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## MALDI-MSI and Ovarian Cancer Biomarkers

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### 1. Introduction

Early diagnostic and disease management is one of the most important challenges facing modern medicine, which is particularly relevant in cancer. The lack of effective assays measuring multiple blood-based biomarkers is lacking in many types of cancer. Moreover, transforming a biomarker into a useful clinical diagnostic test is a complex process, which starts with identification, proceeds through validation, but also requires extensive performance testing metrics (i.e., sensitivity, specificity, positive and negative predictive values, false positive and false negative rates, inter-test reliability and test/retest reliability). Identification can be carried out by various means (gene arrays, purification procedures, proteomics), that focus on observed changes of the marker correlated with the disease progression, either in the tissue/tumor or in a body fluid. Many of these methodologies attempt to identify markers in a non-spatial context, for example in tissue extracts, which results in a higher likelihood of obtaining false positives, which are then discovered as such through further validation methods. To avoid these problems or to validate the potential biomarkers several approaches are used including the development of specific antibodies, using protein microarrays or including more refined techniques to include tissue laser dissection. The process is long, arduous and lacks predictive power. Additionally, these methods often require large amounts of material, such as would occur in studies of tumour tissues. Ideally, the direct detection of a protein within spatial context would provide the best chances of rapidly identifying a potential and useable biomarker. One of the most powerful mass spectrometry applications known to date, MALDI mass spectrometry imaging (MALDI-MSI) <sup>1</sup> does just that. This technology is a major new alternative that combines both biomarker identification and validation in a single step<sup>1, 2</sup>. It has recently successfully been used for *in situ* tracking of biomarkers, as predictors of cancer aggressiveness, and for improved therapeutic strategies<sup>1,3-11</sup>

## 2. MALDI Mass Spectrometry Imaging (MALDI-MSI)

Over these past ten years, important technical improvements in mass spectrometry instrumentation together with the growing importance of this method for compound identification had lead to the development of *direct analysis* of tissue samples. Mass spectrometry has become an analytical tool allowing identification of compounds directly from tissues without any extraction or separation and adding the essential and time saving spatial resolution to the analysis. Furthermore, in a single experiment, molecular information on hundreds of chemical or biological molecules can be retrieved. By automation of this method and powerful data processing, molecular maps are generated from single tissue sections. Another major advantage is the sensitivity of mass spectrometry instruments giving access to hundreds of compound molecular images after one set acquisition. Matrix-assisted laser desorption/ionization (MALDI) ion sources are well suited for this application as they can provide data on a range of biomolecular families ranging from small molecule drugs, peptides, proteins, oligonucleotides, sugars or lipids with a spatial resolution that approaches near cellular resolution. MALDI-imaging mass spectrometry (MALDI-MSI) was first introduced by Caprioli and coll.<sup>12</sup> but major improvements have been developed in by other groups seeking to improve sample preparations, instrumentation, image spatial resolution, as well as develop new fields of applications<sup>2,12-14</sup>. For example, MALDI-MSI technology has been used for biomarkers hunting, drug biodistribution tissue interactions in drug discovery as well as for the molecular diagnosis through biopsy analyses in pathology. The translational nature of this technology provides unique challenges and as yet unimagined opportunities that promise to transform the way disease is detected, treated, and managed.

Rather than focusing on genetic alterations that may lead to a particular disease, it is emerging that changes in protein expression patterns are the most accurate way to identify diseases in their early stages and to determine the most effective course of treatment. Indeed, genome sequences fails to provide certainty for post-translational modification events such as glycosylation, phosphorylation, acylation or partial proteolysis. One of the most common objectives in proteomics is the study of protein expression patterns (e.g., protein profiling) associated with diseases. Pathologies that cause changes in signal transduction pathways generally result in changes in specific cell phenotypes. Using MALDI-MSI in this context does not have knowledge prerequisite of the studied system due to the non-targeted nature of the analysis. Such data leads to the establishment of a classification of cell phenotypic changes at the molecular level and in this way can provide a better understanding of pathologies, can lead to new diagnostic biomarkers or even new therapeutic targets. The capacity of generating multidimensional pictures with a spatial resolution that can approach the cellular level, allows monitoring, in the same analysis, of the localization of drugs compounds and the changes in biomarkers expression<sup>2</sup>.

In the context of the present discussion, there is a single clear advantage of MALDI-MSI, that is the spatial localization of identified compounds, that tremendously increases the predictive potential of which markers are most likely to be successful at the clinical level.

There are additional advantages to the MALDI-MSI approach for biomarker hunting. MALDI ion sources can identify a wide range of biomolecular families including small molecule drugs, peptides, proteins, sugars or lipids with a spatial resolutions that approaches the cellular level. Due to its high data acquisition, MALDI-MSI can permit the establishment of a classification of cell phenotypic changes at the molecular level, which can

be used to complement histology techniques. The correlation between molecular images obtained by MALDI-MSI and the ones obtained by pathologists using classical histocytochemistry can be inclusive of all grades, stages, cancer types, and cell types. However, differently from classic histocytochemistry, MALDI-MSI allows identification at the molecular level, in each cell type. Combined with powerful multivariate analyses like the hierarchical classification and principal component analyses (PCA)<sup>5</sup>, it is possible to identify biomarkers present in carcinoma region from one in a stromal area, from those in an interstitial region. Therefore, in a single analysis we can access multiple biomarkers present in a region of interest, characterize them *in situ*, without any tissue extraction. In regards to cancer tissues, which most often are high heterogeneous, the combination of MALDI-MSI and multivariate analyses are the most powerful and suited tools developed to date. Consequently, we propose that biomarkers uncovered using MALDI-MSI will be more clinically useful than those uncovered by standard methods, such as gene arrays or tissue extraction/fractionation, which lack spatial context. Other predictions also follow from this logic, as biomarkers are known for their potential roles in a disease’s etiology. It therefore follows that they may well represent important therapeutic targets.

In the present chapter, we will focus on a single example, namely in ovarian, cancer, to establish the usefulness of MALDI MSI technology for tracking and validating new biomarkers.

3. Ovarian cancer

Ovarian cancer is the fourth leading cause of cancer death among women in Europe and the United States. Among biomarkers, cancer-antigen 125 (CA-125) is the most studied. CA-125 has a sensitivity of 80% and a specificity of 97% in epithelial cancer (stage III or IV, (Table 1, Figure 1)). However, its sensitivity is around 30% in stage I cancer, its increase is linked to

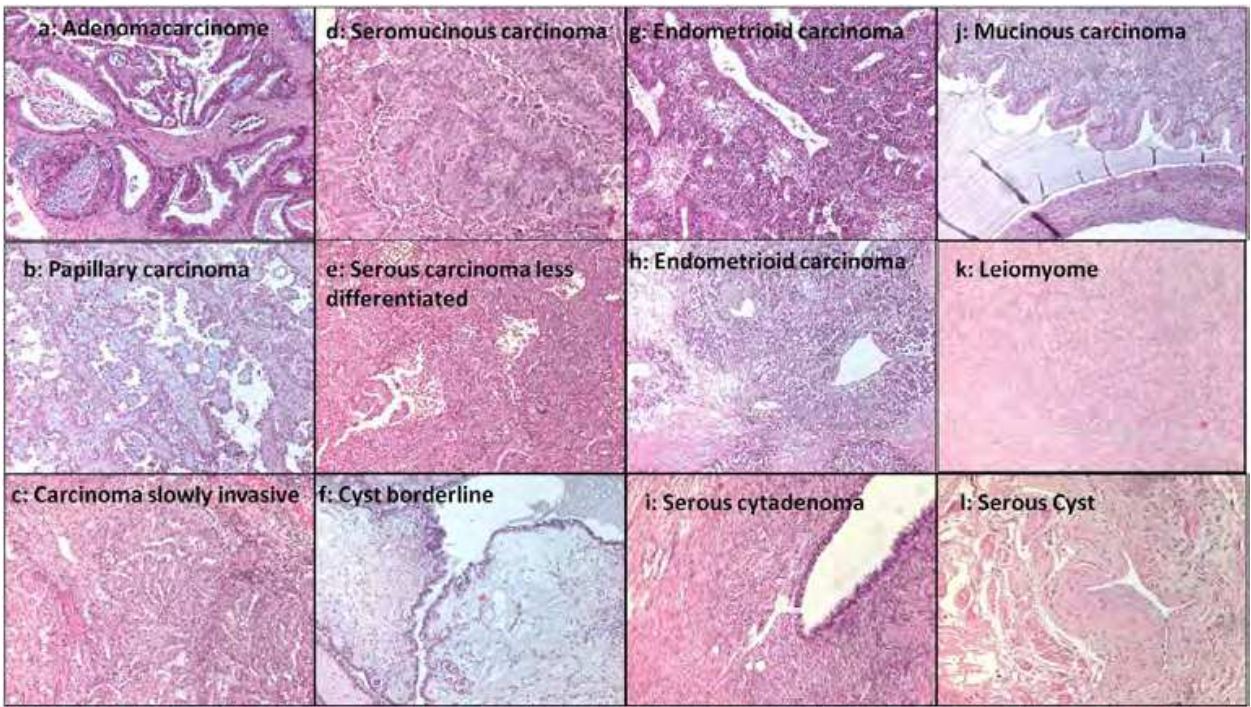


Fig. 1. Hematoxylin eosin staining of different type’s of ovarian cancer and benign tissues.



several physiological phenomena and it is also detected in benign situations<sup>15</sup>. CA-125 is particularly useful for at-risk population diagnosis and following disease progression during therapeutic treatment. In this context, CA-125 is insufficient as a single biomarker for ovarian cancer diagnosis. The alternative is to identify additional biomarkers, using a proteomic strategy, that can better establish the diagnosis and prognosis in regards to the tumor stage (Table 2)<sup>16-24</sup>. Presently, two strategies have been established. First has been the attempt to identify ovarian cancer markers in plasma SELDI-TOF profiling or chromatography coupled to mass spectrometry<sup>17,25-30</sup>. Second, has been the development of classic proteomic strategies using comparative 2D-gels and mass spectrometry<sup>24,31-33</sup> or using genomic methodologies (Table 3).

FIGO STAGE				
TNM	FIGO	Description	Prevalence	% of survey after 5 years treatment
TX		Non evaluable primitive Tumor		
T0		No ovarian lesion		
T1	Stage I	Tumor limited to the ovary	25%	
T1a	Ia	Unilateral, capsule intact, no ascite		80%
T1b	Ib	Bilateral, capsules intact, no ascite		75%
T1c	Ic	Limited to ovaries but presence of ascite		70%
T2	Stage II	Tumor limited to the pelvis	11%	
T2a	Ila	Extensions limited to the uterus and the ducts		60%
T2b	Iib	Extensions to the other pelvic issues		65%
T2c	Iic	Extensions to the other pelvic issues with ascites		65%
T3	Stage III	Tumor limited to the abdomen	47%	
T3a	IIla	Peritoneal microscopic extension		40%
T3b	IIlb	Peritoneal implants less than 2 cm		25%
T3c	IIlc-p	Peritoneal implants more than 2cm		20%
N1	IIlc-g	Lymphatic ganglia colonized: sub-pelvis, para-aortic and inguinal		<10%
M1	Stage IV	Metastasis at distance and pleural effusion	17%	<10%

Table 1. Grading systems of epithelial carcinoma. FIGO 1995: Universal grading nomenclature.

Marker Name	Genomic	Proteomic	MALDI Imaging
Mesothelin-MUC16	99		
STAT3	99		
LPAAT-β (Lysophosphatidic acid acetyl transferase beta)	100		
Inhibin	101		
Kallikrein Family (9, 11, 13, 14)	102		5
Tu M2-PK	103		
c-MET	104-106		
MMP-2, MMP-9, MT1-MPP: Matrix metalloproteinase	107-109		5
EphA2	110-112		
PDEF (prostate-derived Ets factor)	63, 113		
IL-13	114		
MIF (Macrophage inhibiting factor)	63, 113		
NGAL (Neutrophil gelatinase-associated lipocalin)	115		5
CD46	116-118		
RCAS 1 (Receptor-binding cancer antigen expressed on SiSo cells)	64, 119		
Annexin 3	120		
Destrin	121, 122		
Cofilin-1		123	
GSTO1-1		121, 122	
IDHc		121, 122	
FK506 binding protein		124	
Leptin		125, 126	
Osteopontin		120	
insulin-like growth factor-II		127	
Prolactin		128	
78 kDa glucose-regulated protein		129	
Calreticulin		129	
Endoplasmic reticulum protein ERp29		129	
Endoplasmin		129	
Protein disulfideisomerase A3		129	
Actin, cytoplasmic 1		129	
Actin, cytoplasmic 2		129	
Macrophage capping protein		129	
Tropomyosin alpha 3 chain, alpha-4 chain		129	

Marker Name	Genomic	Proteomic	MALDI Imaging
Vimentin		129	5
Collagen alpha 1(VI) chain		129	
Dihydrolipoyllysineresidue succinyltransferase component of 2-oxoglutarate dehydrogenase		129	
Pyruvate dehydrogenase E1 component beta		129	
Superoxide dismutase [Cu-Zn]		129	
Chromobox protein homologue 5		129	
Lamin B1, B2		129	
14-3-3 protein		129	
Cathepsin B		129	
Heterogeneous nuclear ribonucleoprotein K		129	
Nucleophosmin		129	
Peroxiredoxin 2		129	
Prohibitin		129	
Receptor tyrosine-protein kinase erbB-3		129	
Fibrinogen gamma chain		129	
Splicing factor, arginine/serine-rich 5		129	
Elongation factor 1-beta		129	
Lysosomal protective protein		129	
Hemoglobin beta subunit		129	
Transitional endoplasmic reticulum ATPase		129	
Serum albumin		129	
Protein KIAA0586		129	
Similar to testis expressed sequence 13A		129	
SNRPF protein		129	
Fibrinogen gamma chain		129	
Transitional endoplasmic reticulum ATPase		129	
Heat shock 70 kDa protein 1, 60K protein		129	
Heterogeneous nuclear ribonucleoprotein K		129	
Keratin, type I cytoskeletal 7, 9, 18, 19 ?		129	5
Adenylosuccinate lyase		129	
Peroxiredoxin 2		129	
Glutathione S-transferase P		129	
Ras-related protein Rab-7		129	
Prohibitin		129	

Marker Name	Genomic	Proteomic	MALDI Imaging
Cathepsin B		129	
Heterogeneous nuclear ribonucleoprotein K		129	
Tumor protein D54		129	
Rho GDPdissociation inhibitor 1		129	
Annexin A2		129	
ATP synthase beta chain		129	
Heterogeneous nuclear ribonucleoprotein K		129	
Actin, cytoplasmic 1		129	
Heterogeneous nuclear ribonucleoprotein A/B		129	
Immunoprotease activator fragment 11 S			7
Mucin-9			5
Tetranectic			5
Urokinase plasminogen activator			5
Orosomucoid			5
S100-A2			5
S100-A11			5
Apolipoprotein A1			5
Transgelin			5
Prolargin			5
Lumican Precursor			5
Siderophilin			5
Alpha 1 antiprotease			5
Phosphatidyl Ethanolamine Binding Protein			5
Hemopexin			5
Profilin -1			5

Table 2. Biomarkers identified by genomic, classical proteomic or SELDI approaches.

Protein	Patient 1	Patient 2	Patient 3	Virus
VE2-HPV36	X		X	HPV
VE2-HPV39		X		HPV
VE6-HPV56			X	HPV
UL16-EBV	X	X	X	EBV
UL11-EBV	X	X	X	EBV

Table 3. Viral protein detected in patients tumor by NanoLC-IT MS/MS.



Our group has taken a different approach, attempting direct tissue analysis and peptide profiling followed by MALDI profiling and imaging<sup>1,3-11,34-38</sup>. Ovarian carcinomas (stages III and IV) and benign ovaries were directly analyzed by MALDI-TOF-MS after three different treatments for proteins, high hydrophobic proteins and peptides extraction. Hierarchical clustering based on principal component analysis (PCA) as well as PCA-Symbolic Discriminant Analysis (SDA)<sup>34</sup> was carried out using ClinProTools software to classify tissues. Principal component analysis was used in the unsupervised mode to differentiate tumors and healthy spectra based on their proteomic composition as determined by MALDI-MSI. These characterized proteins can be grouped into functional categories such as cell proliferation, immune response modulation, signaling to the cytoskeleton, and tumor progression<sup>1,5,7,37</sup>.

#### 4. Proteins involved in immune response modulation

Recent studies have shown that ovarian cancer-associated ascites may provide an immunosuppressive environment<sup>39</sup> (**Figure 2**). A high CD4/CD8 ratio, which may indicate the presence of regulatory T-cells, is associated with poor outcomes. Recently, Clarke *et al.*<sup>40</sup> have validated in a cohort of 500 ovarian cancer patients that the presence of intraepithelial CD8+T-cells correlates with improved clinical outcomes for all stages of ovarian cancer. Curiel *et al.* demonstrated in 104 ovarian cancer patients that CD4+CD25+FoxP3+ Tregs suppress tumor-specific T-cell immunity and contribute to growth of the tumor *in vivo*<sup>41</sup>. These data point to a mechanism of immune suppression in ovarian cancer either by over-expression of Tregs or by the tumor itself by escaping the immune response by molecular mimicry or by escaping immunosurveillance<sup>42,43</sup>. Additional evidence has reinforced the involvement of Tregs in ovarian cancer. CCL22, a protein secreted by dendritic cells and macrophages, highly expressed in tumor ascites is known to have a role in Treg cell migration in tumors<sup>41</sup>. Over-expression of the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) has also been demonstrated in ovarian cancer<sup>44-47</sup>. IDO suppresses the proliferation of effector T cells or natural killer cells and their killer functions<sup>45,48</sup>. In ovarian cancer, high IDO expression in tumor cells was correlated with a reduced number of tumor-infiltrating lymphocytes<sup>44</sup>. Reduced IL-2 and elevated TGF- $\beta$  and IL-10 levels favor induced Tregs<sup>49</sup>. On the other hand, tumor cells escape the immune response by inducing peripheral mature DCs to induce IL-10 CCR7+CD45RO+CD8+Tregs. Primary suppressive CCR7+CD45RO+CD8+ T cells are found in the tumor environment of patients with ovarian cancer<sup>50</sup>. Another way that tumor cells escape immunosurveillance is through the expression of Human Leukocyte antigen (HLA-G)<sup>51,52,53</sup> (Sheu, 2007 #5782). Recent studies have shown that the expression of HLA-G was detected in 22/33 (66.7%) primary tumor tissues, but was absent in normal ovarian tissues ( $P < 0.01$ ). Cytotoxicity studies showed that HLA-G expression dramatically inhibits cell lysis by NK-92 cells ( $P < 0.01$ ), which could be restored by the anti-HLA-G conformational mAb 87G ( $P < 0.01$ ). HLGA-G5 type has been detected in tumor and soluble form of HLA-G in ascites<sup>54,55</sup> and in the blood of patients<sup>56</sup>. HLA-G seems to be implicated in the immune response modulation through NKT cell inhibition<sup>57</sup>. In the tumor cells expressing a B7 costimulatory family molecule, B7H4 is known to inhibit antigen-dependent induction of T cell proliferation and activation. B7-H4 promotes the malignant transformation of epithelial cells by protecting them from apoptosis and seems to be expressed at an early stage of the tumor<sup>58-60</sup>. In the same way, tumor cells highly express the mesothelin-Mucin 16 (MUC16) which inhibits the formation of immune synapses between NK cells and ovarian tumor targets<sup>61</sup>.

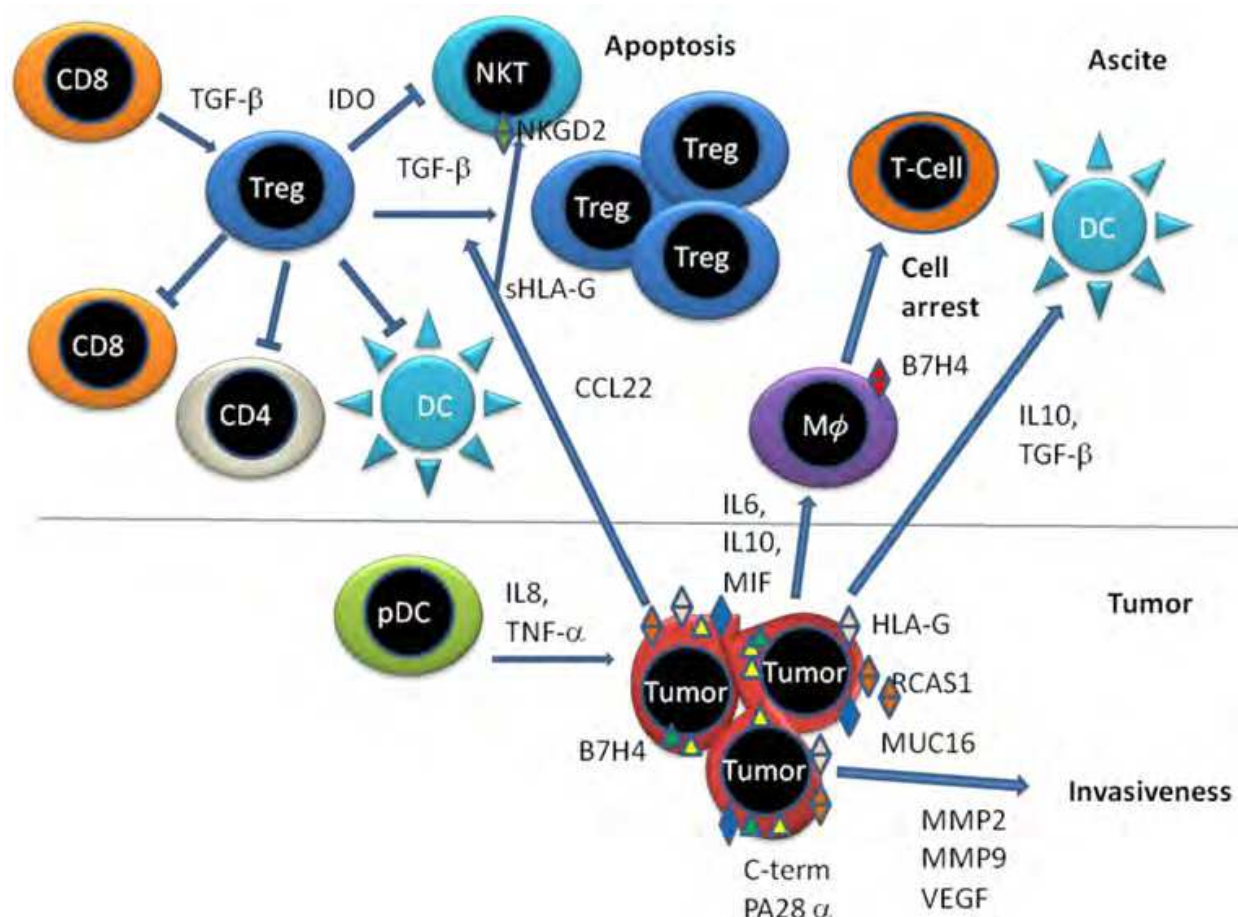


Fig. 2. Immune suppressive pathways in ovarian cancer.

Tregs are attracted to the tumor environment by CCL22 secreted by the tumor. Tregs inhibit CD4<sup>+</sup>, CD8<sup>+</sup> via direct contact or by secretion of IL10 and TGF- $\beta$ . NKT cells are inhibited by sHLA-G, MUC16, RCAS1 and MIF produced by the tumor and by IDO produced by Tregs. MIF acts through NKGD2 activation on NKT cells. The tumor environment expresses molecules that can convert functional APCs into dysfunctional ones. These dysfunctional APCs in turn stimulate Treg differentiation and expansion. The tumor produces IL6, IL8, pDCs are present in tumor environment and stimulate tumor growth by releasing TNF- $\alpha$  and IL8. IL6, IL10 are produced by Tregs and stimulate B7H4 expression in macrophages leading to T-cell cycle arrest. IL10, TGF- $\beta$  suppress APC function by inhibiting the expression of CD80, CD86.

Transcriptomic and proteomic studies performed at the level of the tumors confirm the active role of the tumor cells to escape the immune response. Transcriptomic studies have shown the over-expression of the macrophage migration inhibitory factor (MIF)<sup>62,63</sup>, Receptor-binding cancer antigen expressed on SiSo cells<sup>64</sup> known to be implicated in lymphocyte apoptosis. MIF contributes to the inhibition of antitumoral CD8<sup>+</sup> T and NK cells by down-regulation of NKGD2 (NK cell receptor NK group 2D)<sup>65</sup>.

From our MALDI-MSI studies, five factors involved in immune response modulation in mucinous tumors have been identified, namely a C-terminal fragment of the 11S immunoproteasome (Reg-alpha) (**Figure 3**), orosomucoid, apolipoprotein A1, hemopexin, and lumican which have also been detected in ascites<sup>1,5,7,36,37</sup>.

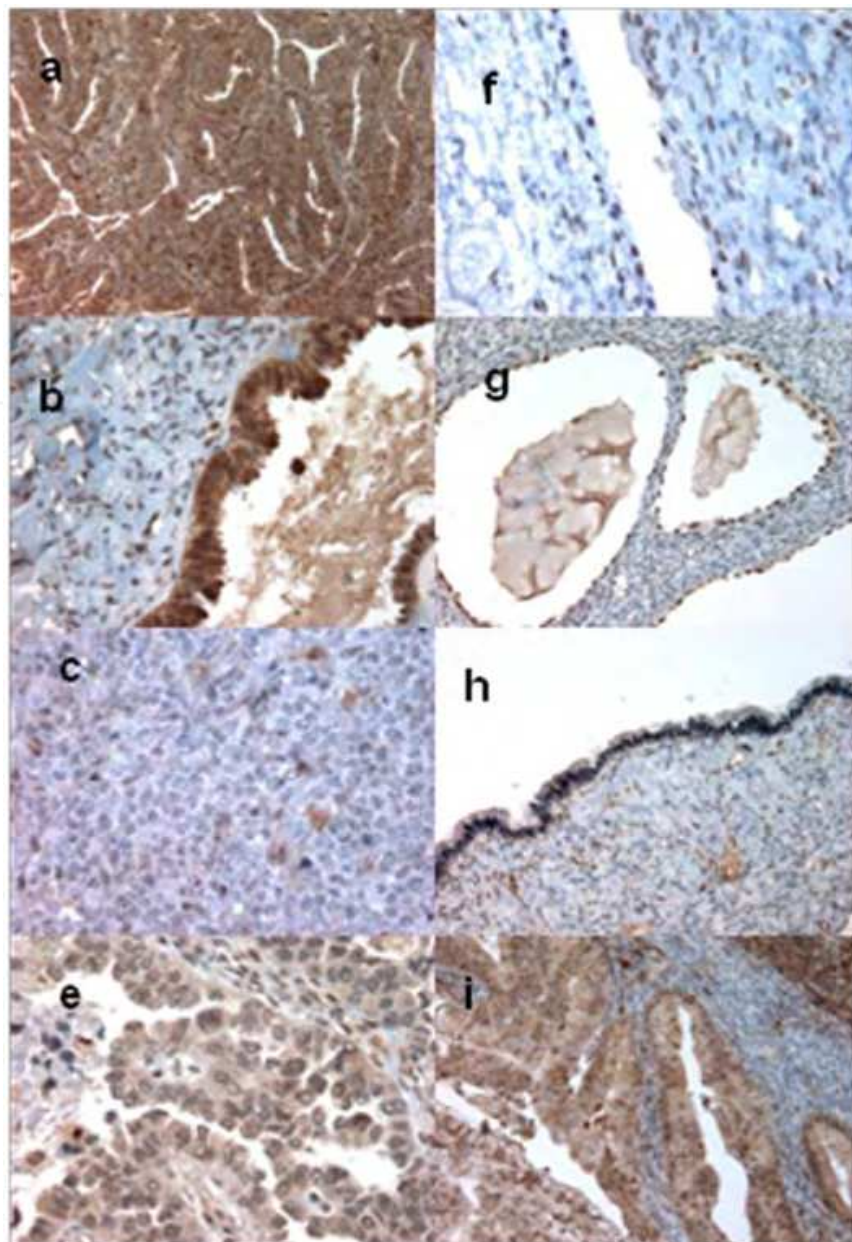


Fig. 3. Immunocytochemical studies with polyclonal antibody rose against the c-terminal part of Reg alpha.

- a. Epithelial cells of immunolabeled differentiated endometrioid carcinoma
- b. Epithelial cells of immunolabeled in carcinoma region
- c. Cytoplasmic epithelial cells immunolabeling of nondifferentiated endometrioid carcinoma.
- d. Epithelial cells of immunolabeled in clear cells adenocarcinoma (mesonephroma)
- e. Nuclear epithelial cells immunolabeling of benign tumor
- f. Nuclear epithelial cells immunolabeling of adenofibromatous tumor

PSME1 (proteasome activator complex subunit 1, 11S regulator complex [syn: PA28 alpha]) cleaved into the Reg-alpha fragment could lead to default self-antigen presentation<sup>7</sup>. PA28 is a regulatory complex associated with 20S proteasome that consists of 3 subunits: alpha, beta, and gamma<sup>66</sup>. Binding of the 11S regulator complex to the 20S proteasome does not depend



on ATP hydrolysis and unlike the 19S regulatory subunit, the 11S regulator complex does not catalyze degradation of large proteins. Rather, it is responsible for MHC-class I antigen processing,<sup>67-69</sup> which is greatly improved by interferon gamma-induced expression of the alpha and beta subunits<sup>70</sup>.

Several viral proteins that interact with these proteasome subunits have been reported, and may interfere with host anti-viral defenses, thereby contributing to cell transformation<sup>71</sup>. The manner in which they bind to the core particle via its subunits' C-terminal tails, and induce an  $\alpha$ -ring conformational change to open the 20S gate, suggests a mechanism similar to that of the 19S particle<sup>66</sup>. No role in ovarian cancer has been demonstrated for the 11 S regulator complexes. Our data demonstrate a high level of expression of PA28 in carcinomas, especially in epithelial cells at stage III/ IV but also at early stages Ia (**Figure 4**).

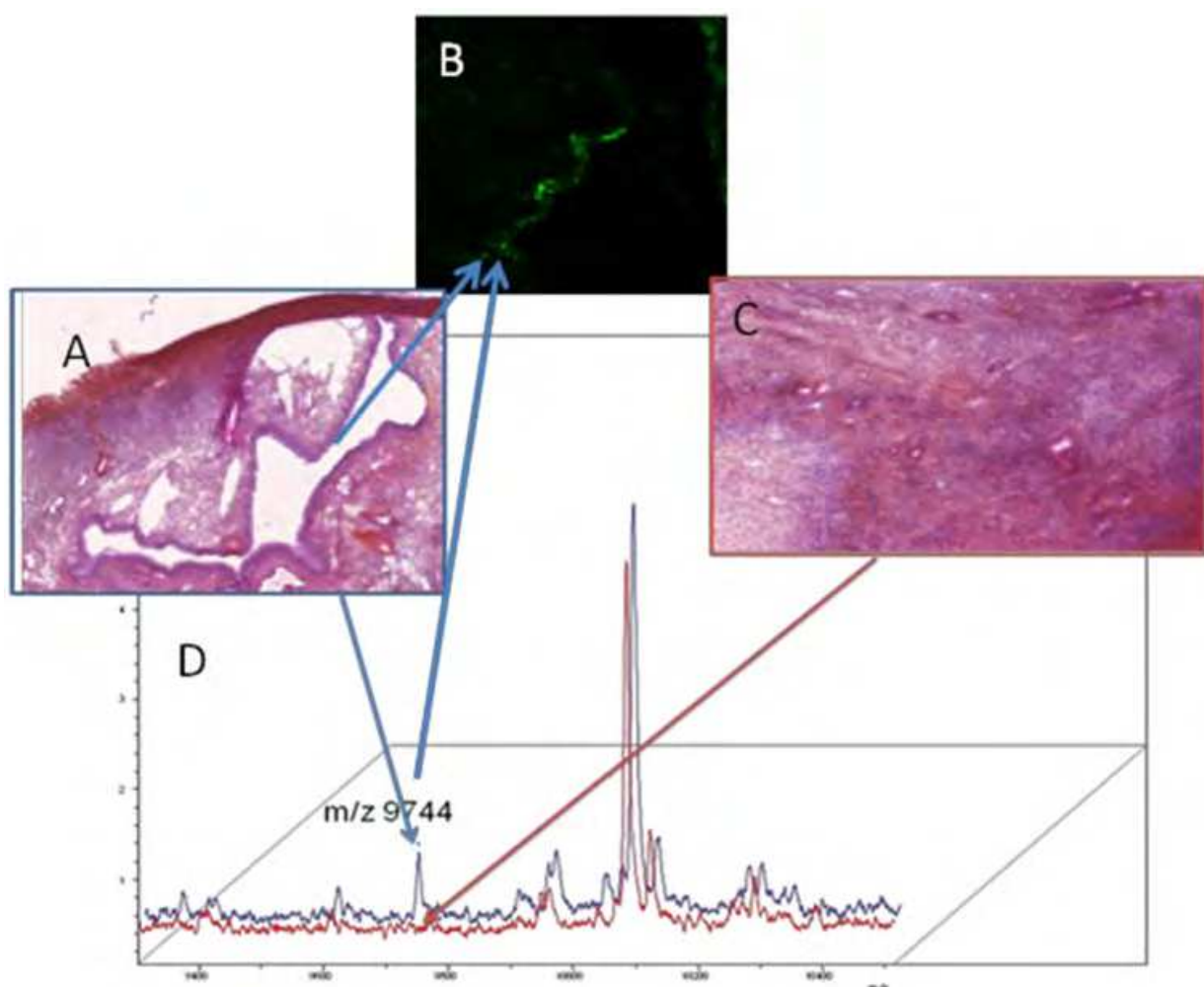


Fig. 4. C-terminal fragment of Reg alpha detection in stage Ia of ovarian cancer.

- Hematoxylin eosin staining of the carcinoma cell (acini)
- Hematoxylin eosin staining of the benign region
- Immunocytochemical studies with polyclonal antibody rose against the c-terminal part of Reg alpha
- MALDI mass spectra obtained from carcinoma cell and from the benign region. The data point out the detection of the m/z of 9744 in carcinoma region in line with the immunocytochemical data.

The PA28 activator belongs to the antigen processing machinery (APM). Its alteration by cleavage in ovarian carcinomas may be a mechanism to evade immune recognition. Such a hypothesis has already been proposed for the case of APM chaperones such as TAP, LMP2, LMP10, and tapasin in colon carcinoma, small cell lung carcinoma, and pancreatic carcinoma cell lines. In fact, IFN- $\gamma$  treatment of these carcinoma cell lines corrects the TAP, LMP, and tapasin deficiencies and enhances PA28  $\alpha$ , LMP7, calnexin and calreticulin expression, which is accompanied by increased levels of MHC class 1 antigens<sup>72</sup>. Recently, PSEM2 (proteasome activator complex subunit 2, PA28 Beta) has also been detected in ascites fluid, implicating its immune cell tolerance toward carcinoma cells and confirms the dysregulation of self-antigen processing in ovarian tumors<sup>73</sup>. Additionally, PA28 alpha seems to be a target for Epstein-Barr virus (EBV) and herpes virus (HV), as our proteomic and qPCR data indicates (**Tables 3 and 4**). Pudney and colleagues<sup>74</sup> have also shown that as EBV-infected cells move through the lytic cycle, their susceptibility to EBV-specific CD8+ T-cell recognition falls dramatically, concomitant with a reduction in transporter associated with antigen processing (TAP) function and surface human histocompatibility leukocyte antigen (HLA) class 1 expression. The implication of virus in the ethiology of ovarian cancer is also sustained by the over-expression of furin enzyme (Figure 5), which is known to be implicated in glycoprotein B cleavage through a motif R-X-K/R-R in both EBV and HV<sup>75,76</sup>.

Tumor Type	EBV (DNA copies/ng tumors)	HHV6 (DNA copies/ng tumors)
Carcinoma		
Serous adenocarcinoma	1.56	0.82
Mucous adenocarcinoma	0.37	0.10
Cytadenoma Carcinoma	0.28	1.16
Adenomacarcinoma highly infiltrated	1.14	0.37
Adenomacarcinoma clear cells	1.46	-
Benign		
Fibrous cytadenoma Benign	-	-
Fibrous cytadenoma Benign	-	-
Serous Cyst Benign	-	-
Yellow body hemorrhagic	-	-

Table 4. Viral DNA quantify by qPCR per ng of tissue.

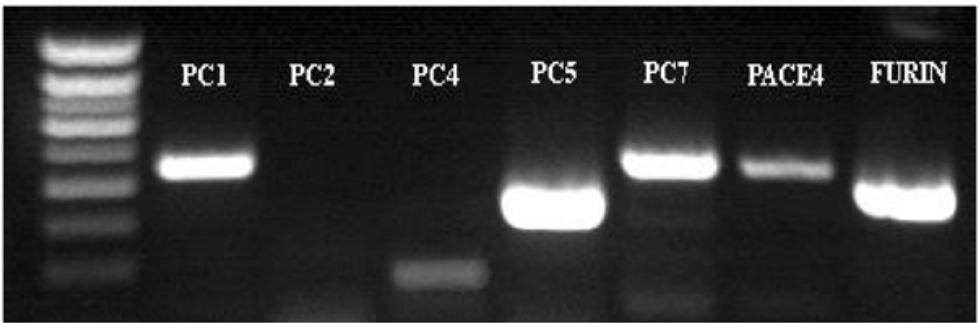


Fig. 5. RT-PCR amplification of prohormone convertase enzymes from serous stage III/IV carcinoma tissues.



Among the other four factors that might participate in the tolerance phenomenon by inhibiting immune activation, the acute phase protein, orosomucoid (ORM, also known as alpha1-acid glycoprotein or AGP), is normally increased in infection, inflammation, and cancer, and it seems to have immunosuppressive properties in ovarian carcinoma ascites through inhibition of IL-2 secretion by lymphocytes<sup>77</sup>. Similarly, apolipoprotein A1 has been detected in conjunction with transthyretin and transferrin in early-stage mucinous tumors<sup>78</sup>. ApoA-I is known to decrease expression of surface molecules such as CD1a, CD80, CD86, and HLA-DR in dendritic cells, and it stimulates the production of IL-10<sup>79</sup>.

Interestingly, hemopexin has recently been demonstrated to reduce TNF  $\alpha$  and IL-6 from macrophages during inflammation and limits TLR4 and TLR2 agonist-induced macrophage cytokine production<sup>80</sup>. We demonstrate that in SKOV-3 epithelial ovarian carcinoma cells, all TLRs are over-expressed with the exception of TLR9 and TLR10 (**Figure 6**). This is in line with the over-expression of lumican, which is a small LRR proteoglycan in the extracellular matrix. Along with other proteoglycans, such as decorin, biglycan, and prolargin, lumican is known to be over-expressed in breast cancer and to play a role in tumor progression<sup>81,82</sup>. However, as demonstrated for biglycan, which interacts with TLR2/4 on macrophages<sup>83,84</sup>, we speculate that lumican is also involved in the activation of the inflammasome through TLR2/4 interaction. The activation of all danger-sensing receptors in carcinoma cells can be explained through the regulation of inflammation by carcinoma cells to facilitate tumor progression. In a sense, this implies that ovarian cancer cells act as “parasites” and use molecular mimicry<sup>85</sup> to escape the immune response, as they produce immunosuppressors to achieve tolerance (**Figure 7**).

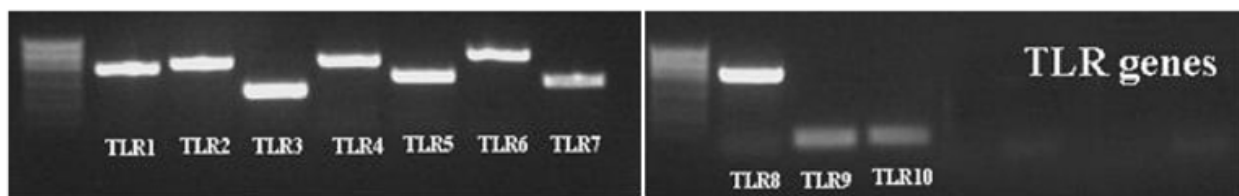


Fig. 6. RT-PCR amplification of Toll-like receptors from serous stage III/IV carcinoma tissues.

## 5. Proteins associated with cell proliferation

The S100 protein family has been previously detected in aggressive ovarian tumors<sup>30</sup>. In our study, we detected S100 A11 and S100 A12 proteins. S100 A11 has been detected in ovarian ascites<sup>73</sup>. S100 A11 (or calgizzarin) is known to regulate cell growth by inhibiting DNA synthesis<sup>86,87</sup>. S100 A12 is known to contribute to leukocyte migration in chronic inflammatory responses<sup>88</sup>. In conjunction with S100 proteins and cytoskeleton modifying proteins, we also detected expression of oviduct-specific glycoprotein (OGP, Mucin-9), a marker of normal oviductal epithelium. Our data are supportive of Woo and associates, who found that OGP is a tubal differentiation marker and may indicate early events in ovarian carcinogenesis. These data also support the hypothesis of oviduct ascini as the origin of serous ovarian carcinoma.

From immune components, stromal cell-derived factor-1 (SDF-1), the ligand of the CXCR4 receptor, is a CXC chemokine that induces proliferation in ovarian cancer cells by increasing the phosphorylation and activation of extracellular signal-regulated kinases (ERK)1/2, which in turn is correlated to epidermal growth factor (EGF) receptor transactivation.

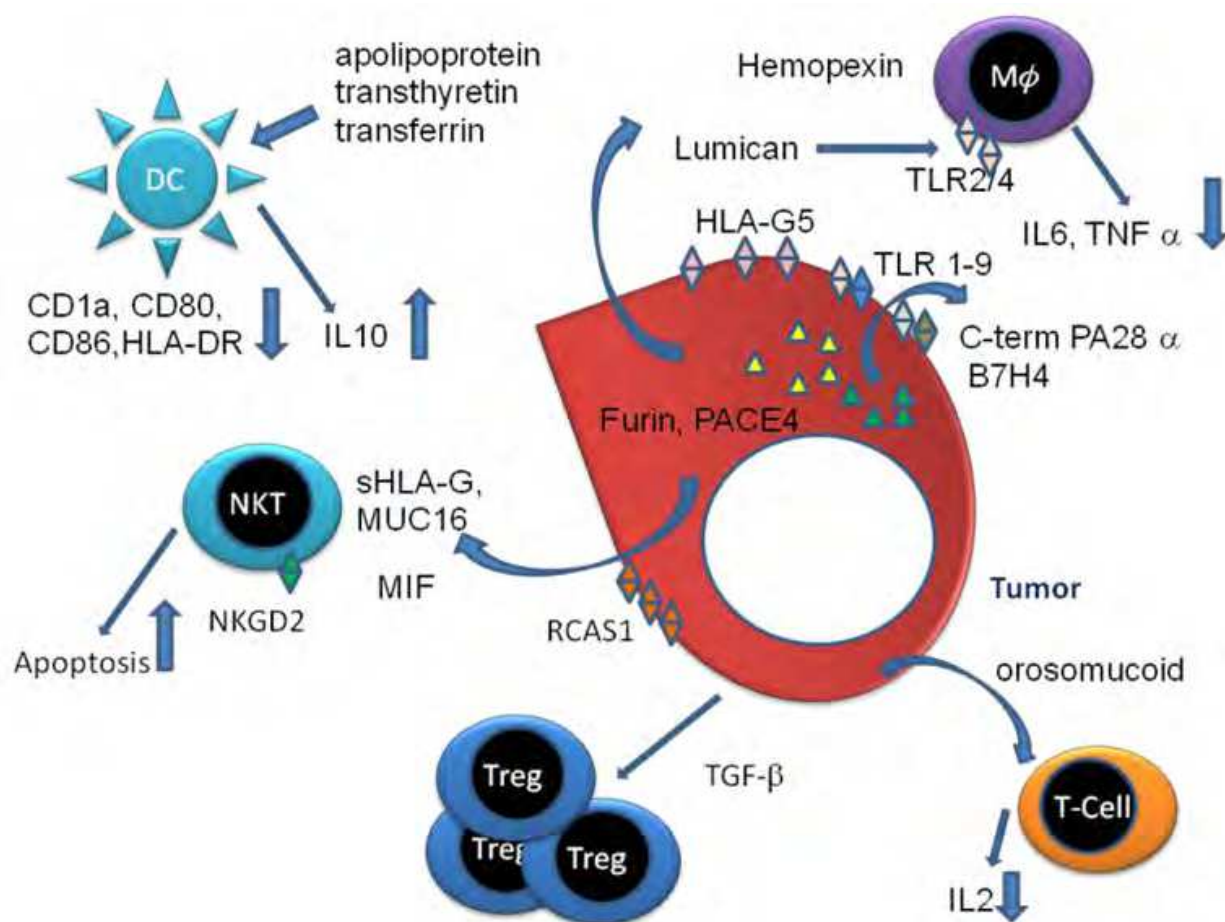


Fig. 7. Tumor cell factors production for escaping immune response.

Apolipoprotein A1 has been detected in conjunction with transthyretin and transferrin in early-stage mucinous tumors. Lumican, which is a small LRR proteoglycan in the extracellular matrix is known to be overexpressed in breast cancer and to play a role in tumor progression. ApoA-I is known to decrease expression of surface molecules such as CD1a, CD80, CD86, and HLA-DR in dendritic cells, and it stimulates the production of IL-10. Hemopexin has recently been demonstrated to reduce TNF and IL-6 from macrophages during inflammation, and it limits TLR4 and TLR2 agonist-induced macrophage cytokine production. Orosomucoid have immunosuppressive properties in ovarian carcinoma ascites through inhibition of IL-2 secretion by lymphocytes. The tumor environment expresses molecules that can convert functional APCs into dysfunctional ones. These dysfunctional APCs in turn stimulate Treg differentiation and expansion. The tumor produces IL6, IL8, MUC18, MIF, RCAS1, sHLA-G exerting negative effects on the T-Cells. PA28 activator belongs to the antigen processing machinery (APM). Its alteration by cleavage by (furin, PACE4) in ovarian carcinomas participates in a mechanism to evade immune recognition.

Similarly, TGF- $\beta$  produced by Treg cells stimulates tumor cell proliferation and increases matrix metalloproteinase's (MMP) production and enhances invasiveness of ovarian cancer cells<sup>89-93</sup>. In ovarian cancer, IL7 acts as a growth factor, like in breast cancer, and has been found in ascites and plasma<sup>39,94,95</sup>. pDcs are also present in tumor environment and stimulate tumor growth by releasing TNF- $\alpha$  and IL8. The sum of these data reflect that cytokines exert pleiotropic effects in ovarian cancer and exert a major role in tumor proliferation.

## 6. Signaling to the cytoskeleton

Several candidate proteins, including profilin-1, cofilin-1, vimentin, and cytokeratin 19 are involved in the intracellular signaling to the cytoskeleton. Changes in cell phenotype, such as the conversion of epithelial cells to mesenchymal cells, are integral not only to embryonic development but also to cancer invasion and metastasis. Cells undergoing the epithelial-mesenchymal transition (EMT) lose their epithelial morphology, reorganize their cytoskeleton, and acquire a motile phenotype through the up- and down-regulation of several molecules, including tight and adherent junction proteins and mesenchymal markers. TGF- $\beta$  has been described to induce EMT in ovarian adenocarcinoma cells<sup>96</sup>. (Figure 8A)

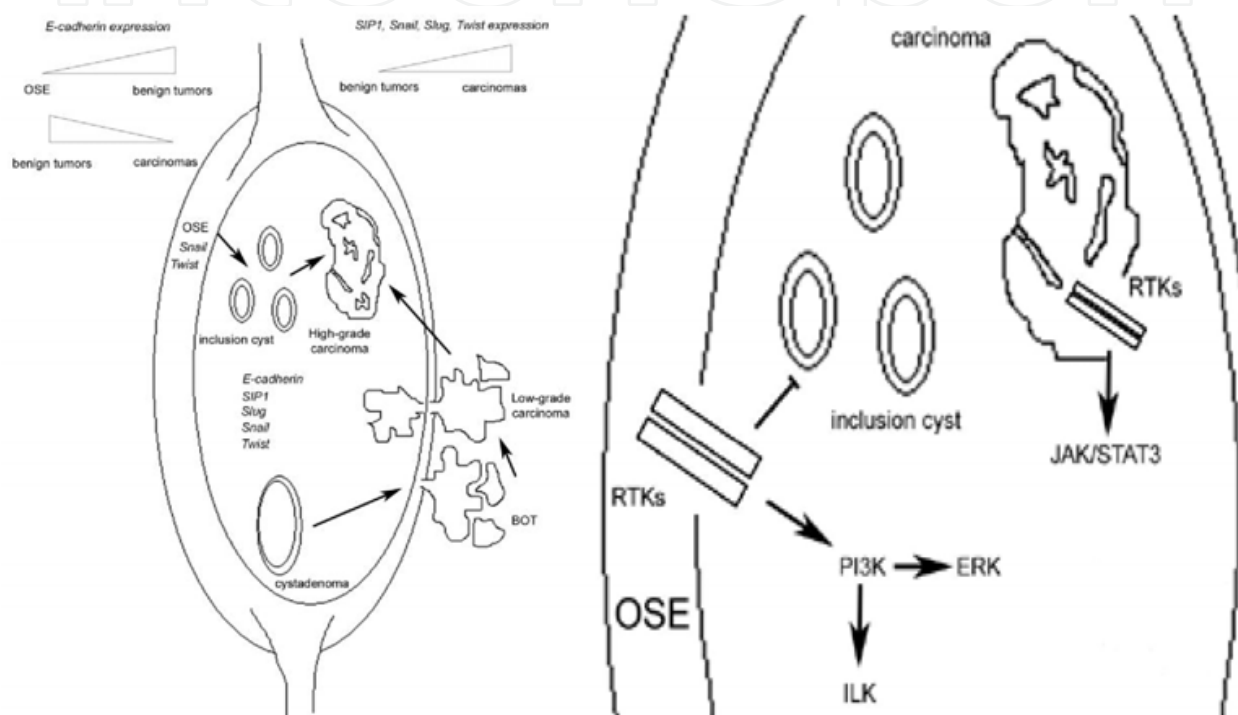


Figure 8.

**A:** Schematic illustration of E-cadherin, SIP1, Snail, Slug and Twist during ovarian progression. In this model, epithelial ovarian tumors have been classified into two broad categories: type I tumors including low-grade serous carcinomas, mucinous, endometrioid, and clear cells carcinomas seem to develop from their precursors, namely borderline ovarian tumors (BOTs), in a stepwise manner; type II including high-grade serous malignancies develop from the OSE or inclusion cysts without a common precursor. OSE cells covering the ovarian surface do not express E-cadherin but are positive for Snail and Twist expression. As depicted, E-cadherin expression changes during ovarian cancer progression showing an inverse correlation compared to SIP1, Snail, Slug and Twist expression<sup>93</sup>.

**B:** A simplified overview of signalling network regulating EGF-induced EMT. In OSE cells, activation of the EGF receptor tyrosine kinases (RTKs) by EGF results in activation of the phosphatidylinositol 3-kinase (PI3K), which activates ILK and ERK pathways. EGF treated OSE cells display a molecular signature characteristic of EMT and are less likely to undergo a conversion in inclusion cysts. JAK/STAT3 pathway is required to induce EMT in ovarian cancer cells. Ovarian cancer cells that undergo EMT lose the expression of E-cadherin and NGAL and show an increased motility.

In the human lung adenocarcinoma cell line A549, this differentiation is accompanied by modification in the expression of several cytoskeleton proteins including  $\beta$ -actin, cofilin 1, moesin, filamin A and B, heat-shock protein beta-1, transgelin-2, S100 A11, and calpactin. These changes presumably increase migratory and invasive abilities<sup>97</sup>. We recently demonstrated that treatment of the ovarian cancer cell line SKOV-3 with TGF- $\beta$  (10 ng/mL, 24 h) increases the expression of cofilin and profilin-1 at mRNA and protein level, and modifies its cytoskeletal organization as assessed by confocal microscopy analysis<sup>98</sup>. After binding to its receptor, TGF- $\beta$  stimulates the reorganization of the actin cytoskeleton and triggers the formation of stress fibers and cellular protrusions<sup>98</sup> (**Figure 8B**).

## 7. Conclusion

A decade after its inception, MALDI-MSI has become a unique technique in the proteomic arsenal for biomarker hunting in a variety of diseases. In this report, we consider the contributions of MALDI-MSI and profiling technologies to clinical studies compared to the ones obtained by genomic and classical proteomic. A stringent analysis of the list of potential biomarkers detected by three technologies reflects little convergence between genomic and proteomic (classical and MALDI MSI) investigations by biomarker comparison. However, when integrating these biomarkers in biological process, a real convergence can be shown. What emerges is picture showing how tumors modulate and escape the immune response. In this context, several biomarkers can be detected. Similarly, immune tolerance forced by the tumor production and interaction with the immune cells also revealed, in ascites and in plasma, some specific immune related biomarkers. In the same way, genes and proteins associated with cell proliferation, cell migration, invasiveness and EMT can be detected. The sum of these data confirm that diagnostics and treatment efficacy can be followed by the modulation of these markers. One of the most exciting finding is based on data obtained with the C-terminal fragment of Reg-alpha, suggested that self modulation mechanism developed by the tumor cells starts very early in the pathogenic process. Antibodies directed against this specific marker can be used to track early stage tumor cells. MALDI-MSI can be used to detect these antibodies in tumors and validate the therapeutic strategy.

A decade after its inception, MALDI-MSI has become a unique technique in the proteomic arsenal for biomarker hunting. At this stage of development, it is important to ask whether we can consider this technique to be sufficiently developed for routine use in a clinical setting or an indispensable technology used in translational research. In this report, we have considered the contributions of MALDI-MSI and profiling technologies for clinical studies, outlining new directions that are required to align these technologies with the objectives of clinical proteomics.

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