The Role of CA 125 as Tumor Marker: Biochemical and Clinical Aspects

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Abstract

CA 125 also known as mucin 16 or MUC16 is a large membrane glycoprotein belonging to the wide mucin family, encoded by the homonymous *MUC16* gene. Following its discovery in the blood of some patients with specific types of cancers or other benign conditions, CA125 has found application as a tumor marker of ovarian cancer. Thirty years after its discovery, use of CA 125 is still FDA-recommended to monitor response to therapy in patients with epithelial ovarian cancer and to detect residual or recurrent disease in patients who have undergone first-line therapy and would be considered for second-look procedures. However, due to its limited specificity and sensitivity, CA 125 alone cannot still be an ideal biomarker. Increased clinical performance, in terms of better sensitivity and specificity in identifying epithelial ovarian cancer relapse, has been obtained by combined use of CA 125 with HE4, another ovarian cancer marker recently introduced in clinical use. Significant advancements have been achieved more recently, due to the introduction of FDA-approved ROMA and OVA1 algorithms to evaluate the risk of ovarian cancer for patients with a pelvic mass.

Keywords

CA 125 • CA 125 clinical use • CA 125 measurement • CA 125 nadir • HE4 • Membrane-associated mucins • MUC16 • MUC16 biochemical structure • Mucins biological functions • OVA1 algorithm • Ovarian cancer • Ovarian cancer monitoring • ROMA algorithm • Secreted mucins

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14.1 Introduction: Biochemical Structure

CA125 is a large transmembrane glycoprotein, the largest of the class of membrane-associated mucins (MAMs) to which it belongs.

For long debated whether the mucin family members were secreted or associated with the cell membrane, it is now known that both two classes of mucins exist, the secreted mucins and the cell-surface associated mucins, including different members that have common structural features. Mucin consists typically of a protein backbone, termed apomucin, covered with many O-linked oligosaccharides and a number of N-glycan chains [1]. The range and chemical composition of structures that are created by branched O-linked oligosaccharides are immense. In addition, many post-translational modifications, including glycosylation, sialylation and sulfation may then occur on mature mucin glycoproteins often in a cell-type specific manner. Structural feature that is common to all mucins and differentiate them from other membrane-bound glycoproteins, the exceptions being MUC14, MUC15, and MUC18 [2], is the centrally located tandem-repeat (TR) domain [3–5], which comprises TRs of identical or highly similar sequences particularly rich in proline, threonine, and serine and for this also known as a PTS domain [6]. The TR regions, whose number and specific sequence is highly variable among different mucins, provides a scaffold on which cells build complex oligosaccharide structures. The TR repeats typically consist from 5 to 100 potential glycosylation sites per repeat, and this peculiar TR arrays contribute increased 'stoichiometric power' to a confined area, thereby creating a locally high concentration of specific molecular structures. Also regions surrounding the TRs can contain carbohydrate structures which can be potential binding sites for interacting partners. The non-glycosylated regions of mucins consist of many structural motifs and domains [7] that might play a pathophysiological role [1].

In addition to glycosylated TR regions, common to all the mucin family members, additional structural motifs are present in the amino and carboxy termini of mucin protein backbones. Based on their biochemical features and physiological fate, mucin class are grouped into two structurally distinct categories: the secreted and the membrane-associated mucins.

14.1.1 Secreted Mucins

Next to the centrally located variable number TR sequences, which are unique to each MUC gene, the secreted mucins contain characteristic cysteine rich, cysteine knot and von Willebrand C and D domains (named D because of its homology with dimerisation domain of von Willebrand factor) at the N- or C-termini of the monomers, which are linked through disulphide bridges.

These domains are deemed to be responsible for oligomerization of the very large core-proteins.

Secretory mucins are further subdivided into those gel-forming and non-gel-forming. The first include the large secretory mucins with cysteine-rich motifs, MUC2, MUC5AC, MUC5B, MUC6, encoded by a cluster of genes at the chromosomal locus 11p15 and evolved from a common ancestor with von Willebrand factor (VWF). More recently has been identified and characterized MUC 19, another cysteine-rich secretory mucin, encoded by a gene showing 12q12 chromosomal location [8]. MUC7 and MUC9, instead, are smaller secreted mucins that do not oligomerize and do not form gels [9]. Also MUC18 do not form gels, and lacks the VWF D4 or C1, C2 domains typically present in the carboxy end of large secretory mucins. For a more detailed description we refer to some exhaustive review [9, 10].

14.1.2 Membrane-Associated Mucins

Cell-surface-associated mucins, which form the largest group of mucins, are bound to cells by an integral transmembrane domain and have relatively short cytoplasmic tails (CT) domain, often containing sites of phosphorilation that interact with mediators of signal transduction and other

cytoskeleton-associated proteins [11]. On the extracellular side of the membrane-associated unit most MAMs have one, but also two or three domains which show significant homology to the epidermal-growth-factor (EGF) family members. The precise functions of EGF-like domains remains to be established, however, experimental evidence support their role in mediating interactions between cell-surface-associated mucins and members of EGF receptors (ERBB) family, which are involved in regulation of cellular growth, differentiation, motility and inflammation [12]. Another important functional module widely distributed among cell surface associated proteins is the SEA domain, so named after the first three proteins in which it was identified (Sperm protein, Enterokinase and Agrin). It is an extracellular domain of ~120 amino acids, of which an about 80-residue conserved region and an about 40-residue segment that separates the conserved region from the subsequent C-terminal domains. Characteristically located between the *O*-glycosylated PTS repeats and the transmembrane domain, SEA module contains autocatalytic proteolytic cleavage site that leads to the release of the large extracellular mucin into the mucus gel layer. This involvement of SEA domain in the cleavage of proteins has been well investigated in MUC1 [13]. Generally present in only one unit in the mucins, SEA domains can also to be present in more modules, such as in MUC16, which contains multiple SEA domains. The presence of multiple SEA domains, but no EGF-like domains, in MUC16 emphasizes the diversity in the evolution and potential function of the different MAMs [10]. Common feature of the ectodomains of MAMs is the heavy O-glycosylation, for which up to 80 % of the mass of the mucin is O-glycans, with galactose, N-acetylgalactosamine, N-acetylglucosamine and sialic acids as the main sugars with minor amounts of fucose, mannose and glucose.

14.1.2.1 MUC16: Biochemical Structure

In the genome, MUC16 is localized in 19p13.2 chromosome and is coded by sequences present within approximatively 179 kb of genomic DNA. MUC16 protein, also known as CA125 antigen, is a MAM, the largest of the class. With a core peptide of 22,152 amino acids and a molecular weight of 2,353,428 Da, MUC 16 is more than twice as long as MUC1 and MUC4.

MUC16 is composed of three different domains: an N-terminal domain, a central tandem repeat region, and a carboxy terminal domain. The N-terminal and tandem repeat domains are entirely extracellular and highly O-glycosylated. In particular, the N-terminal domain consists of 12,070 amino acids rich in serine/threonine residues and has been reported to contain the major O-glycosylation known to be present in CA125. The MUC16 protein back bone is composed of tandem repeat region, which has more than 60 repeat domains of 156 amino acids each. Though not all individually similar, most of the repeat units, which, such as any mucins, are rich in serine, threonine and proline residues, recur more than once inside the sequence. In the tandem-repeat domain of MUC16 there is also a small cysteine ring region on which are thought to be present the epitopes for known anti-CA125 antibodies (OC125 and M11).

The carboxy-terminal domain has 284 amino-acids and consists in an extra cellular region, a transmembrane and a cytoplasmic tail. The extracellular part of the carboxy-terminal domain contains multiple SEA modules, many N-glycosylation sites and some O-glycosylation sites. It has been reported to harbor also a putative cleavage site. The MUC16 cytoplasmic tail is 31 amino acids long and contains several potential phosphorylation sites, in particular a putative tyrosine phosphorylation site (RRKKEGY), which was first recognized in Src family protein. Recently, it has been shown that MUC16 cytoplasmic tail, which contains a polybasic amino-acid sequence, can interact with cytoskeleton through ERM (ezrin/radixin/moesin) actin-binding proteins.

14.2 Biological Functions

14.2.1 Physiology

All mucins are highly O-glycosylated in tissue-specific manner and in function of specific roles that they play at these locations. The properties

and the wide variety of types of mucins expressed by epithelia, reflecting their different structural organization, the nature of their post-translational modifications, the degree of intramolecular and intermolecular crosslinking, has raised interest for further investigation into the biochemical properties and biological implications of mucins and their functional roles in normal and malignant cells.

Mucins produced by secretory epithelial cells of the gastrointestinal, respiratory, and urinogenital tracts contribute to confer normal physiological lubrification and protection to epithelial surfaces ducts and lumens within the human body. Keeping epithelial surfaces hydrated, needed for the lubrication and normal functioning of ducts and passageways, may guarantee to the epithelial cells also an effective protection from infections and injuries [7, 14]. The continuous need for maintaining mucosal protection against all external aggressive forces requires a normal turnover of the barrier. From this it results a dynamic and balanced process of mucin biosynthesis, secretion and degradation at mucosal surfaces that relies on the availability of specific proteases and glycosidases secreted by other mucosal cells or present in the extracellular microflora [9]. In addition, mucins play an important role in renewal and differentiation of the epithelium, in modulation of cell adhesion and cell signalling and immuno suppression [15].

Secreted mucins show patterns of expression that are restricted to specialized organs and cell types that secreted them into the extracellular space. They are key components in most mucus gels that protects underlying epithelia from adverse conditions by forming a chemical barrier that limits exposure to various injuries, including bacteria, virus, pH, ingested toxins, reactive oxygen species (ROS) and proteolytic enzymes in the gastrointestinal tract, and suppresses the inflammatory response. Mucin 2 (MUC2), the major secreted mucin lining the gastrointestinal mucosa, functions by suppressing inflammation in the intestinal tract and inhibiting the development of intestinal tumours [10]. Near the cell surface, the secreted mucin layer might interact with MAMs or other cell-surface molecules, thus contributing also in this way to physicochemical protection of the epithelial cell surfaces by maintaining the local molecular environment with respect to hydration, ionic composition and concentration, and accessibility of macromolecules [7].

Unlike the secreted mucins, MAMs are released from the apical cell membrane by enzymatic cleavage of the N-terminal subunit into the mucous gel; alternative splicing can also produce secreted variants [1]. From an evolutionist point of view, the inclusion of a transmembrane component provides many advantages to the epithelial cell. Informations about the condition of the external environment can be transmitted to the interior of the cell, to indicate that a normal status exists at the cell surface or that inflammation processes and other forms of stress are present. In such way, MAMs behaves as cell-surface receptors and sensors, and conduct signals in response to changes in conformation or ligand status of their extracellular domains. All that leads to coordinated cellular responses that include proliferation, differentiation, apoptosis and secretion of specialized cellular products [7], thus providing an additional level of defence to promote the growth, repair and survival of epithelial cells. In fact, among the multiple biological functions of MAMs, of particular importance is their role in signal transduction. The presence in many MAMs (specifically in MUC3A, MUC3B, MUC4, MUC12, MUC13, and MUC17) of two or three EGF-like domains might allow these membrane mucins to interact with the ErbB receptors and regulate EGF-receptor-mediated cell signalling, whose excessive signalling is well-known to be associated with the development of a wide variety of tumours.

14.2.2 Pathophysiology

Alterations in mucin forms and amounts can occur in numerous pathologic processes, including lung diseases such as asthma, bronchitis, cystic fibrosis and chronic obstructive pulmonary disease.

Mucus and mucins play in fact a fundamental role in disease mechanisms leading to the characteristic mucus hypersecretion, pulmonary obstruction, reduced mucociliary clearance and subsequent infection.

More importantly, mucins have multiple implications in cancer development. It is well known that mucins are overexpressed and aberrantly glycosylated in many adenocarcinomas, including in the breast, lung, gastric, colorectal, pancreatic, cervical and ovarian cancers. These aberrant forms can derive from deregulation of expression of mucin core proteins and alteration of enzymatic glycosylation events, which may be by incomplete synthesis or neosynthesis, with subsequent expression of novel, pathological combinations of different mucins. This results in an enormous selective advantage for tumor cell, which, by acquiring a widest range of potential ligands for interaction with other receptors at the cell surface, modifies its behaviour and enhances its survival ability during invasion and metastatic events. The changes in mucin expression lead in general to a loss of normal epithelial function, with decreased mucosal protection. In addition, the control of local environment by mucins and the capture of growth factors and cytochines can contribute not only to cell proliferation, invasion and metastasis, but also to affect the ability of immune, inflammatory and stromal cells to interact with the tumour, with subsequent impact, more or less direct, on the development and maintenance of immune responses, which are frequently suppressed in malignancies that overexpressed mucins [16].

Also the interactions between transmembrane mucins and several protein partners seems to be very important to regulate different molecular and cellular events, including cell-cell/protein-protein binding, signal transduction and protein stabilization. In fact, carbohydrate structures present in the highly glycosylated TR region or outside the TR region of mucins make them potential candidates to interact with several carbohydrate binding proteins, including the galectin family. Among the 14 known galectins, it has been observed an interaction of galectin-3 with both MUC16 (also called CA125) and MUC1, and of galectin-1 with MUC16 [17, 18]. In particular, following the observation that MUC16 is also a potent inhibitor of natural killer (NK) cell responses in vitro [19], it has been hypothesized that the interaction of the galectin-1 with MUC16 may be important in the attachment of the mucin to the NK cell surface, thus promoting metastasis and evading immune responses [20]. The association of cell surface mucins with galectin-3 has been reported to contribute to the ocular surface epithelial barrier, which is critical to preventing damage to and infection of wet-surfaced epithelia [17]. Furthermore, of particular importance is the direct interaction occurs between mesothelin, a glycosylphosphatidylinositol-linked cell surface protein present on mesothelial/ovarian cancer cells, and MUC16 [21, 22]. This interaction, which relies on MUC16-N-glycosylation, seems to involve the MUC16 TR region and mesothelin residues 296–359 [23, 24]. The biological importance of MUC16-mesothelin interaction in facilitating cell-cell adhesion, thus promoting the metastasis of ovarian cancer cells has aroused lively interest in its potential therapeutic implication. Additionally, the identification of the MUC16-interacting region in mesothelin has favoured the design of antibodies against MUC16 that can be used as potential agents to inhibit the MUC16-mesothelin interaction, thus inhibiting ovarian cancer cell metastasis. Furthermore, the characterization of the interacting domains in both the two partner proteins is opening new ways for the development of specific pharmacological tools against these interactions [23]. Further studies are needed, however, to determine the in vivo effectiveness of all these novel potential therapeutic agents.

14.3 Measurement of CA 125

CA 125 was discovered by Bast et al. in [25], with the development of a murine monoclonal antibody (OC 125) produced by immunizing a mouse with OVCA 433 cell line, derived from a patient with ovarian serous carcinoma. The first immunoassay for CA 125 developed and

commercialized in [26] used the OC 125 antibody for both capture and detection [27].

A second-generation assay (CA125 II) typically uses the monoclonal antibody, M11, as the capture antibody and OC 125 as the conjugate antibody. Other FDA-cleared assays for CA 125, which employ antibodies other than the OC 125 and M11 antibodies, are available on automated immunoassay platforms. Notably, although the majority of manufacturers reports similar reference intervals, the concentrations of CA125 can vary because of differences in calibration, assay design, and reagent specificities [28]. It follows that, at present, results obtained with different assay methods cannot be used interchangeably. As recommended on National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines for Use of Tumor Markers, in the absence of an International Standard for CA125, it is then suitable that manufacturers specify all features of the used method and laboratories indicate them on their clinical reports. For all that, patients should be monitored with a single assay or rebaselined if there is a change in adopted methodology [29].

14.3.1 Sensitivity, Specificity

In a healthy population, CA 125 serum levels are <35 U/mL. This cutoff was determined from the distribution of values in healthy individuals so as to include 99 % of normals [30]. Values tend to decline with menopause and aging. Elevations of CA 125 assay values may be found in approximately 1–2 % of healthy individuals [26] and in 5 % of individuals with nonmalignant conditions such as in women in the follicular phase of the menstrual cycle [31] and in individuals with cirrhosis [32, 33], hepatitis [34], endometriosis [35, 36], first trimester pregnancy [37, 38], ovarian cysts [39], and pelvic inflammatory disease [38, 40]. Increased CA 125 values have been reported in 28 % of subjects with non-ovarian malignancies which include breast, lung liver pancreatic, colon, stomach, biliary tract [34, 41] cervical [34, 40] uterine [33] fallopian tube [40] and endometrial carcinomas [42]. Therefore, CA-125 has poor specificity as biomarker for ovarian cancer because of many inflammatory conditions in the abdominal area that cause fluctuations in CA-125 levels, and other non-ovarian malignancies which result often in false positives. CA 125 has limited sensitivity in detecting ovarian cancer. Elevated levels of CA 125 have be found in about 50 % of patients with early stage ovarian cancer, meaning that CA 125 has particularly poor sensitivity for ovarian cancer before the onset of symptoms. Furthermore, CA 125 is elevated in 90 % of patients with stage II disease, and more than 90 % with stage III and IV, whereas the remainder do not express this antigen. The concentration of CA 125 correlates with tumor size and staging. The use of CA 125 to detect ovarian cancer, especially in early stages of disease, can frequently lead to false negatives with important clinical implications. It follow in fact that patients that receive false negatives could not receive required cares and an appropriate treatment for their disease. CA 125 determination may be then useful in the evaluation of the disease status in patients with advanced endometriosis, but is not useful in screening for ovarian cancer in asymptomatic populations.

Another issue that should be consider in measuring CA 125 is the possible interference which may be observed in presence of heterophilic antibodies in the serum, similarly to other immunoassays [43, 44]. In particular, individuals who follow a therapeutic protocol with monoclonal antibodies by parenteral routes may produce anti-mouse antibodies. Serum specimens from these patients may produce erroneous results in such assay.

14.4 CA 125 Determination in Clinical Practice

14.4.1 Ovarian Cancer Statistics

Ovarian cancer is the seventh most common cancer in women worldwide (18 most common cancer overall) and the second most common gynaecological cancer after uterus. Worldwide, nearly 239,000 women were estimated to have

been diagnosed with ovarian cancer, with incidence rates varying across the world. The highest incidence of ovarian cancer was in Central and Eastern Europe and Northern America, and the lowest in Western Africa and Asia, but this partly reflects varying data quality worldwide. The most striking international difference occurs in Japan, which has lower rates of ovarian cancer than in Europe. In Europe, ovarian cancer is the 13th most common cancer overall and the fifth most common cancer for females with around 65,600 new cases diagnosed in 2012. Some of this variation may be explained by different prevalence of risk factors, use of screening, and diagnostic methods. Morphologically, ovarian cancer is composed of different tumor categories including surface epithelial tumors, sex-cord stromal tumors, and germ cell tumors [45]. Of these, epithelial tumors (carcinomas) are the most common, representing the 80–90 % of overall ovarian malignancies, and are divided into the following histologic types: serous, mucinous, endometrioid, clear cell, and transitional [46]. Data on prevalence and mortality clearly indicate that serous ovarian carcinoma represents the most important of all primary ovarian carcinomas [47]. Importantly, this distinct histological features result in different clinical behavior, tumorigenesis and pattern of gene expression, with subsequent and considerable clinical implications.

The latest statistics available on mortality for ovarian cancer are of 2012 and indicate that, worldwide, around 152,000 women were estimated to have died from ovarian cancer in 2012, with mortality rates varying across the world. Data related to the same year 2012 estimate that in Europe around 42,700 women have died from ovarian cancer [48]. Since ovarian cancer often has no symptoms at the early stages, its diagnosis happens generally when the disease is in advanced stage. That implies that, even though 10-year survival from ovarian cancer has almost doubled over the last 40 years, it remains however still poor, ranging from approximately 30–50% at 5 years after diagnosis (which compares the 5-year survival of people with the cancer to the survival of others at the same age who do not have cancer). As often happens for other types of cancer, early detection is often crucial for a better outcome of the disease. Statistical data recently reported show, in fact, that ovarian cancer survival is highest in younger women, who are more often diagnosed with early cancer.

14.4.2 CA 125 as a Screening and Diagnostic Biomarker for Ovarian Cancer

Due to its limited specificity and sensitivity, CA 125 alone is not useful as a screening assay for ovarian cancer detection in asymptomatic population. A single measurement of CA 125 cannot be interpreted, without use of other diagnostic techniques, as absolute evidence of the presence or absence of disease. Screening is however recommended by the NACB Panel or by other authoritative organizations in at-risk women with a family history of hereditary ovarian cancer, in conjunction with pelvic examination and ultrasound testing [49–51]. To improve the clinical usefulness of CA 125 for screening/early detection, several strategies has been suggested, including approaches combining CA125 with ultrasound, longitudinal measurements of CA125, and measurement of CA125 in combination with other recently proposed multimarker panels [27, 52–55].

The recognition that early detection of ovarian cancer may have the potential to considerably improve prognosis prompted the development, in the last few years, of a number of large prospective trials to evaluate the potential role for CA125 in screening for ovarian cancer in asymptomatic populations. A total of 82,487 low-risk postmenopausal women were screened using an annual ultrasound and CA125 determination in a Japanese Shizuoka Cohort Study of Ovarian Cancer Screening. The trial showed encouraging sensitivity (77.1 %) and specificity (99.9 %) with a more effectiveness in detecting cancer at an early stage (63 %) compared to the control arm (38 %) [56].

The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial was designed to determine the effect of specific cancer screening

tests on cause-specific mortality. Enrollment for this randomized controlled trial began in November 1993 and concluded in July 2001. Planned follow-up was for up to 13 years from randomization. A total of 68,616 women aged 55–74 were enrolled, of whom 30,630 underwent screening, between 1993 and 2007, for serum CA125 and transvaginal ultrasound for 4 years followed by CA125 alone for a further 2 years. Data from this study indicate that annual screening for ovarian cancer as performed in the PLCO trial with simultaneous CA-125 and transvaginal ultrasound does not reduce disease-specific mortality in women at average risk for ovarian cancer but does increase invasive medical procedures and complications in women undergoing surgery for false positive results [57].

If the PLCO trial has reported no mortality benefit of ovarian cancer screening, also the largest prospective randomized trial realized so far, the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), has been designed to assess the effect of screening with serial CA125 measurements and transvaginal sonography on cause-specific mortality. Between 2001 and 2005, a total of 202,638 postmenopausal women aged 50–74 years were randomly assigned to no treatment (control; $n=101,359$); annual CA125 screening (interpreted by a 'Risk of Ovarian Cancer' algorithm, ROCA) with transvaginal ultrasound as a second-line test (multimodal screening [MMS]; $n=50,640$); or annual screening with transvaginal ultrasound (USS; $n=50,639$) alone. The use of longitudinal algorithm ROCA, that compares the CA125 profile of cases to that of healthy women and incorporates age-specific incidence of ovarian cancer in estimating risk, seems to shown encouraging performance characteristics both on prevalence and incidence screening [58]. Data from the UKCTOCS suggest that CA125 rise within normal range can be detected by the ROCA well before any abnormalities are detected on transvaginal imaging. Whether this converts into a mortality impact will be known as soon as the results will be available [59].

If not recommended by NACB for use in screening asymptomatic women, CA 125 is however considered useful in distinguishing benign from malignant disease in women, particularly in postmenopausal women with suspicious ovarian masses, thus facilitating orientation for a more o less extensive surgical intervention. If in premenopausal women several benign conditions that cause increased values of CA 125 may be a confounding factors, thus rendering more difficult the discrimination of benign from malignant disease, in postmenopausal women elevated concentrations of CA125>95 kU/L can discriminate malignant from benign pelvic masses with a positive predictive value of 95 % [27].

14.4.3 CA 125 as Biomarker for Ovarian Cancer Prognosis and Monitoring of Therapeutic Effect

Measuring CA 125 is actually considered standard of care by many for ovarian cancer patient surveillance. Monitoring response to therapeutic treatment is in fact the primary FDA-indicated use for CA 125 in patients with epithelial ovarian cancer. The second FDA-indicated use is to detect residual or recurrent disease during the follow up of patients who have undergone first-line therapy and would be considered for second-look procedures.

Generally, in monitoring studies, elevations of CA 125>35 U/mL after debulking surgery and chemotherapy indicate that residual disease is likely (>95 % accuracy). A persistently rising CA 125 value after three cycles of chemotherapy suggests progressive malignant disease and poor therapeutic response. However, CA-125 levels below 35 U/mL do not rule-out recurrence, because patients with histopathologic evidence of ovarian carcinoma may have CA-125 test concentrations within the range of healthy individuals. Then clinical decisions for these patients should not be based on a CA-125 test concentration below 35 U/mL.

Currently, there is general consensus about the recommendation of CA 125 measurement for monitoring response to treatment and detecting disease recurrence. However, which may be the

best evaluation criteria of CA 125-based response remains still debated [60–62]. In 2011, the Gynecologic Cancer Intergroup (GCIG), in evaluating the criteria of CA 125 use to define progression-free survival after first-line therapy as well as the criteria to define response to treatment in recurrent disease, suggested that CA 125 alone can be used to evaluate the effectiveness of treatment [63]. The commonly accepted response criterion is a 50 % decrease in CA 125 as compared to the pretreated sample, which should be taken 2 weeks before treatment. According to NACB ovarian cancer panel recommendation, subsequent samples should be taken at 2–4 weeks during treatment and at intervals of 2–3 weeks during follow-up [28]. Then, if CA 125 monitoring is actually considered a relatively sensitive and cost-effective test to follow up of ovarian cancer patients, however other methods such as physical examination, CT scan, and ultrasound are also important for detecting residual disease. Of note, the retrospective study of Gadducci et al. [64] assessed the pattern of failures of 412 patients with recurrent ovarian cancer followed up with different surveillance protocols. Follow-up by using clinical examination, imaging technique and serum CA 125 raised the suspect of recurrent disease in the 80 % of patients, while only 23 % of them were detected by CA 125 measurement alone [64]. It is likely that the GCIG progression or recurrence criteria, based on CA 125 monitoring, might be so strict as they do not allow to detect disease progression in those patients whose CA 125 levels are less than two times the upper limit of the reference range or nadir value [62].

The possible advantages related to use of CA 125 nadir have been recently evaluated in several retrospective studies, suggesting that the nadir serum CA-125 level, in women achieving a clinically-defined complete response to primary chemotherapy, accurately defined the risk of relapse [65, 66]. According to these reports, the retrospective analysis of Markman et al. [67] demonstrated that the baseline CA-125 level before initiation of maintenance chemotherapy may be of strong prognostic value. In particular, baseline CA-125 levels distributed into subgroups for values of (A) $<$or$=$10 U/mL, (B) 11–20 U/mL, and (C) 21–35 U/mL, was highly statistically significant in strongly predicting the risk of subsequent relapse. At premaintenance baseline CA-125 values $<$or$=$10 U/mL corresponds to a superior progression-free survival compared with higher levels in the normal CA-125 range. Given findings of different prognostic groupings existing within the commonly regarded normal CA-125 range, Liu et al. [68] evaluated another criterion to detect early signal of progressive disease, by predicting progression if CA 125 $\geq$ 20 U/mL on two consecutive occasions for patients with CA 125 nadir $\leq$ 10 U/mL or if CA 125 $\geq 2 \times$ nadir on two consecutive occasions for patients with nadir more than 10 U/mL. This proposal, which essentially applies the GCIG CA-125 disease progression criterion, lowering however the upper normal limit from 35 to 10 U/mL, obtained a positive predictive value of 93 % (95 % CI, 88–97 %).

To analyze the prognostic value of the CA-125 nadir in the normal range (<35 U/mL), Prat et al. [69] included in their retrospective analysis patients with CA-125 $>$ 35 U/mL at time of diagnosis, treated with optimal cytoreductive surgery and perioperative platinum/taxane-based chemotherapy. By dividing patients that have achieved a complete biochemical (<35 U/mL) and radiological response after primary treatment into the following arbitrary groups, group A $\leq$10 U/mL; and group B, 11–35 U/mL, they have found that the outcome were significantly improved for group A as compared to group B. Similarly to previous findings, also results from this study, with a 96.4 % positive predictive value, demonstrated that the CA-125 nadir value is a strong independent prognostic factor for subsequent disease relapse and overall survival. All together, these results suggest that variations in the CA- 125 levels after primary surgery and, more importantly, the nadir value of the CA-125 after primary chemotherapy, are associated with patient outcome. An appropriate use of CA 125 and a careful evaluation of the variations from CA 125 nadir may be useful to oncologist to early detect ovarian cancer relapse.

14.5 Other Serum Markers for Pancreatic Cancer

14.5.1 HE4

Human epididymis protein 4 (HE4) is another ovarian cancer marker intensely studied in the last years and recently introduced in clinical use. HE4 is a small secretory protein, encoded by the *WFDC2* gene, which resides on human chromosome 20q12-13.1, a region that harbors a locus of 14 genes encoding protein domains that have homology with whey acidic protein (WAP) [70]. This protein is also designated WAP four-disulfide core domain protein 2 (WFDC2) because it contains two WAP domains and a "four disulfide core" made up of eight cysteine residues. The WAP domain is a conserved motif, containing eight cysteines found in a characteristic 4-disulphide core arrangement, that is present in a number of otherwise unrelated proteins. These proteins typically are secreted and are protease inhibitors, although this function has not been ascribed to HE4, and its exact physiologic role has not been characterized.

HE4 protein was initially discovered, by using microarrays, to be overexpressed in epididymal tissue and later in ovarian cancer tissue [71, 72]. Generation of the monoclonal antibodies 2H5 and 3D8 to epitopes on HE4 has allowed development of a sandwich ELISA and measurement of HE4 serum, test which has become available for the routine laboratory repertoire. Subsequent studies have shown that HE4 is not specific for ovarian tumors, although its expression is however restricted to the normal tissue of the reproductive tracts and respiratory epithelium. It has been observed also in a subset of lung tumour cell lines [73].

In the serum of patients with epithelial ovarian cancer, HE4 is overexpressed in 93 % of serous histologic subtype and in 100 % of endometrioid epithelial ovarian cancers, but only in 50 % of clear cell carcinomas and not in mucinous or germ-cell ovarian cancers.

14.5.1.1 Clinical Applications of HE4

At an HE4 concentration of 150 pM, 95 % of healthy women were below this cutoff, while 79 % of women with ovarian cancer were above this cutoff. Elevations in other subjects include breast (13 %), endometrial (26 %), gastrointestinal (16 %), and lung cancers (42 %), as well as benign gynecologic disease (7 %) and other benign disease (24 %). A recent study revising the available literature on biological and lifestyle factors affecting HE4 concentrations in serum highlights that, in contrast to CA-125, higher HE4 concentrations are reported in the elderly, with a strong difference in biomarker biological intra-individual variation according to the fertility status is reported. In addition, the evaluation of HE4 results may be problematic when patients suffer from additional conditions that may alter HE4 level. Other factors, such as smoking and decreased renal function also show a substantial impact on HE4 values, which should be considered in each patient [74].

The great interest aroused by HE4 is motivated primarily by better specificity that this protein seems to have compared with CA125 in discriminating benign diseases. Recent studies demonstrated that the more prominent differences among them are observed in patients with some benign ovarian diseases, such as endometriosis, who showed the 67 % of increased CA 125 values compared with 3 % of HE4 [75]. It has been reported that mean serum concentration of HE4 was significantly higher in serum samples of patients with both endometrial (99.2 pM, $P<0.001$) and ovarian (1125.4 pM, $P<0.001$) cancer but not with ovarian endometriomas (46.0 pM) or other types of endometriosis (45.5 pM) as compared with healthy controls (40.5 pM) [76].

At present, although several studies comparing HE4 and CA 125 performance demonstrated that HE4 is a more specific marker for ovarian cancer than CA 125 [75, 76], the clinical use of HE4 in differentiation of ovarian cancer from other benign gynecologic diseases continues to be evaluated. Certainly, measuring both HE4 and

CA125 serum concentrations may allow more accurate prediction of cancer than use of the individual markers, thus providing valuable information to discriminate ovarian tumours from ovarian endometriotic cysts. Furthermore, the HE4 assay is FDA cleared for monitoring recurrence or progressive disease in patients with epithelial ovarian cancer. Similarly to CA 125, a prompt reduction and subsequent normalization of HE4 levels reflects a response to primary surgery and chemotherapy. But HE4 values that remains elevated are important indicator of the recurrence of the disease. Interestingly, the study by Anastasi et al. [77] showed that, in the follow-up of patients with ovarian cancer, the increased expression of HE4 is detected 5–8 months before CA125 increment, suggesting that HE4 might be a better marker for monitoring disease progression. Hynninen et al. [78] in evaluating response of patients treated with primary surgery and six cycles of chemotherapy demonstrated that HE4 correlated with PET/CT results better than CA 125. Similarly, the study of Manganaro et al. [79] confirmed that HE4 may serve as marker of epithelial ovarian cancer relapse and, more importantly, its values, measured within three time intervals after surgery and adjuvant chemotherapy, were found to increase early compared with CA 125. A percentage of elevated HE4 levels were detected already in patients within the first time interval, while positivity for CA-125 was found later at time interval III and only in 44 % of patients. Combining then HE4 serum evaluation with CE CT imaging may improve the monitoring management of women affected by ovarian cancer.

About the diagnostic test performance, available data are rather limited and still insufficient to conclude that HE4 alone or in combination with CA-125 has significantly better diagnostic performance than CA-125 alone. Moreover, there is not sufficient evidence from prospective or controlled studies demonstrating that HE4 is an effective screening tool for identifying ovarian cancer in asymptomatic women.

14.6 Multiple-Marker Based Algorithms

Due to well-known limitations associated to the use of a single marker, for some years oncologic research has turned to evaluate clinical utility of the combined use of multiple biomarkers associated with ovarian cancers, including biochemical, ultrasound and other imaging techniques. Two algorithms, ROMA and OVA1, have been recently approved by FDA and are used to assess ovarian cancer risk for premenopausal or postmenopausal women with a pelvic mass.

ROMA (Risk of Ovarian Malignancy Algorithm) is a qualitative serum test that generates a numerical score (from 0.0 to 10.0) by incorporating the results of CA-125 (the most widely accepted biomarker for ovarian cancer) and HE4 blood tests, plus menopausal status, to identify patients presenting with an adnexal mass as being at high or low likelihood for having malignancy. Results must be interpreted in conjunction with an independent clinical and radiological assessment (https://www.accessdata.fda.gov/cdrh_docs/reviews/K103358.pdf)

Data from a combined population of pre- and postmenopausal women, published in the instructions for use of ROMA [80], showed for this algorithm a sensitivity of 88.4 %, a specificity of 67.2 %, and an NPV of 96.2 %. The high accuracy and reproducibility characteristic of this regression model in stratifying patients into a high or low ovarian cancer risk is independently confirmed in a number of publications, some of which indicated increased benefit with ROMA vs traditionally measured CA-125 and HE4 [81–83]. It may, furthermore, be improved with inclusion of supplemental data, such as age and ultrasound findings. The performance and clinical utility of ROMA has been described in detail by Chudecka-Głaz [84] in her exhaustive review.

Based on the proteomics biomarker discovery approach using mass spectrometry, Zhang and coworkers [55, 85] identified several proteins that, when combined with CA 125, provide

diagnostic value for ovarian cancer. Data were submitted to the FDA and were cleared for clinical use as the OVA1 test the first in vitro diagnostic multivariate index assay proteomic diagnostic for cancer.

The OVA1 Test is a qualitative serum test that combines the values for 5 analytes (Prealbumin, Apo A-1, β2M, Transferrin, and CA 125) from separately run immunoassays into a single numerical score between 0.0 and 10.0 to indicate the likelihood that the pelvic mass is benign or malignant. The algorithm was derived using two independent training data sets from preoperative serum samples. Two cutoffs, 5.0 and 4.4 for pre- and post-menopausal patients respectively, were identified based on the training data. The cutoff score classifies a patient based on her OVA1™ Test score as low probability or high probability for presence of ovarian malignancy [86]. The FDA reviewed a study of 516 patients, collected from 27 clinical sites and including 269 evaluated by non-gynecological oncologists, which compared OVA1 results with biopsy results. When combined with pre-surgical information, such as radiography and other laboratory tests, results from the OVA1 tests identified additional patients, not identified using pre-surgical information alone, who might benefit from oncology referral (https://www.accessdata.fda.gov/cdrh_docs/reviews/K081754.pdf).

The effectiveness of OVA1 in the preoperative assessment of ovarian tumors has been investigated in a study of Ueland and coworkers [87], who assessed its clinical performance in a prospective, double-blind clinical study of 524 subjects (29 % with ovarian cancer) at 27 demographically diverse collection sites throughout the U.S. The authors reported high sensitivity (93 %) and NPV (93 %) but low specificity (43 %) and low PPV (42 %), demonstrating for OVA1 a higher sensitivity and lower specificity compared with physician assessment and CA 125 in detecting ovarian malignancies. Similar results were reported by Bristow et al. [88], who evaluated the effectiveness of a multivariate index assay in identifying ovarian malignancy compared to clinical assessment and CA125-II. Data from a prospective, multi-institutional trial, enrolling a total of 494 women, scheduled to undergo surgery for an adnexal mass from 27 non-gynecologic oncology practices, showed that, when combined with clinical impression, the sensitivity for OVA1 was 95.7 %, validating its usefulness as a preoperative cancer referral test. Investigators concluded that OVA1 demonstrated higher sensitivity and negative predictive value (98.1 %) for ovarian malignancy compared to clinical impression and CA125-II in an intended

In conclusion, OVA1 test is not intended for ovarian cancer screening or for a definitive diagnosis of ovarian cancer. It should be used as an adjunctive test to complement, not replace, other diagnostic and clinical procedures. Furthermore, interpreting the test result requires to know whether the woman is pre- or post-menopausal.

More recently, Grenache et al. [89] evaluated the clinical performance of OVA1 and ROMA for the prediction of malignancy in women with an adnexal mass, reporting a sensitivity of OVA1 and ROMA of 97 % and 87 %, respectively ($p=0.25$). Results indicated that ROMA was more specific than OVA1 (83 % vs. 55 %, respectively; $p<0.0001$), while the negative predictive values of both tests were similar (98.4 % and 96.0 %, respectively). A sequential testing strategy may improve overall performance, producing a positive predictive value of 69 % when ROMA is performed on all patients identified as high risk by OVA1. The authors concluded that the use of these tests to appropriately triage women with an adnexal mass should be gauged within the context of their respective limitations.

14.7 Conclusions

Although the role of CA 125 in the screening is controversial, CA 125 serum measurement is useful in the differential diagnosis of ovarian masses, and in monitoring response to therapeutic treatment and in detecting residual or recurrent disease during the follow up women with epithelial ovarian cancer. However, due to its limited specificity and sensitivity, CA 125 alone cannot still be an ideal biomarker. From all these considerations arises the need to identify comple-

mentary biomarkers which may be used in association with CA 125, to improve diagnostic performance. HE4 is another ovarian cancer marker intensely studied in the last years and recently introduced in clinical use as marker of epithelial ovarian cancer relapse. Considerable efforts have been applied to the development of multiplexed biomarker-based tests and more than 200 potential markers of ovarian cancer has been proposed so far [90]. Several significant advancements have been achieved recently, including the introduction of FDA-approved HE4, ROMA and OVA1 tests to evaluate the risk of ovarian cancer for patients with a pelvic mass. Results from recent studies are encouraging, in demonstrating that a multi-marker approach seems guarantee a better sensitivity than CA 125 alone, although their real clinical contribution is still under accurate investigations in properly designed clinical trials. Meanwhile major efforts are underway to detect biomarkers capable of recognizing disease in its preclinical phase, in an attempt to improve ovarian cancer risk stratification by identifying populations at greatest risk of disease. It is a very difficult challenge, but the considerable advances in high-throughput technologies over the past decade and their intense use in identifying a characteristic disease-related markers profile clearly indicates that a new era in screening is underway.

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