem Repeats - VNTR) and have a highly conserved nature (35,41,94). The repeat unit structure increases the potential for multivalent interactions with ECM neighbours. Each repeat has 156 amino acids (94). The length of the tandem repeat region is a unique feature of MUC16, which has a longer repeat sequence compared to MUC4 (16 amino acids) and MUC1 (20 amino acids) (31,101). The primary amino acid sequence in each repeat is not identical but is homologous (26). The tandem repeat region features both O- and N-glycans (29,37).

The SEA domains form a major part (approximately 120 amino acids) of those tandem repeats that contain them (102). They are supposed to play an important role in interactions with other glycoproteins and have also been suggested as potential carriers of the CA125 epitope (35,95,102). The high number of MUC16's SEA sequences is unique among other tethered mucins, which only have one SEA domain (with the exception of MUC4 that has none). It is also believed that MUC16 SEA domains have evolved separately from other mucins (99).

The carboxy-terminal domain is the smallest part of the molecule, composed of 284 amino acids (26). It anchors MUC16 at the cell surface and is divided into three major regions: a small extracellular portion consisting of 229 amino acids, the transmembrane domain with 23 amino acids and the cytoplasmic tail with 32 amino acids (61). Its extracellular part contains the last SEA domain and a small number of O- and N-glycosylation sites (26,94,103). The cytoplasmic tail contains one serine, two threonine and three tyrosine amino acid residues that can be potential phosphorylation sites (48,57). An antibody against the cytoplasmic tail of MUC16 has recently been developed (104).

The variable number of tandem repeats contained in each molecule and differential glycosylation gives rise to several MUC16 isoforms, probably conferring functional heterogeneity (41,105). The presence of multiple MUC16 isoforms was supported by Reinartz *et al.*, who found diverse binding patterns of OC125 and M11 antibodies in ovarian and breast cancer cell lines with variable MUC16 expression, suggesting the existence of different MUC16 binding epitopes (63).

MUC16 glycosylation

As mentioned previously, MUC16 is abundant in O- and N-linked glycans. The process by which mucin O-linked glycosylation occurs is well characterised, though we know relatively little about its regulation. It involves complex post-translational modifications on the protein backbone, whose initiating step is the addition of N-acetylgalactosamine carbohydrates; this forms the so-called Tn antigen (106). The Tn antigen can be further extended to form core 1 (T-antigen), 2, 3 and 4 structures, based on the identity of linked carbohydrates and type of linkage (107). In normal cells, these structures are elongated further and often become branched (108). In contrast, cancer cells express only their early biosynthetic intermediates (109). For example, Tn and T can be capped immediately with sialic acid by sialyltransferases, producing the sialyl-Tn (STn) and sialyl-T (ST) antigens respectively. This occurs only rarely in normal tissues but is common in cancer (110,111). Core 1 and 2 are the major O-glycans present on MUC16, both in normal conditions and ovarian cancer (100,112). Core 3 and 4 O-glycans are synthesised in healthy states (mainly by colonic and respiratory tissues) but are down-regulated in malignancy (113).

Many tumours exhibit aberrant O-glycans and the aberrant expression of truncated O-glycans is a hallmark of epithelial cancers (114). In many instances, expression of these truncated structures is driven by alterations to the expression of enzymes involved in the glycosylation process. For example, the extension of core 1 structures relies on a single enzyme, T-synthase (115). T-synthase is known to regulate O-glycan expression on glycoproteins of ovarian cancer cells, and loss of its activity leads to increased Tn and STn levels (115,116). Another potential factor is deregulation of enzymes that

extend or terminate extension of O-glycans (*e.g.*, sialyltransferases), which has also been observed in a variety of cancers, including serous ovarian carcinomas (117-120).

The presence of truncated core 1 O-glycans in ovarian tumours has been investigated in several studies. T, Tn and STn are almost absent in benign ovarian tumours and normal ovarian tissues but are clearly expressed in most ovarian carcinomas and the serum of ovarian cancer patients. Increasing expression of these epitopes could correlate with the progression from benign ovarian cysts to invasive carcinomas (121-127). In most cases more than one antigens are expressed, indicating that mucin glycoproteins exist at different stages of glycosylation in a given tumour (125). In the study by Akita *et al.*, MUC16 and STn co-localised in ovarian serous and endometrioid carcinomas, whereas STn was mostly carried on mucins other than MUC16 in mucinous ovarian carcinomas (possibly MUC1 or MUC4) (128). The same study discriminated the level of STn expressed on MUC16 (STn/MUC16) in peritoneal fluid from patients with EOC and endometriosis with 44% sensitivity and 100% specificity. Remarkably, the elevation of STn/MUC16 with advancing stages of ovarian cancer was more evident than that of MUC16 (128). Chen *et al.* developed a glycoprofiling microarray assay, in which the combined serum levels of STn/MUC16, ST/MUC16 and STn/MUC1 glycoforms distinguished benign ovarian tumours from EOCs with a specificity of 61%, whereas the serum CA125 assay specificity was 41% (127). Finally, truncated core 1 O-glycans can protect tumours from immune system attack, by inhibiting cytotoxic effects of Natural Killer (NK) cells and T-lymphocytes (129,130). Of note, core 2 O-glycans can also suppress immune responses by blocking the binding of NK to cancer cells (113).

A very interesting hypothesis is that truncated O-glycans exhibit diverse properties at different stages of ovarian cancer with an impact on tumours' biological behaviour. In the study by Coelho *et al.*, an EOC cell line with induced expression of homogeneously truncated Tn and STn O-glycans exhibited decreased cell proliferation and increased apoptosis compared to its parental cell line (derived from a peritoneal metastasis). Cells with truncated O-glycans exhibited increased cell migration but decreased invasiveness and capacity to form metastases (131). Partially similar findings were reported by Chou *et al.*, where T-synthase knockdown led to decreased growth but also decreased migration, whereas its overexpression had opposite results (116). This discrepancy in cell migration might be due to different ovarian cancer cell lines used in these two studies. Interestingly, Chou *et al.* reported significantly lower overall survival for ovarian cancer patients with overexpressed T-synthase (116). A possible explanation for this finding could be high levels of T-synthase in patients with advanced metastatic ovarian cancer. Indeed, in the study by Davidson *et al.*, the expression of Tn and STn was significantly up-regulated in pleural and peritoneal effusions compared with both primary and metastatic ovarian solid lesions, confirming that truncated O-glycans are more frequently observed at the migratory front and less often in metastases. The authors hypothesised that this up-regulation is

transient if a metastatic sequence of primary tumour → effusion → solid metastasis is assumed. They concluded that Tn and STn may have a role in the progression from primary tumour to malignant effusions (132). Therefore, it can be speculated that the expression of truncated core 1 O-glycans facilitates the detachment of individual cells from the primary ovarian tumour, but does not favour their settlement to metastatic sites (131). On the other hand, in a study with pancreatic cancer cells, truncated O-glycans promoted their growth and invasive properties (114). These differences may reflect varied behaviours of truncated O-glycans in different tumour types.

With regards to N-glycosylation, changes in cancer involve increases in size, branching and sialylation (133). In the study by Saldova *et al.*, CA125 from ovarian cancer patients' sera displayed increased sialylated biantennary N-glycans and decreased bisecting biantennary ones compared to healthy controls (112). On the other hand, in the study by Nagata *et al.* predominantly biantennary and triantennary bisecting type glycans were present on CA125 isolated from serum and ascites of ovarian cancer patients (134). Of note, the bisecting type biantennary chains can inhibit the cytotoxic responses of NK cells (135). Complex multi-antennary chains on N-glycans of glycoproteins have been implicated in tumour metastasis (136).

MUC16's core 1 O- and N-glycans bind with two members of the family of galectins, respectively galectin-1 and galectin-3 (137,138). Galectins consist part of the group of lectins, acting as ligands between glycoproteins and glycolipids at the cell surface (139). Both galectin-1 and galectin-3 are up-regulated in malignancies (including EOC) and can contribute in cancer progression by enhancing transmembrane signalling, with subsequent activation of oncogenic transduction pathways (137,138,140,141).

Finally, extended core 1 O-, core 2 O- and N- MUC16 glycans display sialofucosylated oligosaccharides, such as sialyl Lewis^x (sLe^x) and its isomer, sialyl Lewis^a (sLe^a – also known as the tumour marker CA19-9) (100,142). These epitopes bind with selectins, a family of lectins acting as vascular cell adhesion molecules (143). Selectins promote lymphatic and haematogenous metastasis, by mediating interactions between their ligands on tumour cells and selectin-expressing endothelial cells in the microvasculature (142,143). In the study by Chen *et al.*, MUC16 on pancreatic cancer cells was found to be the main ligand to selectins, through the sLe^a and sLe^x antigens (142). Aberrant expression of these epitopes on glycoproteins is associated with pancreatic cancer progression (144). In another study by Ricardo *et al.*, MUC16 was also found to be one of the main carriers (together with MUC1) of sLe^a and sLe^x in ovarian serous adenocarcinomas. Although these epitopes were present in the majority of benign cystadenomas as well, they were mainly carried by unidentified proteins and not by MUC16 or MUC1 (145). Combined, the aforementioned findings suggest that the increased metastatic potential inferred by sLe^a and sLe^x is mostly associated with their expression on

particular carrier proteins, with MUC16 being a major representative of these. Indeed, the binding affinity of selectins for isolated sLe^x and sLe^a antigens is markedly low (142). They also suggest that MUC16 could play a significant role in distant lymphatic and haematogenous spread in ovarian cancer, a speculation that needs to be validated by future studies.

Figure 1 demonstrates differences in MUC16 structure between normal/benign conditions, solid tumour growths and migratory EOC, focusing on glycosylation changes. Figure 2 presents these changes in the context of early EOC tumourigenesis and in correlation with cellular phenotypic shifts.

MUC16 cleavage

Cell surface expression and release of MUC16 proteolytic fragments appear to be associated with the conversion of ovarian cells from benign to cancer (137,146). The penultimate and final SEA domains have been proposed as putative MUC16 cleavage sites (94,103,147). Potential cleavage locations as close as only 12 amino acids distal to the transmembrane domain have also been suggested (94,103,147,148). The kinetics and dynamics of MUC16 cleavage/shedding from the tumour cells is not well understood, part of the problem being the enormous size and complexity of this glycoprotein (27). In non-migratory EOC, most MUC16 remains intact on the cells while a small proportion is cleaved, leaving a carboxy-terminal fragment (consisting of a small extracellular portion of various lengths, the transmembrane domain and the cytoplasmic tail) on the cell membrane. The rest of MUC16 ectodomain is released from the cells, while a fraction of the two fragments remains non-covalently associated on the cells (i.e. these fragments remain associated with each other) (103,147). Antibodies directed to the extracellular portion of MUC16 membrane-bound carboxy-terminal domain have been developed, confirming its presence on the surface of ovarian cancer cells (147,148).

Regulation of MUC16 cleavage has been extensively investigated, although most studies have included non-ovarian cancer cell lines. Yet no firm conclusions can be drawn about what factors potentiate it. For example, there is controversy about the role of extracellular factors (such as proteases, cytokines, growth factors and hormones) or post-translational modifications (such as glycosylation) around the cleavage site, and intramembrane proteolysis has been ruled out (49,103,149-153). An endogenous protease activity inherent to the molecule appears to exist, leading to autoproteolysis (103,154,155). This takes place at the Golgi apparatus *en route* to the cell membrane, dictated by a change in MUC16 structure as it encounters Golgi's acidic pH, and not by a primary amino acid sequence (103,137). Finally, although it has been argued that cleavage is independent of intracellular cues, it is triggered by the phosphorylation of the serine, threonine and tyrosine residues present on MUC16's cytoplasmic tail (94,103,156,157).

As previously mentioned, EOC classically spreads through peritoneal dissemination, with epithelial to mesenchymal transition (EMT) being a hallmark event in the metastatic process. Two important molecules in EMT are epithelial (E)-cadherin and neural (N)-cadherin. Cadherins are a family of transmembrane glycoproteins, which have a fundamental role in the formation of cell-to-cell adhesions. Their intracellular domain binds with another group of proteins called catenins, which are mainly located at the cytoplasmic part of the membrane, and stabilise cadherin-mediated cell contacts through linkage with the actin cytoskeleton. Among catenins, β -catenin also plays a significant role in EMT (158). Even more importantly MUC16 associates with E-cadherin/ β -catenin junctional complexes, being bound to the former extracellularly and to the latter intracellularly (159,160). E-cadherin is a feature of the epithelial phenotype (as well as the expression of predominantly uncleaved MUC16) and generates tight cell-to-cell contacts in the normal fallopian tube, ovarian inclusion cysts, primary ovarian tumours and solid metastases. N-cadherin is a marker of mesenchymal differentiation, expressed in the normal ovarian surface epithelium and metastatic lesions; its up-regulation is associated with tumour progression and produces loose bonding between cells, which enhances their migratory and invasive properties (11,16,17,161-163). Both cadherins contribute to the formation of MCAs but increased N-cadherin expression multiplies their aggressiveness and resistance to apoptosis (162,163). As a rule cadherin subtype-specific junctions are formed, whereas cells expressing both cadherins (such as the hybrid ones) can create contacts with epithelial and mesenchymal cells (163). The cadherin/ β -catenin complex is expressed on cells even when they are not involved in cellular adhesions or aggregates (*i.e.*, on single cells) (158).

E-cadherin down-regulation with concomitant N-cadherin up-regulation is a key step in EMT, described as the ‘‘cadherin switch’’ phenomenon (162). The loss/reduction of E-cadherin expression or function is associated with progression from well to poorly differentiated EOC and promotes metastasis (10,11,164,165). It can be triggered by soluble factors present in the ascites (such as proteases, cytokines and growth factors that cause shedding of its extracellular part) but is also associated with overexpression of MUC16’s carboxy-terminal domain (CTD) (17,62,166). The latter consists a MUC16 isoform that promotes migration and invasion of tumour cells, with increased N-cadherin expression and acquisition of mesenchymal phenotype (62,167). Additionally, it attenuates cellular apoptosis and sensitivity to platinum chemotherapy agents (61,64). Ectodomain shedding triggered by extrinsic factors (such as proteases and cytokines), enhanced autoproteolytic MUC16 cleavage and phosphorylation of its cytoplasmic tail have been proposed as potential mechanisms for MUC16 CTD overexpression (103,160,168). Indeed, Src phosphorylation of a tyrosine residue at MUC16’s cytoplasmic tail has been demonstrated in ovarian, colon and breast cancer cells, which leads to deregulation of E-cadherin/ β -catenin junctional complexes and increases cellular migration (62,103,160). Src is a non-receptor tyrosine kinase, whose activity enhances metastasis. (169). It is

also known that Src activity negatively regulates the E-cadherin/ β -catenin cell adhesion system (170). More specifically Src-mediated phosphorylation of E-cadherin induces its endocytosis and degradation, while Src inhibition increases E-cadherin levels and can induce EMT reversal (171,172). β -catenin is also phosphorylated by Src, which negatively regulates its binding to E-cadherin (173). Taken together the above suggest that Src phosphorylation initiates a cascade of events, including MUC16 shedding with overexpression of MUC16 CTD and breakdown of E-cadherin junctional complexes (174). It is also evident that increased MUC16 cleavage occurs in metastatic EOC, which would contribute to the higher serum CA125 levels detected in advanced compared to early stage disease.

MUC16 CTD is sufficient to increase the metastatic potential of ovarian cancer cells (175). Following MUC16 cleavage the CTD fragment can remain on the cell membrane to exert its actions, or undergo endocytosis to affect signalling in other cellular compartments (103). The latter is confirmed through binding antibodies to MUC16 CTD ectodomain, demonstrating internalisation by ovarian cancer cells (148). MUC16 CTD can also translocate to the nucleus and is present within chromatin, suggesting its function as a transcriptional co-regulator (103,167). N-glycosylation at its ectodomain appears to be associated with an altered gene-expression profile and increased expression of critical invasion genes, through activation of the PI3K (phosphatidylinositol 3-kinase) / Akt (protein kinase B) and MAPK (mitogen-activated protein kinase) / ERK (extracellular-signal regulated kinase) cell survival signal transduction pathways (175,176). Both of them induce EMT in carcinomas (159,177-179). The mechanisms by which MUC16 CTD activates these pathways include interactions of its N-glycans with galectin-3, which in turn binds with N-glycans expressed by two transmembrane glycoproteins, the epidermal growth factor receptor (EGFR) and β 1-integrin (part of the integrins family of cell adhesion receptors). The bonds in the formed tripartite heteromolecular junctions are prerequisites for activation of both agents (138). For EGFR this occurs after binding of its ligands (such as the epidermal growth factor – EGF) whereas for β 1-integrin through intracellular recruitment and phosphorylation of Focal Adhesion Kinase (FAK – a non-receptor protein tyrosine kinase) and Src complexes. These events trigger signalling cascades that can eventually activate both PI3K/Akt and MAPK/ERK (180-182). Disruption of MUC16 CTD's N-glycan-mediated binding with galectin-3 abrogates these effects, demonstrating its crucial role for their conduct (138).

Another example of MUC16 CTD's role in intracellular signalling is its interaction with β -catenin (167). β -Catenin is a dual function protein involved in regulation of intracellular adhesion (as highlighted previously) but also in gene transcription (183,184). It accumulates in the nucleus of many malignant cell types (including ovarian cancer), acting as an intracellular signal transducer in the Wnt (Wingless-related integration site) pathway (183,185). The Wnt/ β -catenin transduction is known to enhance EMT in carcinomas, up-regulating transcription factors that decrease E-cadherin and

increase N-cadherin expression, along with mesenchymal genes' activation. In the presence of E-cadherin, β -catenin locates at the cell membrane to participate in the formation of their junctional complexes. Loss of E-cadherin leads to β -catenin release to the cytoplasm and its quick degradation. Binding of Wnt ligands to their receptor stops this turnover, leading to increased levels of cytoplasmic β -catenin, which is then transferred to the nucleus (158). In ovarian cancer, overexpressed MUC16 CTD also inhibits β -catenin clearance hence up-regulating Wnt downstream genes transcription, which enhances tumour proliferation and migration (166,167). Of note, MUC16 CTD can increase the cytoplasmic levels of another catenin participating in E-cadherin complexes, the p120 catenin, with the same impact on tumour behaviour, but its mechanism of action is unknown (186).

Similar effects have been described through the interaction of MUC16 CTD with a non-receptor tyrosine kinase called Janus kinase 2 (JAK2). In pancreatic cancer cells, MUC16 CTD overexpression leads to JAK2 translocation from the cytoplasm to the nucleus, with increased transcription of its target genes (187). Activation of the JAK2 signalling has been reported in EOC as well (188). This could therefore be another pathway that MUC16 CTD activates to enhance ovarian cancer cells' metastatic properties.

Finally, MUC16 CTD contributes in the formation of MCAs (166). Although not experimentally validated, it can be presumed that this occurs through interactions with N-cadherin, as MUC16 CTD induces a relocation of cell surface E-cadherin to the cytoplasm. Additionally, apart from the aforementioned increase in cytoplasmic β -catenin levels, MUC16 CTD increases its cell membrane pools as well (166). It is therefore likely that MUC16 CTD recruits β -catenin at its cytoplasmic tail to stabilise junctional complexes with N-cadherin, leading to MCA formation with other N-cadherin expressing cells. At the same time it increases MCAs migratory and invasive properties, by enhancing β -catenin's transcriptional activity through the Wnt/ β -catenin pathway. Overall the interactions with β -catenin indicate the importance of MUC16 CTD's cytoplasmic tail in intracellular signalling. This has been confirmed in experiments that induced its deletion, leading to a reduction of MUC16 CTD's oncogenic potential and its ability to form MCAs (62,176).

Figure 3 demonstrates the EMT mechanisms induced by MUC16 CTD overexpression in EOC.

MUC16 knockdown

MUC16 knockdown has been associated both with increased and decreased ovarian cancer aggressiveness. When induced at the surface of cancer cells it inhibits tumour growth (either by cellular apoptosis or by arrest in proliferation) and reduces their metastatic potential (62,63,167). Additionally, it increases their sensitivity to platinum chemotherapy agents (64). However, ovarian cancer cells inherently lacking MUC16 expression do not undergo apoptosis (63). It has also been

reported that MUC16 knockdown leads to decreased cell surface expression of E-cadherin. Binding of MUC16 to E-cadherin results in the latter's surface localisation whereas, in the absence of MUC16, E-cadherin re-localises to the cytoplasm. Ovarian cancer cells with down-regulated MUC16 display a mesenchymal phenotype with increased N-cadherin expression, cell motility and invasion. Therefore, MUC16 knockdown could be another EMT-promoting mechanism in EOC metastasis (159). On the other hand, MUC16 knockdown ovarian cancer cells do not form MCAs and exhibit increased β -catenin degradation with attenuation of its transcriptional activity (159,166). These effects are contrary to what is observed in MUC16 CTD overexpression, strengthening the assumption that MUC16 CTD participates in MCAs through enhanced β -catenin recruitment in N-cadherin complexes.

It is also apparent that oncogenic pathways other than the Wnt/ β -catenin signalling are up-regulated by MUC16 knockdown. Indeed, EGFR activation has been demonstrated even in the absence of MUC16. More specifically, E-cadherin extracellularly interacts with the EGFR and negatively regulates the ligand-dependent activation of this receptor (189). MUC16 knockdown (with subsequent loss of extracellular E-cadherin) allows for EGF binding to EGFR, thus initiating the PI3K/Akt and MAPK/ERK pathways transduction relay (159). Therefore, MUC16 down-regulation triggers EMT-associated signalling, consistent with the finding that MUC16 tissue loss is associated with poor prognosis in EOC (65).

MUC16 and formation of peritoneal metastases

The primary target of the metastasising EOC is the mesothelial cell monolayer, which covers the abdominal peritoneum (149). MUC16 binds with very strong affinity to mesothelin, a mesothelial cells' membrane-bound glycoprotein, which also has a soluble form produced by proteolytic cleavage (77,190-192). Importantly it is expressed on EOCs of all histological types (particularly serum ones), in significantly higher levels compared to benign ovarian tumours (77,193-195). Of note, it may be able to trap MUC16 in the tissue, thus providing another possible explanation for the discrepancies noted in tissue and serum CA125 levels in ovarian cancer patients (77).

MUC16 is the only ligand for mesothelin on ovarian cancer cells and this binding is not shared by other mucins (77,190). Both single cells and MCAs attach to the peritoneal mesothelium (196). The ovarian cancer cell-to-mesothelin binding occurs via MUC16's N-glycans (190-192). It is questionable whether cleaved MUC16 could inhibit or decrease binding of cell-bound MUC16 to mesothelin. Mesothelin interacts with both shed and cell-surface MUC16 but its affinity is higher for the latter. This could be due to proteolytic processing after MUC16's tumour release or digestion of N-glycans by glycosidases in the ascites (190). Additionally, mesothelial cells produce and shed

MUC16 in the peritoneal fluid, in benign and malignant conditions (70,151). It has been argued that the co-expression of MUC16 and mesothelin in both ovarian cancer and mesothelial cells could competitively inhibit their binding. However, the multivalent nature of mesothelin's binding on MUC16 likely promotes rather than inhibits the attachment of ovarian cancer cells to mesothelium, even involving expressed MUC16 CTD in these contacts (77). Nevertheless, co-expression of MUC16 and mesothelin on ovarian cancer cells leads to recruitment of additional tumour load at metastatic sites and MCAs. On the other hand, it has been argued that tumour cell attachment to mesothelium *via* the MUC16-mesothelin interaction may not be adequate to maintain this heterotypic adhesion (190). Indeed, it has been found that other adhesion molecules mediate ovarian cancer and mesothelial cells binding as well. For example, β 1-integrin expressed by ovarian cancer cells binds to fibronectin expressed by mesothelial cells. However, the ovarian cancer cells-mesothelin binding is only partially inhibited by anti- β 1-integrin antibodies (196-198). Interestingly, when β 1-integrin is blocked, cells with low surface MUC16 do not adhere to mesothelin, whereas adhesion of cells with high surface MUC16 remains unaffected (149). It is therefore likely that the mesothelin-MUC16 interaction provides the necessary first step for metastasis transitioning to β 1-integrins or other molecules, either in the absence of MUC16 or to stabilise contacts (149,190).

The interaction between mesothelin and tumour cell-surface MUC16 represents the initial adhesive event. Once attached, ovarian cancer cells undergo EMT to start clearance of the mesothelial cell layer and spread to the sub-mesothelial matrix. Subsequently, invasion through degradation of the sub-mesothelial matrix collagen occurs, in order to form firm metastases through MET (11,15,149,161,165). These events seem to be strongly linked to the mesenchymal phenotype and N-cadherin expression or acquisition, as cells maintaining E-cadherin exhibit reduced mesothelial retraction, migration and collagen invasion (161,163,165). MUC16 participates in this invasive process as well, through interactions with members of a family of proteolytic enzymes called matrix metalloproteinases (MMPs). Examples of metalloproteinases in ovarian cancer are the membrane type 1 matrix metalloproteinase (MT1-MMP), MMP-2 and MMP-9, all degrading interstitial collagen in the sub-mesothelial matrix. MMP-2 and MMP-9 are activated by MT1-MMP and MMP-9 is involved in ovarian cancer's EMT, as it catalyses the shedding of E-cadherin's extracellular domain (199-201).

An inverse relationship between MT1-MMP and MUC16 has been demonstrated by Bruney *et al.* Ovarian cancer cell lines which express high MUC16 levels do not exhibit surface expression of MT1-MMP. On the other hand, cells overexpressing MT1-MMP exhibit loss of surface MUC16 but increased levels of soluble forms. These findings suggest that MT1-MMP may catalyse MUC16 cleavage. Increased peritoneal adhesion in the absence of MT1-MMP has been shown and, inversely, a loss of adhesion in MT1-MMP expressing cells with reduced cell-surface MUC16 (149). Therefore, it is likely that ovarian cancer cells express MUC16 for mesothelin attachment, but subsequent

invasion and metastasis formation is achieved through MT1-MMP-mediated MUC16 shedding. The aforementioned relationship between MT1-MMP and MUC16 has been indirectly confirmed by Comamala *et al.*, who found increased activation of MMP-2 and MMP-9 in MUC16 knockdown ovarian cancer cells (159). To that end, it is plausible to conclude that MUC16 down-regulation enhances the activity of these three metalloproteinases, thus potentiating the formation of solid peritoneal metastases.

However, partially contrary findings to the above were reported by Reinartz *et al.* (63). Although ovarian and breast cancer lines with high MUC16 expression demonstrated loss of invasiveness in collagen matrix, MUC16 knockdown did not alter this attitude. MUC16 knockdown also abolished MMP-2 activation and this was accomplished by suppression of β 1-integrin expression. The discrepancies in the outcomes of these studies could be explained by different cell lines and experimental approaches used.

Finally, MUC16 expression correlates with pancreatic cancer cells binding capacity to mesothelin, and this interaction activates matrix metalloproteinase 7 (MMP-7) promoting metastasis (202). Interestingly, Chang *et al.* found that mesothelin-overexpressing ovarian cancer cells demonstrate enhanced migration and invasion, also through the induction of MMP-7 (203). It is therefore possible that MMP-7 activation by mesothelin is mediated through MUC16 in ovarian cancer as well.

MUC16 and immune interactions

Immune regulation plays an important role in controlling ovarian cancer growth and EOCs have been recognized as highly immunogenic tumours. They exhibit strong expression of HLA (Human Leukocyte Antigen) class I and class II molecules, which are major prerequisites for effective T-lymphocyte-mediated killing (204). EOCs also express ligands that can activate or inhibit NK cells receptors and ovarian carcinoma cells are sensitive to NK mediated lysis (205). However, it is well established that ovarian cancer patients exhibit immune suppression (206). With regards to NK cells, expression of HLA class I antigens on ovarian tumour cells abrogates their cytotoxic responses, as these antigens are strong inhibitory ligands for several receptors on their surface, such as the Killer Immunoglobulin-like Receptors (KIR) (207). These inhibitory receptors are also present on subsets of T-lymphocytes (208). Additionally the malignant microenvironment, including ascites, inhibits NK cells anti-tumour functions. Of note, ascites NK cells are hypo-responsive to tumour targets compared to NK cells from peripheral blood of EOC patients (209).

The role of MUC16 in EOC's interactions with immune factors has been investigated in several studies. MUC16 protects ovarian cancer cells from complement attack by trapping effectors of the complement cascade (210). Cytokines (interferon-gamma and Tumour Necrosis Factor-alpha) can increase the expression and shedding of MUC16 from ovarian cancer cells (36,151). MUC16 was also found to possess the highest number of HLA class I ligands compared to all other antigens present on the surface of EOC cells (204), which as mentioned previously inhibit NK activation. The role of ovarian cancer cell-surface MUC16 in interactions with NK cells was investigated by Gubbels *et al.* Low MUC16 expression was associated with significantly increased NK conjugation compared to medium or high MUC16 levels. Variable expression of activating NK receptors' (DNAM-1 and NKG2D) ligands was ruled out for this effect, as all ovarian cancer cells had comparable levels. Following this observation MUC16 knockdown cells were employed and compared with MUC16 positive ones. These two lines were also similar in NKG2D and DNAM-1 ligands expression. Although HLA class I antigens levels (presented by molecules other than MUC16) were slightly elevated on MUC16 knockdown cells, they were lysed to a significantly greater extent than the MUC16 positive ones. A similar effect was observed when KIR negative NK cells were used as effectors, which markedly enhanced their cytolytic capacity, as the inhibitory effect of HLA class I antigens was abolished (211).

These findings suggest that MUC16 uses its HLA class I antigens to attenuate NK cells activation, but this is not its sole immunoprotective mechanism. In this respect, Gubbels *et al.* noted reduced NK binding not related to expression levels of other mucins (MUC1, MUC4) present on the surface of ovarian cancer cells, which can block immune synapses (211-213). The authors suggested that MUC16's extensive O-glycosylation could be at least partially responsible for this anti-adhesive effect. Indeed as mentioned before, MUC16's core 2 O-glycans offer protection from NK immune attack. As MUC16 is much larger and more heavily glycosylated than MUC1 and MUC4, it could use its size and abundant longer O-glycan chains to inhibit NK binding, irrespective of the presence of those two other mucins (29,174,211). Finally, MUC16 overexpression protects pancreatic cancer cells not only from NK but also from cytotoxic T-lymphocytes (130). It was highlighted previously that HLA antigens are strong stimulants of T-cells activation, but also that subsets of them possess inhibitory receptors for HLA class I ligands. Therefore, it can be speculated that MUC16 uses its HLA class I antigens to regulate T-cell binding too, favouring a mostly inhibitory effect.

Apart from the aforementioned mechanisms, cell-surface MUC16 binds with T-cells and monocytes through siglec-9, an inhibitory receptor present on the surface of T-cells, B-cells, monocytes and NK cells (214-216). Siglecs (sialic acid-binding immunoglobulin-type lectins) bind with sialic acid residues on proteoglycans, glycolipids and glycoproteins (217). As already mentioned, these residues are abundantly present on MUC16's glycan chains in ovarian cancer, particularly on truncated core 1

O- and N-glycans. In EOC, shed MUC16 following cleavage binds with NK cells, B-cells and monocytes through siglec-9 as well. With regards to NK cells, siglec-9 is selectively expressed on a subset that possesses the highest cytotoxicity, and MUC16 binding may inhibit their activation (209,215). Exposure of healthy donors' peripheral blood NK cells to ovarian tumour-derived MUC16 induces a robust inhibition of even their most potent forms, an effect that as previously stated can be exerted through MUC16's truncated core 1 O- and N-glycans. Additionally, it induces a severe down-regulation of the activating receptor CD16, which is present on the highly cytotoxic NK cells, and makes them acquire a less cytotoxic phenotype (218,219). This phenotypic shift is also noted when peripheral blood NK cells from healthy donors are treated with peritoneal fluid from EOC patients (209). Indeed, ascites NK cells obtained from ovarian cancer patients show significant reduction in CD16 expression compared to NK cells derived from healthy donors' peripheral blood (220). This change in phenotype is not caused by increased apoptosis of the more cytotoxic NK group or selective proliferation of the less cytotoxic one (209,218).

It is therefore plausible to assume that shed MUC16 - siglec-9 binding, mediated mainly through the former's truncated core 1 O- and N-glycans, inhibits NK cytotoxicity and induces a phenotypic shift to the less cytotoxic group in ascites. Competent siglec-9-positive T-cells and monocytes enter the tumour microenvironment and adhere via cell-surface MUC16 glycans, but this binding also suppresses immune responses. Finally, the inhibitory (through HLA class I antigens) and anti-adhesive (through core 2 O-glycans) cell-surface MUC16 properties promote hindering and evasion, respectively, of siglec-9-negative immune cells. By using these mechanisms, MUC16 could substantially protect ovarian cancer from immune responses and potentiate its progression.

Conclusion

We propose a model for MUC16's evolving role in relation to EOC progression and ovarian cancer cells' phenotypic shifts (Figure 4). At early stages, predominantly uncleaved MUC16 contributes to tumour growth and stabilises contacts between cells with complex epithelial phenotypes. At the same time, it provides immunoprotection through its O- and N-glycans, thus supporting tumour survival. Increased MUC16 cleavage participates to disruption of cells' adhesions and initiates their release in the peritoneal cavity. This process is facilitated by up-regulated expression of truncated core 1 O-glycans, which is maintained throughout cell migration, whereas solid tumour growths mainly express their extended forms. Detached cells circulate in the ascites and undergo epithelial to mesenchymal transition (EMT) as well as differentiation shifts between epithelial, mesenchymal and hybrid (mixed epithelial/mesenchymal) phenotypes. Mesenchymal cells overexpress MUC16's carboxy-terminal domain (CTD), which is retained on the cell membrane following cleavage, and increases their migratory potential as well as their resistance to chemotherapy. The released MUC16 fragment and

MUC16 present on free-floating ovarian cancer cells continue inhibiting immune cytotoxicity through their glycan chains. Sialofucosylated oligosaccharide epitopes may bind to blood and lymph vessels to promote EOC's haematogenous and lymphatic metastasis. Some mesenchymal cells undergo complete MUC16 knockdown, which might enhance their aggressiveness, but can also increase their susceptibility to chemotherapy. Uncleaved MUC16 and MUC16 CTD contribute to the stability of multicellular aggregates (MCAs), formed by contacts between single circulating cells, which enhance their survival in the ascitic environment. MCAs and single cells attach to the mesothelial layer covering the peritoneal surfaces through binding of MUC16 and MUC16 CTD's N-glycans with mesothelin, expressed by mesothelial cells. MUC16 knockdown cells attach through β 1-integrin, which also stabilises the initial mesothelin-mediated contacts in MUC16 expressing cells. Mesothelin is present on ovarian cancer cells as well, and its interactions with MUC16 leads to further tumour recruitment on MCAs and metastatic sites. Following anchoring to peritoneal surfaces, mesenchymal cells or cells undergoing further EMT induce mesothelial clearance, spread to sub-mesothelial matrix and start degrading the sub-mesothelial matrix collagen. These cells overexpress MUC16 CTD or exhibit MUC16 knockdown, which both enhance their invasive properties. Invasion is also facilitated by matrix metalloproteinases, which promote MUC16 cleavage and are up-regulated in the absence of MUC16. Finally, these early metastatic lesions undergo mesenchymal to epithelial transition to proliferate and form solid peritoneal metastases, with up-regulated uncleaved MUC16 expression promoting their growth and stabilising intercellular contacts.

MUC16's participation in the evolution of ovarian cancer has attracted attention to its potential use as a therapeutic target. Monoclonal antibodies against MUC16 (221-223) and agents targeting the MUC16-mesothelin interaction (224-226) have been developed, but have shown limited benefit in clinical trials (227-230). This could be due to MUC16 cleavage, leading to increased binding of therapeutic agents by shed MUC16 in the serum and reducing the amount of intact MUC16 on tumour cells (27). The recently developed antibodies against MUC16 CTD could help overcome these hurdles (104,147,148). Better understanding of MUC16's structural diversity and aberrant glycoforms, as well as targeting other molecules involved in its interactions (*e.g.* galectins, selectins, siglec-9) or the oncogenic signalling pathways it activates, could also contribute in the development of more effective treatment strategies (27,34).

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Key points

- **CA125 corresponds to the transmembrane glycoprotein MUC16, the largest mucin identified to date.**
- **In normal conditions MUC16 is not present on ovarian surface epithelium but it is overexpressed in epithelial ovarian cancer (EOC).**
- **The variable number of tandem amino-acid repeats in MUC16's structure, the differential glycosylation and the expression of its carboxy-terminal domain give rise to a high number of MUC16 isoforms.**
- **MUC16 exhibits a wide range of interactions with other molecules, which contribute to EOC's neoplastic potential, growth and metastasis.**
- **MUC16 undergoes proteolytic cleavage proximal and upstream to its transmembrane domain.**
- **In solid tumours MUC16 is mostly expressed in its uncleaved form, stabilising contacts between cells with complex epithelial phenotypes.**
- **Enhanced MUC16 cleavage leads to disruption of intercellular junctions and overexpression of its carboxy-terminal domain (CTD), associated with mesenchymal cellular phenotype as well as increased EOC migration and invasion.**
- **Only the released MUC16 fragment following cleavage partially enters the circulation and gets detected by the serum CA125 assay, which therefore underestimates the total amount of MUC16 present in the body. Increased MUC16 cleavage in metastatic EOC contributes to higher serum CA125 levels measured in advanced stages of disease.**
- **Both uncleaved MUC16 and MUC16 CTD contribute to the stability of adherens junctions in multicellular aggregates, which help migrating ovarian cancer cells survive in ascites.**
- **MUC16 knockdown is associated with the aggressive mesenchymal phenotype but can increase cellular chemotherapy-induced apoptosis.**

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Figure Captions

Figure 1: MUC16 structure

Demonstration of MUC16 domains (N-terminal, variable number tandem repeat and carboxy-terminal), glycosylation patterns and putative cleavage sites. The tandem repeat domain consists of 45 to more than 60 repeat amino acid sequences, interspersed by 16 SEA domains. The N-terminal domain is O-glycosylated whereas the tandem repeat domain has both O- and N-glycans. The extracellular part of the carboxy-terminal domain also has a few sites for O- and N-glycosylation.

In normal and benign gynaecological conditions, MUC16 features extended and often branched core 1 and core 2 O-glycans, whereas N-glycans are short with limited branching. In EOC there is aberrant expression of truncated core 1 based O-glycans and core 2 ones are likely shorter. Truncated core 1 structures are mostly up-regulated in the migratory front of EOC compared to solid primary and metastatic tumours, which maintain expression of extended core 1 O-glycans. N-glycans become larger, extensively branched and poly-antennary. Extended core 1 O-, core 2 O- and N-glycans express sialofucosylated oligosaccharides (such as the sialyl Lewis epitopes).

Abbreviations: TR: tandem repeat, SEA: sea urchin sperm protein, enterokinase and agrin, EOC: epithelial ovarian cancer.

Figure 2: MUC16 in epithelial ovarian cancer tumourigenesis

Resting cells in normal OSE exhibit mesenchymal phenotype and do not express MUC16. In contrast, normal fallopian tube cells have predominantly epithelial features and express MUC16 in its benign isoforms. Inclusion cysts formed from OSE invaginations can develop early neoplastic changes and progress to EOC. OSE cells trapped in inclusion cysts undergo MET and produce malignant MUC16 isoforms. Alternatively, epithelial cells at the fimbrial end of the fallopian tube undergo partial EMT converting to a hybrid epithelial/mesenchymal phenotype, and form STICs that express malignant MUC16. Cells in STIC lesions undergo further EMT to acquire a purely mesenchymal phenotype and migrate to the ovarian surface, with subsequent MET and EOC formation.

Abbreviations: OSE: ovarian surface epithelium, EOC: epithelial ovarian cancer, STIC: serous tubal intraepithelial carcinoma, MET: mesenchymal to epithelial transition, EMT: epithelial to mesenchymal transition.

Colours and shapes of MUC16 glycans follow the labelling in Figure 1.

Figure 3: EMT mechanisms induced by MUC16 CTD overexpression

The hallmark event in EMT is the switch from E- to N-cadherin expression, which enhances cancer migration and invasion. In EOC cells with epithelial phenotype most MUC16 is expressed in its uncleaved form, participating in junctional complexes with E-cadherin and β -catenin. E-cadherin's interaction with EGFR inhibits binding of the latter's activating ligands (such as EGF). MMPs and cytokines promote E-cadherin shedding and MUC16 cleavage. Src phosphorylation of E-cadherin, β -catenin and MUC16 deregulates their complexes, leading to E-cadherin degradation, β -catenin release and MUC16 cleavage. These mechanisms initiate EMT with MUC16 CTD overexpression. The latter is enhanced by increased autoproteolytic MUC16 cleavage at the Golgi apparatus. MUC16 CTD can stay on the cell surface or translocate to the nucleus, to act as a transcriptional co-regulator. On the cell surface MUC16 CTD interacts with EGFR and β 1-integrin through N-glycan-mediated tripartite junctions with each of these two glycoproteins and galectin-3. These bonds activate EGFR and β 1-integrin, which subsequently up-regulate EMT-enhancing gene transcription through intracellular signalling pathways (MAPK/ERK, PI3K/Akt, FAK/Src). Furthermore, MUC16 CTD reinforces the Wnt/ β -catenin signalling, by promoting β -catenin's nuclear transfer to exert transcriptional activity. The Wnt/ β -catenin transduction also enhances EMT. MUC16 CTD recruits pools of β -catenin at the cell membrane as well, which contribute to the formation of MCAs, possibly through stabilising junctional complexes with N-cadherin.

Abbreviations: EOC: epithelial ovarian cancer, CTD: carboxy-terminal domain, EMT: epithelial to mesenchymal transition, MMPs: matrix metalloproteinases, EGFR: epidermal growth factor receptor, EGF: epidermal growth factor, PI3K: phosphatidylinositol 3-kinase, Akt: protein kinase B, MAPK: mitogen-activated protein kinase, ERK: extracellular-signal regulated kinase, Wnt: Wingless-related integration site, FAK: Focal Adhesion Kinase, MCA: multicellular aggregate. Dashed lines indicate not experimentally validated molecular interactions.

Colours and shapes of MUC16 glycans follow the labelling in Figure 1.

Figure 4: MUC16 in the evolution of epithelial ovarian cancer

MUC16 protects EOC from immune cytotoxicity with the following mechanisms: 1) HLA class I antigens bind with NK receptor KIR with an inhibitory effect 2) Core 2 O-glycans inhibit NK binding 3) Truncated core 1 O- and N-glycans bind with immune cells through their inhibitory receptor siglec-9 4) Shed MUC16 - siglec-9 binding down-regulates NK cells activating receptor CD16. Cytokines (IFN- γ and TNF- α) enhance MUC16 cleavage.

In early EOC, cells display epithelial phenotypes and join through E-cadherin, in complexes involving uncleaved MUC16. Disruption of these contacts promotes cellular release. Overexpression of MUC16

truncated core 1 O-glycans begins and is maintained throughout EOC migration. Solid tumours mainly express extended core 1 chains. Cells in ascites undergo differentiation shifts and display epithelial, hybrid and mesenchymal phenotypes. Mesenchymal cells mainly express MUC16 CTD or exhibit MUC16 knockdown, which can sensitise them to platinum chemotherapy. They also express N-cadherin, whereas hybrid cells express E- and N-cadherin. Single cells form MCAs, in cadherin subtype-specific junctions supported by MUC16. MCAs and single cells reach peritoneal surfaces and bind to mesothelin through MUC16 N-glycans. Mesothelin is also present on EOC cells, and its interaction with MUC16 leads to additional tumour recruitment on MCAs and metastatic sites. Mesothelial cells express and shed MUC16. MUC16-mesothelin contacts are stabilised by β 1-integrin, which in MUC16 knockdown cells remotely mediates their attachment to mesothelium. Further EMT enhances EOC cells invasion with mesothelial clearance, spread to sub-mesothelial matrix and degradation of its collagen. The latter is facilitated by MMPs, which catalyse MUC16 cleavage. EOC cells then undergo MET to form firm metastases, with up-regulated uncleaved MUC16 expression in E-cadherin complexes. Finally sialyl Lewis oligosaccharides, present on MUC16 core 2 O- and N-glycans, may bind to selectins on vessels, promoting haematogenous and lymphatic spread.

Abbreviations: EOC: epithelial ovarian cancer, EMT: epithelial to mesenchymal transition, MET: mesenchymal to epithelial transition, NK: Natural Killer, MC: monocyte, KIR: Killer Immunoglobulin-like receptor, IFN- γ : interferon-gamma, TNF- α : Tumour Necrosis Factor-alpha, MCA: multicellular aggregate, CTD: carboxy-terminal domain, MMPs: matrix metalloproteinases, sLe^x: sialyl Lewis^x, sLe^a: sialyl Lewis^a. DNAM-1, NKG2D and CD16 are NK activating receptors. Double lines indicate inhibitory receptors.

Colours and shapes of MUC16 glycans and ovarian cancer cells follow the labelling in Figures 1 and 2.

Figure 1

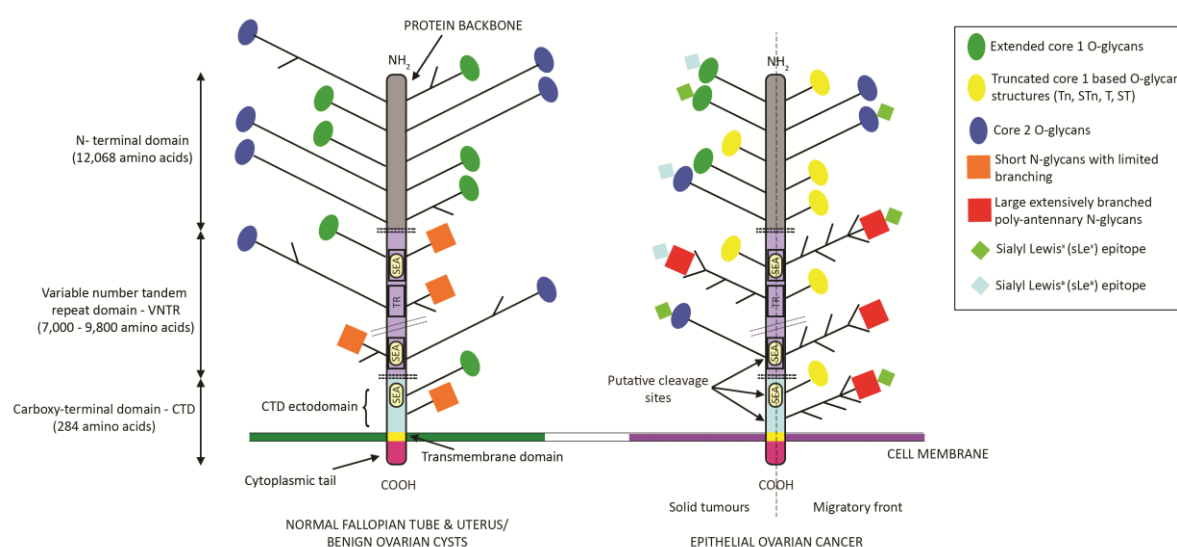


Figure 2

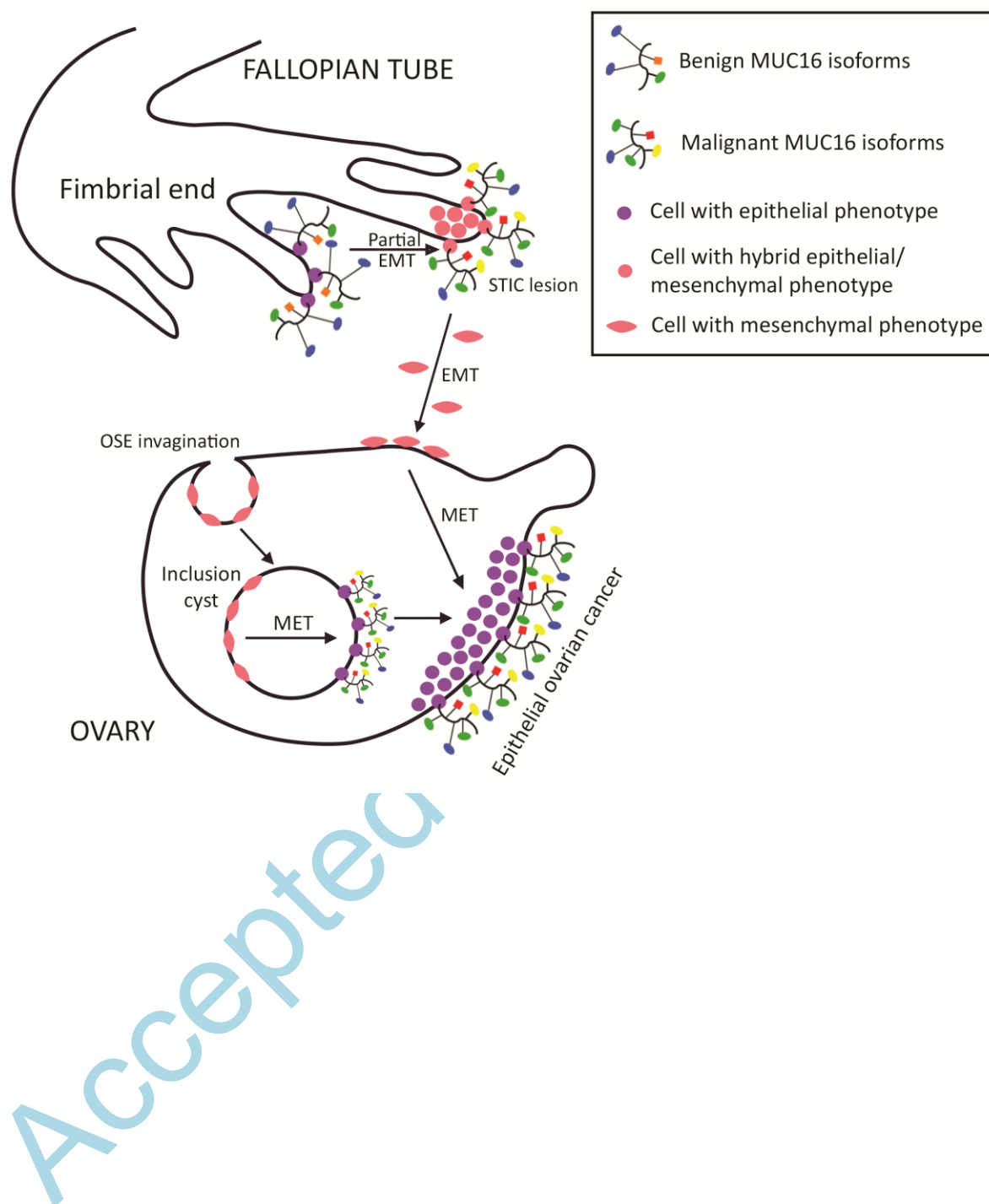


Figure 3

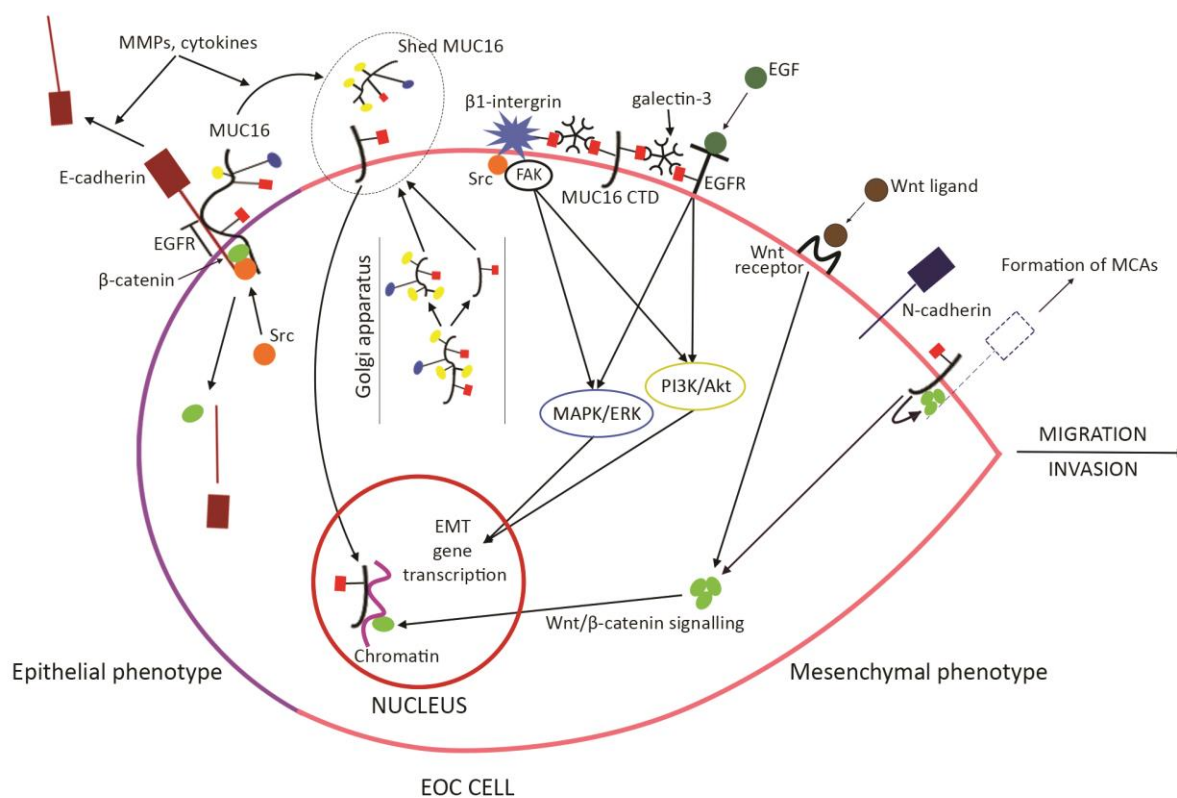


Figure 4

