Katholieke Universiteit Leuven
Group Biomedical Sciences
Faculty of Medicine
Department of Oncology
Division of Gynaecologic Oncology

The evaluation of new biomarkers in Gynaecological Tumours

The emerging role of Proteomics

Toon Van Gorp



Doctoral thesis in Biomedical Sciences
Leuven, 30-03-2012

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THE EVALUATION OF NEW BIOMARKERS IN GYNAECOLOGICAL TUMOURS

THE EMERGING ROLE OF PROTEOMICS.

Toon VAN GORP

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Abbreviations

2-DE	Two Dimensional gel Electrophoresis
95%CI	95% Confidence Interval
ACN	Acetonitrile
AFP	Alpha-Fetoprotein
AUC	area under the curve
BOT	Borderline Ovarian Tumour
CA125	Cancer Antigen 125
CEA	Carcinoembryonic Antigen
CHCA	α–cyano–4–hydroxy cinnamic acid
CID	Collision Induced Dissociation
CIN	cervical intra–epithelial neoplasia
CM10	weak cation exchanger surface
Cystad	Ovarian cystadenoma or cystadeanofibroma
Da	Dalton
ECL	electrochemiluminescence
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immuno Sorbent Assay
EOC	Epithelial Ovarian Cancer
EORTC	European Organisation for Research and Treatment of Cancer
Eosis	Endometriosis
ESI	Electrospray Ionization
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
Fibr	Ovarian fibroma
FIGO	International Federation of Gynecology and Obstetrics
Funct	Functional cyst
hCG	human Chorionic Gonadotropin
H50	hydrophobic surface
HE4	Human Epididymis protein 4
HPLC	High Performance Liquid Chromatography
HRT	hormone replacement therapy
HS	Heperan Sulfate
HSP	Heat Shock Protein
Ig	Immunoglobin
IMAC30	metal-binding surface
IOTA	International Ovarian Tumour Analysis
IR	interquartile range
LC	Liquid Chromatography
LDL	Low Density Lipoprotein
LMP	Low Malignant Potential

LOO	Leave-One-Out
LSSVM	Weighted Least Squares Support Vector Machine
LVSI	lymphvascular space involvement
m/z	mass-to-charge ratio
MALDI	Matrix-Assisted Laser Desorption and Ionization
MARS	Multiple Affinity Removal System
Meta	Metastatis
MIA	Multivariate Index Assay
MMS	Multimodality screening
MRC	Medical Research Council
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	tandem mass spectrometry
NA	not applicable
NanoLC	Nano–liquid chromatography
OC	oral contraception
PGF	Placental Growth Factor
pI	isoelectric point
PI	Predictive Index
PP	Predicted Probability
Q1	first quadrupole
Q2	second quadrupole
Q3	third quadrupole
Q10	strong anion exchanger surface
RMI	Risk of Malignancy Index
ROC	receiver operator characteristic
ROMA	Risk of Ovarian Malignancy Algorithm
SEA	Sperm protein, Enterokinase and Agrin
SELDI	Surface-Enhanced Laser Desorption and Ionization
SCC	Squamous Cell Carcinoma antigen
SPA	sinapinic acid
SRM	Selected Reaction Monitoring
TFA	Trifluoroacetic acid
Thec	Ovarian thecoma
TOF	Time-Of-Flight
UKCTOCS	UK collaborative trial of ovarian cancer screening
VEGF	Vascular Endothelial Growth Factor
WAP	Wey Acidic Protein
Yrs	years

General introduction

Definition of a tumour marker

A tumour marker can be defined as a substance, released into the circulation by tumour tissue, whose detection in the serum indicates the presence of a specific type of tumour [1]. Although most of the currently used tumour markers would comply with this definition, it does not encompass the complete meaning of a tumour marker.

First, not all tumour markers are detected in serum. More and more researchers are looking for tumour markers in different kinds of body fluids, such as ascites, pleural effusion, cyst fluid, urine, saliva, mucus, etc. [3-6]. Some of these body fluids can be sampled quite easily, making tests easy to perform, less invasive and more cost-effective. Human Epididymis protein 4 (HE4), for example, is overexpressed in ovarian carcinomas and can be detected in serum by an Enzyme-Linked Immuno Sorbent Assay (ELISA). HE4 can also be detected in the urine with a similar sensitivity and could therefore be used in the diagnosis and the monitoring of response to therapy [6]. Tumour markers can even be detected in cells of the immune system such as monocytes or macrophages [7-9]. Since these mononuclear cells phagocytise apoptotic or necrotic tumour cells, this could be an early sign of tumour development.

Second, not all tumour markers are produced by tumour tissue or cancerous cell itself. The tumour micro-environment or stroma, and the immune system play an important role in tumour development by facilitating growth, angiogenesis and the development of metastases [10]. Changes in adhesion molecules, growth factors, proteases, and receptors can be observed in the stromal tissue surrounding the cancerous cells, but also in body fluids such as ascites or serum. Angiopoietins, for example, are expressed by pericytes, smooth muscle cells, and fibroblasts and are associated with tumour angiogenesis. They can be produced in response to tumour hypoxia. Angiopoietin-1 and angiopoietin-2 were significantly elevated in serum samples of patients with ovarian cancer compared with healthy controls [11]. Another example are the host-response proteins. Auto-antibodies against p53 have been detected in serum of ovarian cancer patients and as a consequence it can be used to detect ovarian cancer. It is believed that the immune system can be triggered to develop these auto-antibodies by very small amounts of mutant p53 [12]. Osteopontin is a glycophosphoprotein secreted by activated T-lymphocytes, macrophages,

and leukocytes at the site of inflammation. It initially was detected in cDNA micro-array studies and was subsequently found to be higher in patients with ovarian cancer than normal control groups [13]. It can be argued that non-tumour derived tumour markers might play a role in different types of cancer. Consequently, one may question whether these tumour markers can be used as a tumour specific biomarkers. However, these substances can act as small pieces of the puzzle, and when different - more or less tumour specific - tumour markers are combined in logistic regression models, it is quite possible that the combination of markers will act as a tumour specific marker algorithm with significant diagnostic value.

Third, not all tumour markers are upregulated. Since the start of the omics era, several down regulated markers have been discovered in the presence of a malignant tumour. Instead of using a one-by-one approach, high-throughput omics technology is able to perform a multidimensional analytical approach. Multiple samples can be analysed in a short time frame and substantial differences in protein, RNA or DNA content can be detected rapidly. This direct comparison of samples will not only detect upregulated, but also downregulated markers. Examples of downregulated markers are insulin-like growth factor 2 and leptin. Both were downregulated in ovarian cancer [12;14-16].

Fourth, proteins are not the only substances that can be used as tumour markers. Also genetic material (DNA, RNA, and mRNA) can be found in biological fluids. MicroRNAs are small non-coding pieces of RNA that can inhibit gene translation by binding to the mRNA molecules or by causing mRNA degradation [17]. MicroRNAs can also be detected in serum of ovarian cancer patients. In ovarian cancer patients upregulated as well as downregulated microRNAs have been described [17-19]. Not only transcription products are being detected in serum. Tumour DNA has been extracted from serum and peritoneal fluid of ovarian cancer patients. Tumour specific genetic alterations (loss of heterozygosity or microsatellite instability) were found in this DNA, proving that this DNA originated from the malignant tumour [20].

Fifth, diagnosis is not the only clinical use of a tumour marker. Besides diagnosis, tumour markers can also be used in screening, prognosis, tumour staging, monitoring of disease treatment and detecting recurrence. Since tumour markers can be raised in presence of different types of cancer or even benign diseases, most markers lack specificity. The use of serial measurements to monitor the course of a patient's disease seems

particularly useful. Once an abnormal tumour marker level is detected in the presence of a malignant tumour (e.g. Cancer Antigen 125 (CA125) in the presence of ovarian cancer), the marker usually correlates well with tumour mass volume; consequently, these tests can be used to determine whether a tumour is responding to therapy or whether a recurrence of the cancer has occurred following treatment [21].

Taking all these facts together a more comprehensive definition of a tumour marker would be: *Any biological substance found in abnormal amounts in body fluids or tissues in patients with cancer, used for screening, diagnosis, prognosis, staging, monitoring treatment or follow-up.*

Commercially available tumour markers in gynaecological oncology

Ovarian / tubal cancer:

CA125, also known as MUC16, is the most widely used tumour marker in ovarian cancer. Although the MUC16 gene was not cloned until 2001 [22], a monoclonal antibody was developed against the CA125 molecule as early as 1979 and was first reported in 1981 [23]. CA125 is a high molecular mass (1,000,000 Da) glycosylated transmembrane mucin, with multiple identical epitopes on the repetitive sequences that can be recognised by the monoclonal antibody OC125 [24]. CA125 is raised in approximately 80% of all epithelial ovarian cancers and 50% of stage I epithelial ovarian cancers [25;26]. It was already appreciated in the earliest publications that CA125 could elevated in numerous ovarian and non-ovarian, benign and malignant conditions, including cancers of the endometrium, lung, breast, pancreas, and colon [25;27]. The poor sensitivity and specificity for the detection of early stage cancer explains the inadequacy of CA125 as a tumour marker for screening [28]. Even as a diagnostic test, CA125 is not FDA approved due to its poor performance. CA125 seems to be more useful in monitoring patients for which it was cleared by the Food and Drug Administration (FDA) in 1987. Persistent elevation of CA125 correlates with persistent disease at second look surgery in 95% of cases and rising values of CA125 correlated with disease progression in approximately 90% of cases. CA125 is therefore used as a surrogate marker for response during treatment in trials [21]. However, CA125 has limitations. Up to 20% of ovarian cancers fail to express the marker, normalisation of CA125 does not mean that the disease is cured, and a rising CA125 does is not always precede a recurrence. A recent Medical Research Council (MRC)/European Organisation for Research and Treatment of Cancer (EORTC) trial also

refuted the use of CA125 in the follow-up of ovarian cancer patients because there was no overall survival benefit in the early detection and thus early start of second line treatment. However, a certain subset of patients might still benefit from CA125 surveillance, and CA125 surveillance is not abandoned completely [29;30].

HE4 is a novel tumour marker and was found by cDNA micro-array studies to be upregulated in ovarian cancer. HE4 belongs to the family of whey acidic four-disulfide core proteins and is a rather small 20,000-25,000 Da glycosylated protein with suspected trypsin inhibitor properties. Recently, HE4 was reported to inhibit tumour proliferation and metastasis [31]. HE4 was first identified in the epithelium of the distal epididymis and has since been reported to be expressed in several normal tissues including epithelia of respiratory and reproductive tissues. HE4 is expressed in 90% of ovarian cancers [32;33]. The use of HE4 alone was FDA approved for the monitoring of response to therapy for patients with invasive epithelial ovarian cancer. A recent study also showed that an increased pre-chemotherapy serum HE4 level was also associated with a worse progression-free and overall survival, independently from CA125 [34]. HE4 therefore also seems to be a prognostic marker.

To improve the diagnostic performance of CA125 and HE4, they were both combined in the Risk of Ovarian Malignancy Algorithm (**ROMA**). The combination of CA125 and HE4 was first evaluated in 233 patients with pelvic mass [35]. The combination resulted in a 76.4% sensitivity and 95% specificity for the differentiation between benign and malignant ovarian masses. Subsequently, a multicenter prospective study was performed, including 531 women diagnosed with a pelvic mass who underwent surgery. Patients were classified in high and low risk for ovarian with a specificity of 75.0% and a sensitivity of 92.3% for the postmenopausal patients and a specificity of 74.8% and a sensitivity of 76.5% for the premenopausal patients [36]. Based in these studies, the FDA approved ROMA as a diagnostic tool in patients with an ovarian mass. Validation studies of this ROMA algorithm are a subject of the current PhD thesis.

In 2009, **OVA1** or the Multivariate Index Assay (MIA) was approved as a triage tool for ovarian tumours. OVA1 consists out of a biomarker panel, including CA125, transthyretin (prealbumin), apolipoprotein A1, beta 2 microglobulin, and transferrin. Together with a software algorithm it calculates a risk index, range 0-10. High risk patients (premenopausal: ≥ 5.0 or postmenopausal ≥ 4.4) should be referred to a gynaecological oncologist. The OVA1 assay was recently investigated within a prospective trial [37;38]. However, the results have been criticised [39], and a recent study that

compared the OVA1 to CA125 did not show an improvement by adding the additional markers to CA125, fuelling the already existing scepticism [40].
Visintin et al [14] described a six marker blood test which was commercialized as **OvaSure** and determines leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor and CA125. They claimed that the test had a sensitivity of 95.3% and a specificity of 99.4% for the detection of ovarian cancer. However, several methodological concerns were raised [41-45], and after 4 months the test was pulled from the market. Millipore continues to market the biomarker panel for use in research, not by patients.
Other markers, such as alpha-fetoprotein (**AFP**), human chorionic gonadotropin (**hCG**), and **inhibin B** are used in diagnosis and follow-up of non-epithelial ovarian cancers: AFP and hCG for germ cell tumours and inhibin B for sex cord stromal tumours. AFP is a glycoprotein and the single most discriminating laboratory test indicative of malignant disease now available. It is rarely elevated in healthy persons, and a rise is seen in only a few disease states (liver diseases or hepatocellular carcinoma) or pregnancy. It is used in both the diagnosis and follow-up of germ cell tumours. hCG is also a glycoprotein consisting out of subunits, which are non-convalently linked. hCG is essential in managing trophoblastic germ cell tumours. Inhibin B is especially useful for granulosa cell tumours [46].

Endometrial cancer:
The significance of **CA125** in endometrial cancer is limited since the diagnosis of endometrial cancer is usually based on an endometrial biopsy. However raised CA125 levels were significantly correlated with higher stage, higher grade, increased depth of myometrial invasion, lymph node metastases, the presence of lymphvascular space involvement and progression free and overall survival [47-49]. **HE4** is more elevated in endometrial cancer compared to CA125, however the sensitivity is limited and HE4 is therefore also not useful for diagnosis [50]. HE4 does correlate with higher stage, tumour size, myometrial invasion, progression free and overall survival [51-53]. However, neither CA125, nor HE4 affect current clinical practice.

Cervical cancer:
For squamous cell cervical cancer, squamous cell carcinoma antigen (**SCC**) is the marker of choice. SCC is expressed in normal epithelium and epithelial tissues. It is a glycoprotein with a molecular weight between 45,000 and 55,000 Da. Although SCC is not suitable for screening or diagnosis of cervical cancer, serum SCC levels correlate with tumour stage, tumour size,

presence of lymph node metastases, extra-cervical spread, residual tumour after treatment, recurrent or progressive disease, and survival [54-59]. However, there is no evidence that more aggressive treatment in case of elevated pre-treatment SCC levels will improve local-regional control, disease free survival or overall survival, nor is there any evidence that earlier detection of recurrent disease influences treatment outcome or prognosis.
CEA and **CA125** have demonstrated possible utility in patients with cervical adenocarcinoma [59;60]. Both markers correlate with tumour stage, tumour volume, presence of lymph node metastases, and prognosis in this histological subtype. Again, there is no evidence that treatment of patients with an adenocarcinoma of the cervix should be adapted when elevated levels of CA125 or CEA are found.

Vulvar and vaginal cancer:
Vulvar and vaginal cancer are relatively rare and primarily local diseases that are easily diagnosed by physical examination. An effective tumour marker would benefit only the rare patient with distant but not local disease. **SCC** is only investigated in small studies and little conclusions can be drawn from these studies.

Proteomics in the discovery of tumour markers

What is proteomics?
The proteome, all proteins that are possibly present at a certain time point in a certain tissue or body fluid (e.g. serum), is characteristic for a specific examined sample and is influenced by physiological and pathological conditions of the tissue (or organism). The presence of a malignant tumour in a human body will also influence the proteome with over- or underexpression of certain proteins. These changes in protein concentrations can be detected and/or measured, and be used as a biological marker for the absence or presence of malignancy. It speaks for itself that the nature of a certain tumour (histopathological type, tumour grade, resistance for certain types therapy…) will also influence this proteome, causing a different protein profile in these changed circumstances. The proteome is dynamic and in constant flux due to a combination of variables. There are an estimated 25,000 genes in the human genome, and each gene can yield multiple protein products because of variable splicing, posttranslational modifications and the natural metabolism of proteins. It is estimated that there are between 300,000 and 10,000,000 proteins in serum or plasma [41]. The genome is in a relatively

stable condition, whereas the proteome can change rapidly from one moment to another. This makes it increasingly difficult to study the proteome. Robust techniques are needed to measure small differences in proteins. The field of proteomics has developed rapidly in recent years. Until the mid-1990s scientists studied individual or a handful of genes and proteins, with the aid of proteomics this has evolved to hundreds or thousands of proteins per analysis. The aims of proteomics may, at present, be divided into three areas: (a) large scale identification of proteins and their post-translational modifications, (b) differential expression comparisons of specific proteins in healthy and diseased states, and (c) studies of protein-protein interactions [61].

Proteomics tools

Two dimensional gel electrophoresis (2-DE)

In 2-DE the proteins are separated in two dimensions (Figure 1). First, the proteins are separated according to their isoelectric point (pI) with an immobilized pH gradient in the first dimension. In a second step the proteins are separated according to their molecular size. The gels can be stained with Coomassie brilliant blue or silver staining, or with radioactive or fluorescent labelling. The spot intensities are compared among different gels and the differentially expressed proteins can be excised from the gel. After destaining, in-gel digestion with trypsin and extraction, the mixture of peptides can be analyzed with mass spectrometry, and finally identified with peptide mass fingerprinting. The advantage of 2-DE is that hundreds to thousands of proteins can be separated in a single run, with a high level of separation (up to one single kind of protein in one spot). The disadvantages of this technique include the lack of automatisation, the need for large amounts of sample, limited reproducibility, and a smaller dynamic range than some other separation methods. Certain proteins are difficult for 2-DE to separate, including low abundant, very acidic or basic, very hydrophobic, very large or small proteins. Nevertheless, 2-DE is one of the basic techniques in proteomics.

Figure 1: Principle of 2D gel electrophoresis

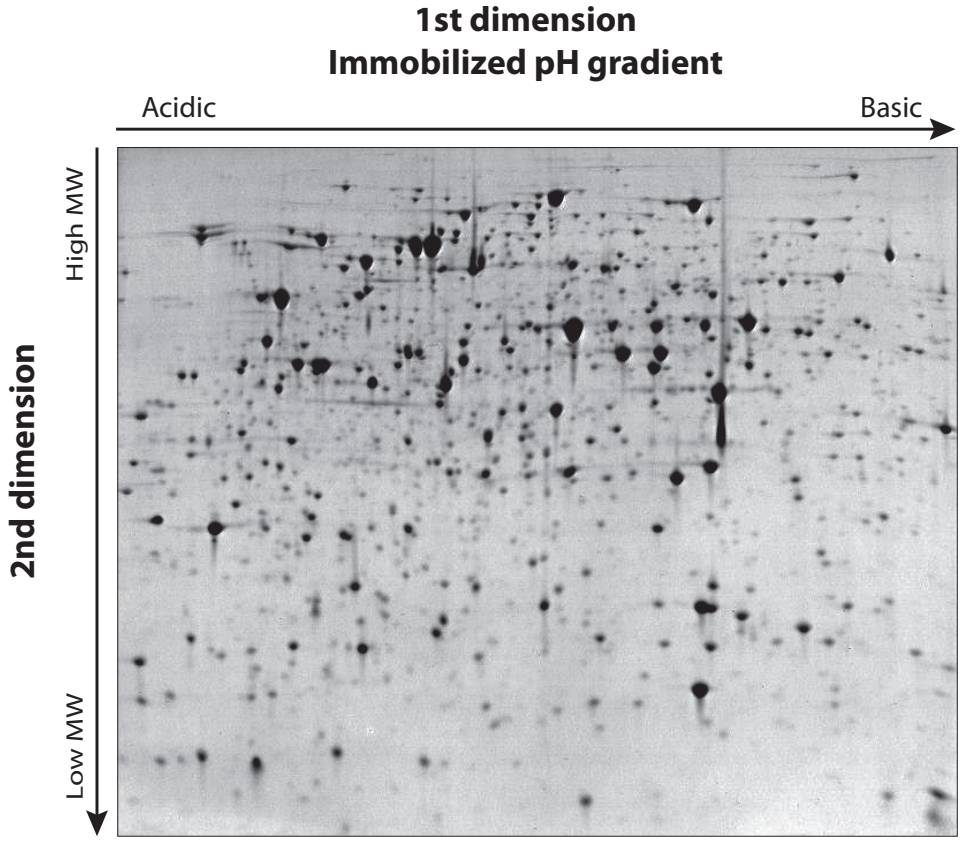
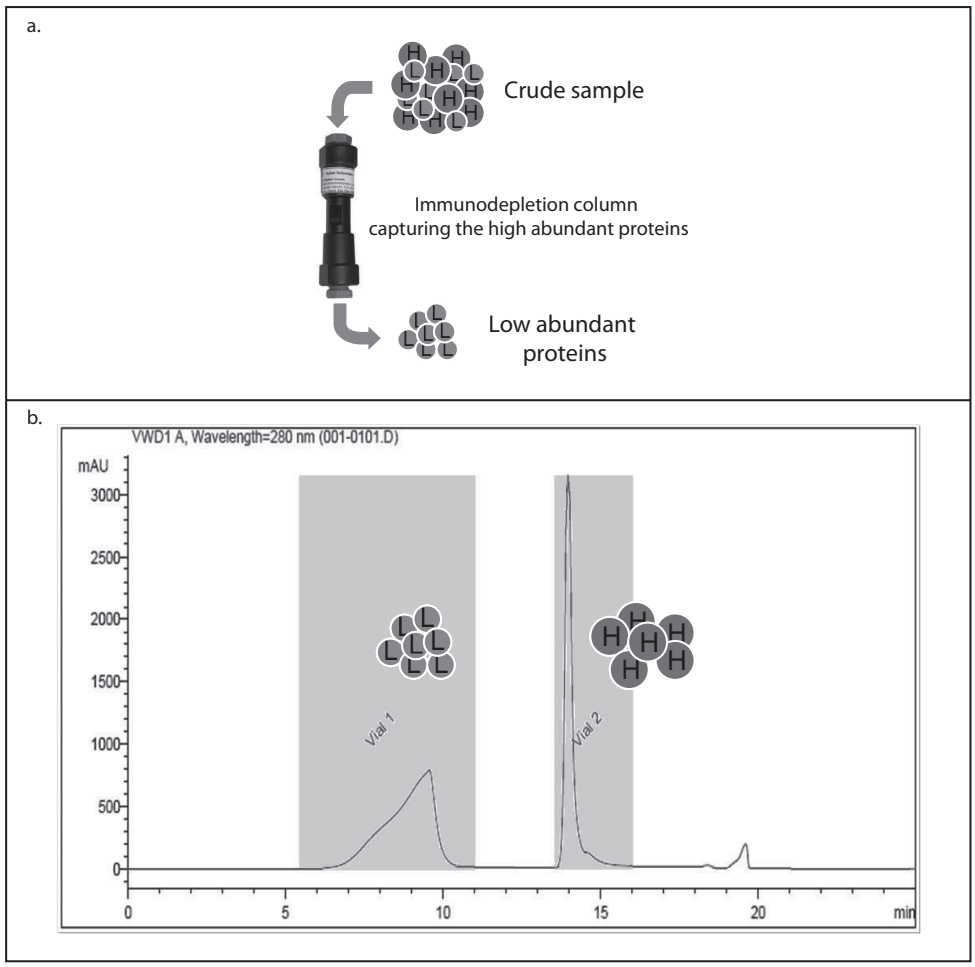


Table 1: Most often liquid chromatography methods for separating proteins and peptides

Principle of chromatographic separation	Type of chromatography
Net charge	Ion-exchange
Size and shape	Size-exclusion
Hydrophobicity	Reversed-phase
Biological function (bioaffinity)	Affinity
Carbohydrate content	Lectin
Antigenicity	Immunoaffinity
Metal binding	Immobilized metal ion affinity
Isoelectric point	Chromatofocusing

Figure 2: Example of immunoaffinity chromatography.



(a) Principle of immunodepletion: The crude sample is pushed through a column filled with immobilized antibodies against the high abundant proteins (e.g. albumin, antitrypsin, transferrin, haptoglobin, immunoglobin, etc.). The high abundant proteins will be captured and the low abundant proteins will not bind and will flow trough.
(b) Chromatogram of an immunodepletion experiment: The first peak will contain the low abundant proteins that flow through the column without binding. Afterwards the high abundant proteins can be eluted from the column and a second peak appears. The low abundant proteins (vial 1) can now be analysed without interference of the high abundant proteins (vial 2).

Liquid chromatography (LC)

Liquid chromatography is a separation technique in which a sample is injected and forced through a column that is packed with particles (the stationary phase) by a liquid at high pressure (the mobile phase). The particles in the column can have different physical and chemical properties. Therefore they will be able to retain proteins based on their intrinsic properties. By changing the mobile phase the proteins will be released from the column. A detector is needed to '*see*' the separated protein bands as they elute from the column. The sample exits the detector and can be sent to waste, or collected for further study. Table 1 illustrates the different types of liquid chromatography based on different protein or peptide properties. Liquid chromatography is also known with the acronym HPLC. Due to improvements in pumps, injectors, detectors, and columns the name liquid chromatography was first changed to *high pressure* liquid chromatography and later to *high performance* liquid chromatography, illustrating the change in performance over the last decades. Figure 2 illustrates the separation of a sample with an immunoaffinity column.

Mass spectrometry (MS).

Although various types of mass spectrometers serve in different applications, they all share the same operating fundamentals. A mass spectrometer consists of three basic modules: (1) an ion source, (2) a mass analyzer, and (3) a detector. Typically, a mass spectrometry experiment goes as follows: The sample is loaded on the mass spectrometer and vaporized. The proteins or peptides in the sample are ionized and become charged particles. The mass analyser will apply an electromagnetic field and the ions will start moving towards a detector. The detector will "count" the ions arriving at the detector at each time point. The ion signals are subsequently converted into mass spectra.

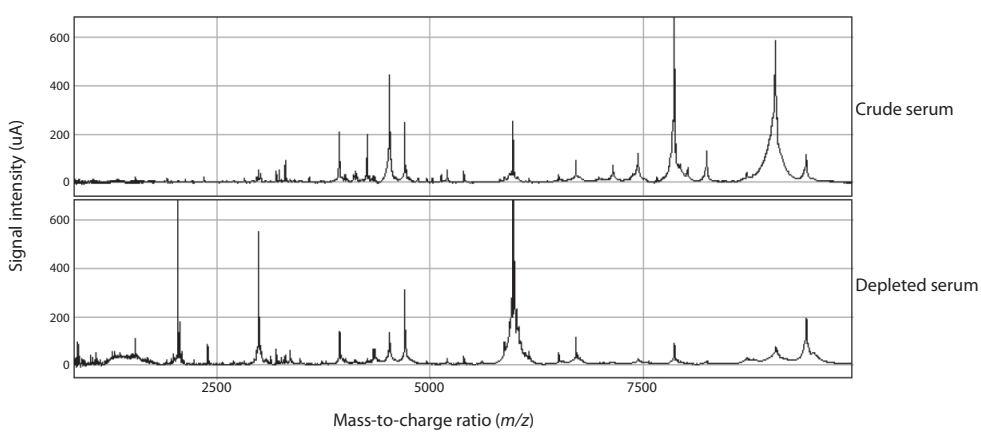
Numerous mass spectrometers exist and explaining all of them would be beyond the scope of this introduction. However, three **ionization methods** are often used in mass spectrometry experiments and need some additional attention: matrix-assisted laser desorption and ionization (MALDI), surface-enhanced laser desorption and ionization (SELDI), and electrospray ionization (ESI).

In MALDI, the protein sample is co-crystallized with a matrix (e.g. sinapinic acid) on a target plate. When a laser is fired upon the co-crystallized sample, the matrix will absorb the photons of the laser. This sudden input

of energy will cause the matrix to evaporate and ionize. The ionized matrix will in turn ionize the proteins that become charged particles. Once the proteins are ionized, they will be picked up by the mass analyzer.
SELDI is a variation of MALDI. In SELDI, the protein mixture is spotted on a surface modified with a chemical functionality. Some proteins in the sample bind to the surface, while the others are removed by washing. After washing the spotted sample, the matrix is applied to the surface and allowed to crystallize with the sample peptides. Binding to the SELDI surface acts as a separation step and the subset of proteins that bind to the surface are easier to analyze. SELDI is an approach that tries to overcome requirements for purification and separation of proteins prior to mass spectrometry analysis. Common surfaces include CM10 (weak cation exchanger), H50 (hydrophobic surface), IMAC30 (metal-binding surface), and Q10 (strong anion exchanger).
In ESI, the sample is dissolved in a polar solvent, pumped into a capillary where it receives a high voltage (between 2000 and 4000 V). The liquid aerosolizes as it exits the capillary at atmospheric pressure. The droplets dissolve and shed ions that subsequently flow into the mass analyzer.

Although there are different **mass analyzers**, time-of-flight (TOF) analyzers are certainly the simplest and most often used. After being ionized, an electric field will be applied to the ions and these ions will travel through a vacuum drift tube. The ions will separate according to their mass (lighter ions drift more quickly, heavier ions more slowly) and their charge (the higher the charge, the quicker the drift). The time-of-flight corresponds to the mass-to-charge ratio (*m/z*).

Figure 3: Example of two mass spectra. The x-axis represents the mass-to-charge ratio (*m/z*) and the y-axis the corresponding signal intensity.



The ions in each ion beam arrive at a collector (**detector**) where they are counted to yield analog signals. The analog signals are in turn converted into spectra that represent ion intensities at particular *m/z* values. The ion intensities are representative of the number of collected ions or proteins. An example of a spectrum is shown in Figure 3.

How can we use proteomics to discover tumour markers?

There are two possible approaches for examining a biological problem: (a) global proteomics, i.e. analyses of the entire protein content, or (b) targeted proteomics, i.e. analyses of a particular protein.

In **global proteomics**, 2D gel electrophoresis or mass spectrometry will be used to screen the total protein content in a sample. A series of matched samples obtained from disease-affected and normal patients are collected. The proteomes are extracted and by comparing different gels or spectra between the disease affected and normal patients, differentially expressed spots or peaks can be identified. The ultimate goal is to identify as many differentially expressed proteins as possible. Different kinds of samples can be used with each having its own advantages and disadvantages (Table 2). Global proteomics experiments are also considered as fishing experiments because there is no prior knowledge on the quantity and nature of the identified proteins [62].

Targeted proteomics is more focused on the function of the individual proteins and their relationships with other proteins. Targeted proteomics can also be used for antibody free quantitation of known proteins [63;64]. A critical step in targeted proteomics is, of course, that the protein which is being investigated is known to the researcher.

After an initial “proteomics-hype” in the first part of the last decade, during which proteomics was mainly focused on global proteomics [65], there is nowadays a shift towards a more targeted proteomics. This is mainly due to the fact that global proteomics had its limitations [66]:

- Bearing in mind the fact that the human body may contain somewhere between 300,000 and 10,000,000 proteins, it is, at least at present, technically impossible to analyse all proteins in one experiment. Until these technical limitations are resolved, there will always be a need for prefractionation techniques. As a

consequence, “global” proteomics will only be examining a “subset” of proteins within a sample.

- Serum or plasma, the most often used samples in global proteomics, contains a majority of high-abundance proteins, accounting for almost 99% of the total protein mass [41;42]. These high-abundance proteins are true serum or plasma proteins that carry out their functions in the circulation, rather than proteins or peptides that leak into the blood.
- Sources of variability or artefacts have contributed to false discoveries. These include erroneous methodology, lack of standardized sample collection and storage, differences between cases and controls in terms of sex, age and physiological states (for example, fasting, weight gain or loss and hormonal status), changes in inflammation and acute-phase reactants, changes in metabolic states, etc. [66].
- Diseases, such as cancer, are heterogeneous. For example, epithelial ovarian carcinomas are malignancies exhibiting great diversity in histological phenotypes. As such, cancers are a to be considered as a collection of different tumours rather than one disease [67].

Table 2: Advantages and disadvantages of different materials used in proteomics experiments [2].

Material	Advantages	Disadvantages
Serum		
	• Easy to obtain • Low cost	• Presence of high abundant proteins • High inter- and intra-individual changes • Spontaneous or accelerated clotting is difficult to standardize • 40% of peptides in serum are specific to serum
Plasma		
	• Easy to obtain • Low cost	• Presence of high abundant proteins • High inter- and intra-individual changes
Urine		
	• Easy to obtain • Low cost • More stable compared to serum and plasma regarding pre-analytical handling procedures	• Presence of high abundant proteins • High inter- and intra-individual changes • Low concentration of proteins • High mass proteins are not cleared by the kidneys
Ascites		
	• Easy to obtain • Contains tumour-derived cells	• Only present in advanced stage disease; not useful in diagnostic setting
Tissue		
	• Tumour derived	• Surgery needed • Non homogeneous cells; min 75% of tumour cells needed in the biopsy
Laser micro-dissected cells		
	• Tumour derived • Homogeneous cells • Enrichment of protein profile in comparison with whole tissue	• Surgery needed • Time consuming • Expensive equipment • Small volume; not compatible with all downstream applications

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General objectives

The objective of this research project is to validate newly discovered biomarkers (*Part I*) and to discover new biomarkers by the profiling of proteomes of ovarian and cervical tumours, using serum or plasma samples of patients with one of these tumours (*Part II*). And finally we review and comment the current achievements of proteomics in the field of gynaecological cancers (*Part III*)

We deliberately chose for two diagnostic problems in, respectively, two different gynaecological tumours. The first diagnostic problem is the **differentiation between a benign and a malignant ovarian mass**. Even when a pelvic mass is present, it is difficult to discriminate between benign and malignant disease. Yet, a correct preoperative discrimination between benign and malignant ovarian tumours is extremely important since this will influence the surgical approach and treatment of these tumours. Benign ovarian tumours can be approached by minimal invasive techniques, minimizing the patients morbidity and damage to the ovary. Malignant ovarian tumours need to be treated aggressively with a debulking or staging procedure by a gynaecological oncologist. To be able to study this diagnostic problem, we started a prospective collection of serum and plasma samples since 2006 of more than 650 patients planned to undergo a surgical procedure for a pelvic mass. The collection of samples was performed according to a strict standard operating procedures. The clinical data of all patients was screened for possible confounding conditions and a routine blood sample for standard laboratory tests, including haematology, biochemistry, clotting and CA125, was taken. Furthermore, all patients underwent a preoperative ultrasound. All ultrasound examinations were performed by a standardized examination technique that employed standardized terms and definitions and high-quality ultrasound equipment. During the course of these studies, we expanded our collection to an international multicenter study with the cooperation of hospitals in Malmö (Sweden), Rome (Italy), and Prague (Czech Republic), using the same strict standard operating procedures, leading to an additional collection of 150 samples.

In a first study (*Part I – Chapter 1*) the ROMA algorithm, a combination of CA125 and HE4, was validated and compared to the gold standard CA125. This study was the first and biggest independent validation study of the ROMA algorithm. After this publication, a letter to the editor was published to which we replied accordingly (*Part I – Chapter 1b*).

The differentiation of benign and malignant masses is not purely based on tumour markers alone. This is why a second study was performed, in which the diagnostic accuracy of ROMA was assessed and compared to the diagnostic accuracy of the two most widely used ultrasound methods, namely the risk of malignancy index (RMI) and subjective assessment by ultrasound (*Part I – Chapter 2*).

Angiogenesis is one of the key events in ovarian tumour growth. It is a balance between endogenous stimulators and inhibitors. Endorepellin, the C-terminal fragment of perlecan, is one these angiostatic peptides. A prospective cohort study was conducted to investigate whether low levels of plasma endorepellin, as a single marker, or in combination with known markers (CA125 and/or HE4), could differentiate between benign and malignant ovarian masses (*Part II – Chapter 1*).

The second diagnostic problem is the **diagnosis of positive lymph nodes in cervical cancer**. Lymph node involvement is a strong prognostic factor for cervical cancer. Whenever positive lymph nodes are found in patients with clinical stage Ib1 cervical cancer, the overall 5-year survival drops from +/- 95% to +/- 78% {Quinn, 2006 743 /id}. Patients with a cervical carcinoma with positive lymph nodes usually receive adjuvant radiotherapy. The combination of surgery and radiotherapy can cause serious side effects, such as lymphoedema of the legs, fistulas and wound breakdown. Hitherto, positive lymph node are difficult to detect with radiological examinations {Choi, 2010 866 /id}. There is a strong need for biomarkers that predict lymph node involvement in cervical cancer. These biomarkers can facilitate the selection of patients requiring pelvic lymphadenectomy or irradiation. We investigated whether we could predict lymph node status with proteomic profiling using immunodepletion and SELDI–TOF MS (*Part II – Chapter 2*).

Finally, the literature was critically reviewed. This resulted in an overview of the utility of proteomics in gynaecologic cancers (*Part III – Chapter 1*). Since methodology in proteomic experiments is of uttermost importance we also included a letter to the editor commenting on this subject (*Part III – Chapter 2*).



Part I
Validation studies

Chapter 1: HE4 and CA125 as a diagnostic test in ovarian cancer: prospective validation of the Risk of Ovarian Malignancy Algorithm

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Abstract:

Background: Recently, a Risk of Ovarian Malignancy Algorithm (ROMA) utilising HE4 and CA125 successfully classified patients as presenting a high or low risk for epithelial ovarian cancer. We validated this algorithm in an independent prospective study.

Methods: Women with a pelvic mass, who were scheduled to have surgery, were enrolled in a prospective study. Preoperative serum levels of HE4 and CA125 were measured in 389 patients. The performance of each of the markers, and that of ROMA was analysed.

Results: When all malignant tumours were included, ROMA (AUC = 0.898) and HE4 (AUC = 0.857) did not perform significantly better than CA125 alone (AUC = 0.877). Using a cut-off for ROMA of 12.5% for premenopausal patients, the test had a sensitivity of 67.5% and a specificity of 87.9%. With a cut-off of 14.4% for postmenopausal patients, the test had a sensitivity of 90.8% and a specificity of 66.3%. For epithelial ovarian cancer *vs* benign disease, the AUC of ROMA increased to 0.913 and for invasive epithelial ovarian cancer *vs* benign disease to 0.957.

Conclusion: This independent validation study demonstrated similar performance indices to those recently published. However, in this study, HE4 and ROMA did not increase the detection of malignant disease compared to CA125 alone.

Interpretation: Although the initial reports were promising, measurement of HE4 serum levels does not contribute to the diagnosis of ovarian cancer.

Introduction:

The majority of women who undergo surgery for an ovarian cyst or pelvic mass are treated in a community hospital by a gynaecologist or general surgeon. Although this is appropriate for patients who have a benign cyst, patients with a malignancy should be referred to a tertiary care centre with multidisciplinary teams specialised in ovarian cancer treatment. A recent systematic review showed an improved outcome for patients with ovarian cancer when they were referred to, and surgically treated by, gynaecological oncologists [2]. Therefore, it is important to triage women with increased risk for ovarian cancer to the appropriate surgeon and centre.

CA125 is the most widely used tumour marker in ovarian cancer [3]. The sensitivity and specificity of CA125 is far from ideal since its levels are raised in approximately 80% of all epithelial ovarian cancers and in only 50% of stage I epithelial ovarian cancer [6]. Therefore, CA125 is rarely used as a unique parameter in the prediction of malignancy. Usually, a combination of a patient's medical history, clinical examination results, imaging data and tumour marker profile is used to differentiate malignant ovarian masses from their benign counterparts. Ultrasound plays an important role in differentiating between benign and malignant adnexal masses, but experience and proper training is of paramount importance in distinguishing both adnexal masses [7]. This highlights a major problem in that the centre with the least experience in dealing with malignant disease requires substantial experience in ultrasound to triage patients to a gynaecological oncologist. This explains the tremendous amount of effort that has been expended over the past few decades to find new ovarian cancer biomarkers that could be used together with, or instead of, CA125. In 1999, the Human Epididymis secretory protein 4 (HE4) gene was found to be overexpressed in ovarian cancer [8]. It is a member of the Wey Acidic Protein (WAP)-gene family [9] and is expressed in normal tissues of the reproductive and respiratory tract [10;11]. The first report mentioning HE4 as a potential serum biomarker for ovarian cancer was published in 2003 [12]. Recently, Moore *et al* published a series of papers that used a combination of CA125, HE4 and menopausal status to predict the presence of a malignant ovarian tumour [4;13]. Originally, nine potential biomarkers were evaluated, of which HE4 was the most effective in detecting ovarian cancer. When CA125 was combined with HE4, the prediction rate was higher, showing a sensitivity for detecting malignant disease of 76.4% at a specificity of 95% [4]. Subsequently, Moore *et al* performed a multicentre

prospective study, including 531 women diagnosed with a pelvic mass who underwent surgery. Patients were classified as being at a high or low risk for ovarian cancer with a specificity of 75.0% and a sensitivity of 92.3% for postmenopausal patients, and a specificity of 74.8% and a sensitivity of 76.5% for premenopausal patients [13].

In this study, we aimed to independently validate HE4 and the combination of HE4 with CA125 using the Risk of Ovarian Malignancy Algorithm (ROMA) for the diagnosis of ovarian cancer.

Materials and methods:

Patients:

From August, 2005 until March, 2009, 389 patients were included in a prospective study conducted at the University Hospitals Leuven. All patients were diagnosed with a pelvic mass of suspected ovarian origin and were scheduled for surgical intervention. Women with a prior bilateral oophorectomy were not eligible. All patients underwent imaging by pelvic ultrasound to document the presence of an ovarian mass. Clinical information was retrieved from the patients' hospital notes. All patients underwent surgical removal of the ovarian mass, and if a patient was diagnosed with an ovarian cancer, surgical staging was performed.

Prior to the collection of biological samples and surgery, all patients were required to give fully informed consent. The protocol was approved by the Local Ethics Committee. The Ethical Committee released the authors from the obligation to obtain an insurance contract because of the character of this study. Patient participation in the study was concluded once the final surgical pathology reports were obtained.

Serum samples:

Immediately before surgery, blood samples were obtained. Blood samples were collected in 10 ml clothing activating tubes (BD Vacutainer® Serum Tube, ref. 369033; Becton-Dickinson, Erembodegem, Belgium). Serum tubes were centrifuged at 800 *x g* for 10 minutes. Serum was collected, dispensed into multiple cryotubes, and frozen at -80°C. The time between blood sampling and freezing of the serum and presence of haemolysis was noted. The targeted time limit between sampling and freezing was 4 hours.

Marker assays:

Serum CA125 concentrations were measured using the CanAg CA125 EIA assay (Fujirebio Diagnostics, Göteborg, Sweden) and serum HE4 concentrations were measured using the HE4 EIA assay (Fujirebio Diagnostics, Göteborg, Sweden). Both assays are solid-phase, non-competitive immunoassays based on the direct sandwich technique and were run according to manufacturer's instructions. Each ELISA was performed manually and in duplicate for calibrators, controls and patient samples. The appropriate controls were within the ranges provided by the manufacturer for all runs. For CA125, the upper normal limit was 35 U/ml, while that for HE4 was 70 pM (as suggested by Moore *et al* [4]) or 150 pM (as suggested in the product insert). A cut-off point that gave the best accuracy (minimal false negative and false positive results) in the study was also determined We also determined our own ideal cut-off, corresponding to the highest accuracy.

Statistical analysis:

The ROMA classifies patients as being at a low or high risk for malignant disease using the following algorithms:

- *Premenopausal:*
  *Predictive Index (PI) = - 12.0 + [2.38*LN(HE4)] + [0.0626*LN(CA125)]*
- *Postmenopausal:*
  *PI = -8.09 + [1.04*LN(HE4)] + [0.732*LN(CA125)]*
- *Predicted Probability:*
  *(PP) = 100 * exp(PI) / [1 + exp(PI)]*

According to the manufacturers insert [5], the following thresholds were selected for the ROMA:

- Premenopausal women:
  - PP ≥ 12.5% = High risk of finding epithelial ovarian cancer
  - PP < 12.5% = Low risk of finding epithelial ovarian cancer
- Postmenopausal women:
  - PP ≥ 14.4% = High risk of finding epithelial ovarian cancer
  - PP < 14.4% = Low risk of finding epithelial ovarian cancer

Statistical analysis was performed with MedCalc v11.1.1.0 (MedCalc Software, Mariakerke, Belgium) and with PASW Statistics v17.0 (SPSS, Brussels, Belgium). The mean age of the patients was compared using Student's t-test, and categorical variables were compared with the Chi-square test. Tumour marker levels were compared using the Wilcoxon rank-

sum test (Mann–Whitney two sample statistic) or Kruskal–Wallis rank test (multiple sample statistic).

Receiver operator characteristic (ROC) curves were constructed, and the area under the curve (AUC) with a 95% Confidence Interval (95%CI) was calculated. The sensitivity and specificity were calculated in pre- and postmenopausal women separately and independently of menopausal status. Subgroups were analysed according to the histological subtype of the tumour, stage, grade, use of hormonal drugs, smoking habit, familial history, presence of haemolysis, and the time between sampling and freezing. The method described by DeLong *et al* [1] was used for the calculation of the difference between two AUCs. For all statistical comparisons a *P* value of < 0.05 was considered statistically significant.

Results:

Patient characteristics

The serum of 389 patients was analysed: 228 (58.6%) patients had benign disease and 161 (41.4%) patients had malignant disease (Table 1). Patients with benign disease were younger and more likely to be premenopausal. Patients with malignant disease were more likely to have a family history of breast and ovarian cancer.

Table 1: Distribution of patient characteristics for patients with a benign or malignant pelvic mass.

Variable	Numerical display	Benign	Malignant	*p*-value
Number of cases	n (%)	228 (58.6)	161 (41.4)	NA
Age (in years)	Mean (SD)	46.3 (16.0)	57.8 (12.6)	< 0.001
Postmenopausal	n (%)	86 (37.7)	119 (73.9)	< 0.001
Smoking	n (%)	53 (23.2)	31 (19.3)	0.457
OC*	n (%)	44 (31.2)	12 (30.0)	0.682
HRT†	n (%)	14 (16.3)	9 (7.6)	0.053
Family history				
Breast cancer	n (%)	35 (15.4)	41 (25.5)	0.009
Ovarian cancer	n (%)	4 (1.8)	8 (5.0)	0.028

Abbreviations: OC, oral contraception; HRT, hormone replacement therapy; NA, not applicable.

*For pre-menopausal patients; †For post-menopausal patients

Sample characteristics

Forty samples were not frozen within a time limit of 4 hours after sampling, of which 24 (10.5%) were from benign cases and 16 (9.9%) from malignant cases (p = 0.851). Haemolysis was noted in 38 cases, of which 23 (10.1%) were from benign cases and 15 (9.3%) from malignant cases (p = 0.801).

Tumour characteristics

The most common benign ovarian tumours were cystadenomas (n = 52), cystadenofibromas (n = 26), endometriomas (n = 66), and mature teratomas (n = 29)(Table 2 and 3). Mixed tumours (n = 16) contain two or more different histological subtypes, making it impossible to categorise these tumours into a specific subtype. The cystadenomas and cystadenofibromas included 47 serous, 26 mucinous, and five other histological types or mixed cystadenomas/cystadenofibromas. The majority of the malignant tumours were epithelial ovarian cancer. Most of the epithelial ovarian cancer were high grade and were diagnosed at an advanced stage. Other primary non-epithelial ovarian tumours included two sex cord stromal tumours and two sarcomas. All other malignant tumours of the ovary (n = 26) were metastases from extra-ovarian primary tumours. These tumours were mainly of an endometrial or gastro-intestinal origin.

Table 2: Histological type and distribution of benign disease.

Histological type	**n**	**%**
Cystadenoma/Cystadenofibroma*	78	34.1
Endometriosis	66	28.9
Mature teratoma	29	12.7
Fibroma/Thecoma	15	6.6
Functional cyst	13	5.7
Hydrosalpinx	3	1.3
Abces	2	0.9
Parasalpingeal cyst	2	0.9
Struma ovarii	2	0.9
Leydig cell tumour	1	0.4
Unknown	1	0.4
Mixed	16	7.0
Total	228	100.0

*Including 47 serous, 26 mucinous, and 5 other or mixed cystadenomas/cystadenofibromas.

Table 3: FIGO stage, differentiation grade and histological type of malignant disease.

	n	%
Histological type		
Epithelial	131	81.4
Serous	84	52.2
Mucinous	21	13.0
Endometrioid	7	4.3
Clear cell	6	3.7
Mixed	6	3.7
Carcinosarcoma	4	2.5
Undifferentiated	3	1.9
Granulosa cell	2	1.2
Sarcoma	2	1.2
Metastatic	26	16.1
Endometrium	11	6.8
Colon	5	3.1
Appendix	3	1.9
Mesothelioma	2	1.2
Breast	1	0.6
Lung	1	0.6
Lymphoma	1	0.6
Pancreas	1	0.6
Stomach	1	0.6
Total	*161*	*100.0*
FIGO Stage*		
I	43	32.8
II	8	6.1
III	66	50.4
IV	14	10.7
Total	*131*	*100.0*
Differentiation grade*		
Borderline	31	23.7
Grade 1	13	9.9
Grade 2	14	10.7
Grade 3	73	55.8
Total	*131*	*100.0*

*For epithelial ovarian cancer only

Tumour marker levels

The median CA125, HE4 and ROMA serum levels differed significantly between benign and malignant cases for the whole group, and for the premenopausal and postmenopausal groups separately ($p < 0.0001$ for all comparisons)(Table 4, Figure 1 and 2). Within the benign group, the most frequent tumours were analysed. With the Kruskal–Wallis rank test we found the median tumour marker levels to be statistically different for CA125 ($p < 0.0001$), HE4 ($p = 0.0043$), and ROMA ($p = 0.0006$). Post-hoc pairwise comparisons with the Wilcoxon rank-sum test showed that CA125 was significantly elevated in endometriosis and ovarian fibromas/thecomas compared to cystadeno(fibro)mas ($p < 0.0001$ and $p = 0.0111$), functional cysts ($p = 0.0160$ and $p = 0.0281$) and mature teratomas ($p = 0.0002$ and $p = 0.0169$). For HE4, the only significant comparison found was the pairwise comparison between cystadeno(fibro)mas and endometriosis ($p = 0.0002$). ROMA was significantly elevated in cystadeno(fibro)mas ($p < 0.0001$) and ovarian fibromas/thecomas ($p = 0.0111$) when compared to endometriosis, and in cystadeno(fibro)mas when compared to mature teratomas ($p = 0.0349$).

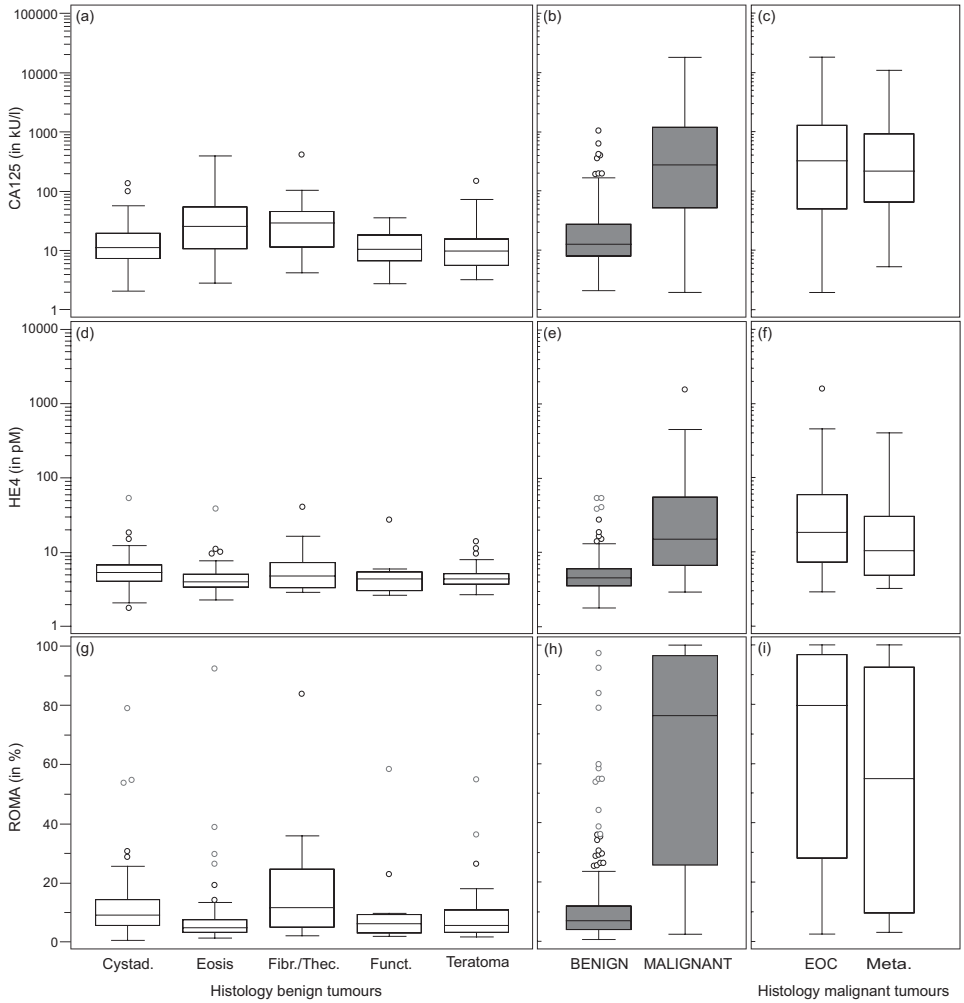
Within the group of malignant tumours, there was no significant difference between the CA125, HE4 and ROMA levels of epithelial ovarian cancers and metastatic tumours. There was no significant difference between FIGO stage I and II tumours, nor between FIGO stage III and IV, although the difference between early stage (FIGO I–II) and advanced stage (FIGO III–IV) was significant for CA125, HE4 and ROMA. There was a significant difference between borderline and invasive disease (grade 1–3) for all of the markers, but there was no difference between grade 1, 2 and 3 for the different markers.

Table 4: Serum CA125, HE4 and ROMA levels according to histology, FIGO stage and tumour grade.

	CA125 (U/ml)		HE4 (pM)		ROMA (%)	
	Median	**IR**	**Median**	**IR**	**Median**	**IR**
Benign histology	12.8	8.0 – 27.6	45.4	35.6 – 60.8	6.8	3.9 – 11.9
Cystadeno(fibro)ma	11.3	7.4 – 19.5	53.7	40.8 – 68.0	9.1	5.7 – 14.4
Endometriosis	25.5	10.7 – 54.9	40.0	34.3 – 50.7	4.9	3.3 – 7.5
Fibroma / thecoma	29.1	11.6 – 45.5	48.1	33.9 – 73.2	11.6	5.0 – 24.7
Functional cyst	10.5	6.8 – 20.7	43.7	30.8 – 54.6	6.2	3.0 – 9.4
Mature teratoma	9.8	5.7 – 15.9	43.9	37.5 – 52.5	5.6	3.4 – 10.9
Malignant histology	276.5	52.4 – 1195.9	151.8	67.4 – 560.4	76.1	25.7 – 96.5
Epithelial ovarian cancer	321.3	50.2 – 1291.4	184.6	72.9 – 589.3	79.6	28.1 – 96.7
Metastatic	222.9	64.9 – 913.5	103.5	48.9 – 302.4	55.0	9.7 – 92.5
FIGO stage						
I	38.4	16.9 – 182.6	73.2	52.6 – 126.5	25.6	11.8 – 64.3
II	60.2	20.6 – 254.8	69.0	44.3 – 152.6	45.5	26.1 – 56.2
III	757.3	227.9 – 1640.0	308.0	135.0 – 712.5	91.2	70.3 – 98.4
IV	1260.7	790.6 – 2905.1	578.7	274.6 – 2612.9	98.0	93.0 – 99.5
Tumour grade						
Borderline	38.3	14.8 – 170.4	67.6	52.6 – 116.4	23.4	10.0 – 50.1
Grade 1	379.4	83.2 – 1120.4	119.9	72.7 – 336.5	74.5	52.2 – 94.9
Grade 2	755.7	124.2 – 945.4	552.9	125.0 – 844.3	94.3	67.8 – 97.6
Grade 3	748.7	112.1 – 1580.9	274.6	97.8 – 716.7	90.3	59.2 – 98.4

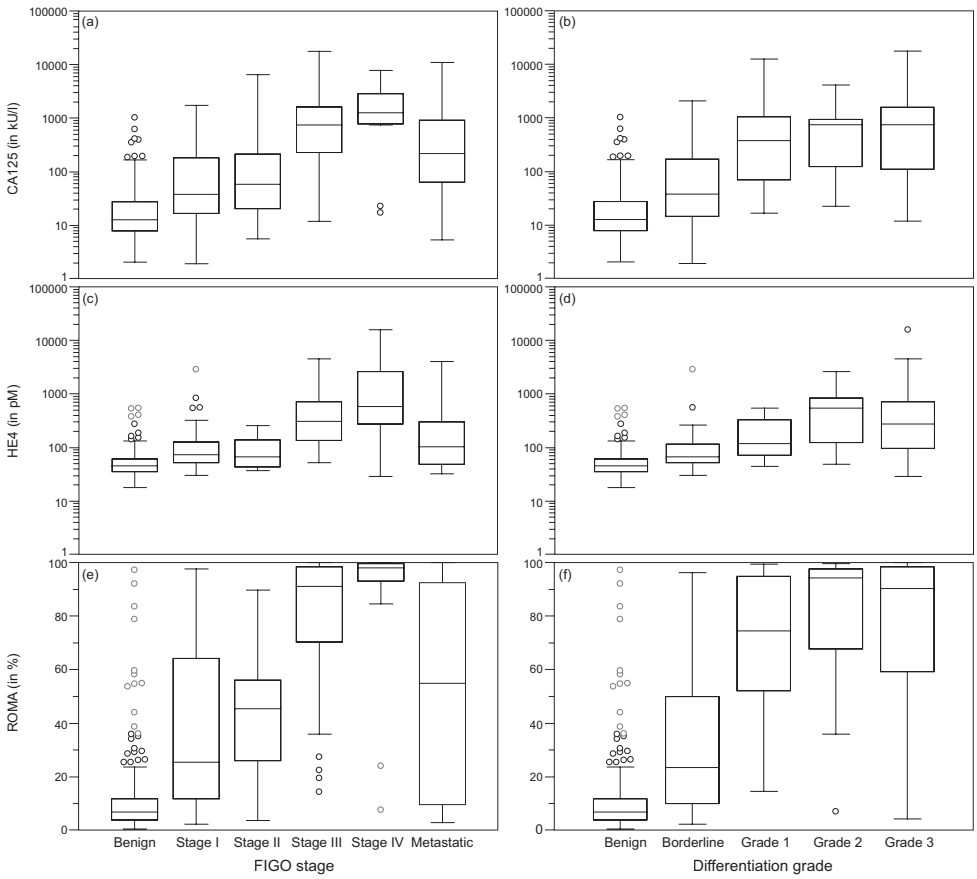
Abbreviations: IR: interquartile range

Figure 1: Box-and-whisker plots representing median levels and interquartile range (box) of CA125 (in U/ml), HE4 (in pM) and ROMA (in %) according to histology.



(a), (d) and (g): Plots for benign histology subtypes
(b), (e) and (h): Plots for benign *vs* malignant histology.
(c), (f) and (i): Plots for malignant histology subtypes
Y-axis is a logarithmic scale for CA125 and HE4.
Abbreviations: Cystad.: cystadenoma or cystadeanofibroma; Eosis: endometriosis; Fibr./Thec.: ovarian fibroma or thecoma; Funct.: functional cyst; EOC: epithelial ovarian cancer; Meta: metastatic disease to the ovary.

Figure 2: Box-and-whisker plots representing median levels and interquartile range (box) of CA125 (in U/ml), HE4 (in pM) and ROMA (in %) according to FIGO stage and grade.



(a), (c) and (e): Plots for benign disease, different FIGO stages and metastatic disease.
(b), (d) and (f): Plots for benign disease, borderline disease and different grades of epithelial ovarian cancer.
Y-axis is a logarithmic scale for CA125 and HE4.

ROC curves and performance indices for all tumours

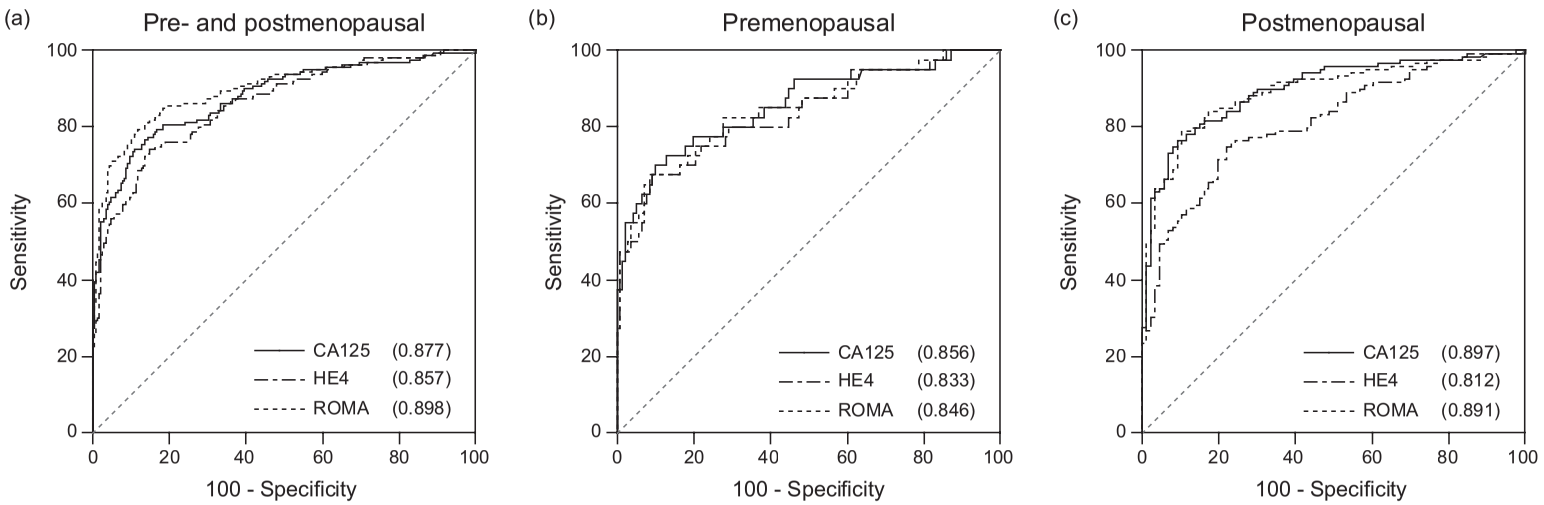
The AUC of CA125 was not significantly different from HE4 or ROMA for all malignant diseases (including epithelial ovarian cancer, non-epithelial ovarian cancer, and metastases) *vs* benign disease (Table 5 and Figure 3). Pairwise comparison of AUCs showed that only the difference between HE4 and ROMA was significant. For the premenopausal patients, again, only the pairwise comparison between HE4 and ROMA was significant. In the postmenopausal population, there was a significant better performance of CA125 *vs* HE4, and of ROMA *vs* HE4. Overall, ROMA did not perform significantly better than CA125 alone for either the whole group of patients, or for the pre- or postmenopausal patients separately.

At the ideal cut-off, corresponding to the highest accuracy (minimal false negative and false positive results), CA125, HE4, and ROMA gave a similar sensitivity and specificity within the different menopausal groups. Sensitivity and specificity, using the cut-off values in the manufacturers protocol, for HE4 and ROMA are also shown in Table 5, together with the sensitivity and specificity of HE4 at a cut-off of 70 pM.

ROC curves for subgroups:

When epithelial ovarian cancer was analyzed alone and non-epithelial ovarian cancer and metastatic tumours were excluded, the AUC of ROMA was higher (Figure 4, Table 6). The AUC was even higher when all borderline tumours were excluded. Concerning histological subtypes, a comparison of AUC of (pure) serous with that of non-serous epithelial ovarian cancer (excluding all mixed serous tumours) showed that AUC of ROMA was higher for the serous subtype. By contrast, all markers performed significantly worse when only the mucinous subtype was examined.

Figure 3: ROC curves for CA125, HE4 and ROMA among patients with all types and stages of ovarian tumours. Total AUC values for each assay are listed in parentheses.



(a) malignant *vs* benign disease in pre- and postmenopausal patients together.
(b) malignant *vs* benign disease in premenopausal patients.
(c) malignant *vs* benign disease in postmenopausal patients.

Table 5: Comparison of AUCs, sensitivity and specificity for CA125 (U/ml), HE4 (pM) and ROMA (%) among patients with all types and stages of ovarian tumours according to menopausal status.

	Marker	AUC (95%CI)	Pairwise comparison of AUC* HE4 *vs* CA125	HE4 *vs* ROMA	CA125 *vs* ROMA	Ideal cut-off‡ Cut-off	Sens (%)	Spec (%)	Suggested cut-off Cut-off	Sens (%)	Spec (%)
All	ROMA	0.898 (0.863–0.926)				22.2	79.2	88.1	12.5/14.4**	84.9	79.7
	CA125	0.877 (0.840–0.908)	*p* = .306	*p* < .001	*p* = .172	62.5	73.9	89.0	35.0	79.5	81.6
	HE4	0.857 (0.819–0.891)				72.2	73.9	85.1	70.0§	74.5	83.3
									150.0#	50.3	96.5
Pre	ROMA	0.846 (0.785–0.895)				16.6	67.5	91.5	12.5**	67.5	87.9
	CA125	0.856 (0.796–0.904)	*p* = .570	*p* = .044	*p* = .782	83.8	70.0	90.1	35.0	75.0	80.1
	HE4	0.833 (0.771–0.885)				66.0	67.5	90.8	70.0§	67.5	90.8
									150.0#	42.5	99.3
Post	ROMA	0.891 (0.840–0.930)				35.9	79.0	89.5	14.4**	90.8	66.3
	CA125	0.897 (0.847–0.935)	*p* = .001	*p* = .001	*p* = .487	51.2	76.5	90.7	35.0	81.5	83.7
	HE4	0.812 (0.752–0.863)				74.2	74.8	77.9	70.0§	77.3	70.9
									150.0#	52.9	91.9

† Differences in AUCs were calculated by using the method as described by DeLong *et al* [1].

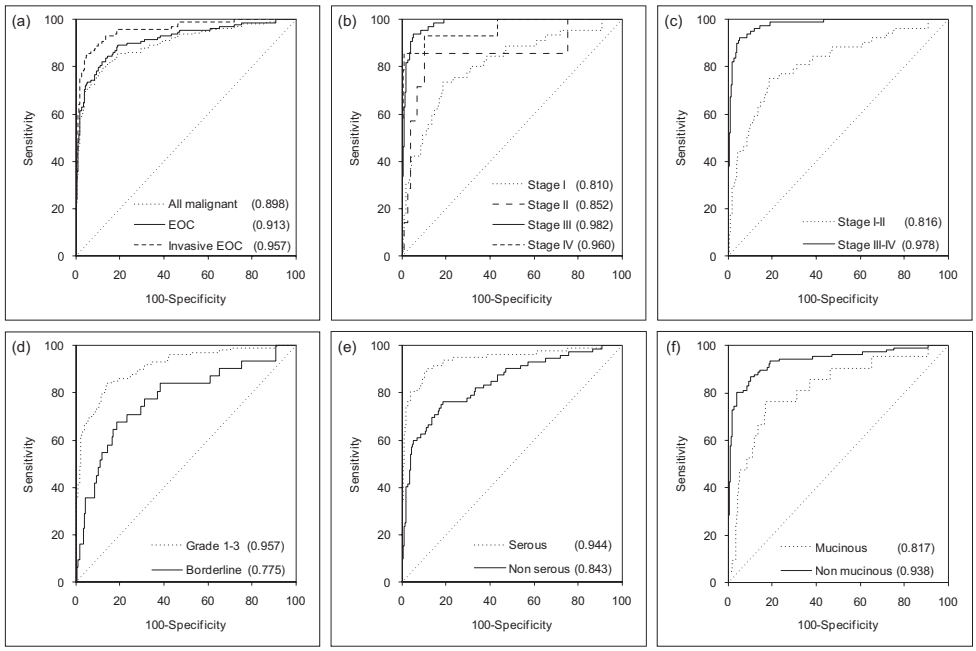
‡ Cut-off value corresponding to the highest accuracy (minimal false negative and false positive results).

§ Cut-off value for HE4 at 70 pM as suggested by Moore *et al* [4].

Cut-off value for HE4 at 150 pM as suggested in the product insert [5].

** Cut-off values for ROMA: 12.5% for the premenopausal patients and 14.4% for the postmenopausal patients as suggested in the product insert.

Figure 4: ROC curves for ROMA. The ROC curves in the different subsets represent different groups of ovarian cancer (cases) compared to benign ovarian tumours (non-cases). Total AUC values for each assay are listed in parentheses.



(a) All malignant tumours, epithelial ovarian cancer (EOC), and invasive EOC (excluding borderline tumours) *vs* benign disease.
(b) FIGO stage I, II, III and IV *vs* benign disease.
(c) Early stage (stage I and II combined) and advanced stage (stage III and IV combined) *vs* benign disease.
(d) Grade 1-3 EOC and borderline EOC *vs* benign disease.
(e) Serous EOC and non-serous EOC *vs* benign disease (all mixed serous tumours were excluded from this analysis).
(f) Mucinous EOC and non-mucinous EOC *vs* benign disease (all mixed mucinous tumours were excluded from this analysis).

Table 6: Comparison of AUCs for CA125, HE4 and ROMA among different patient subgroups according to tumour, personal and sample characteristics. Differences in AUCs were calculated according to DeLong *et al* [1].

	HE4		CA125		ROMA	
	AUC (95%CI)	***p***	**AUC (95%CI)**	***p***	**AUC (95%CI)**	***p***
Type of cancer						
All malignant*	0.857 (0.818 – 0.890)		0.877 (0.840 – 0.908)		0.898 (0.863 – 0.926)	
EOC†	0.871 (0.831 – 0.903)	NA	0.886 (0.848 – 0.917)	NA	0.913 (0.879 – 0.941)	NA
Invasive EOC‡	0.914 (0.878 – 0.942)		0.937 (0.905 – 0.961)		0.957 (0.929 – 0.976)	
FIGO Stage						
I	0.767 (0.713 – 0.816)		0.755 (0.700 – 0.805)		0.810 (0.758 – 0.855)	
II	0.705 (0.642 – 0.762)	NA	0.769 (0.710 – 0.821)	NA	0.852 (0.800 – 0.895)	NA
III	0.954 (0.923 – 0.975)		0.969 (0.943 – 0.986)		0.982 (0.959 – 0.994)	
IV	0.914 (0.871 – 0.946)		0.950 (0.915 – 0.974)		0.960 (0.927 – 0.981)	
Stage						
Early (I-II)	0.758 (0.704 – 0.807)	< 0.0001	0.757 (0.703 – 0.806)	< 0.0001	0.816 (0.765 – 0.859)	< 0.0001
Late (III-IV)	0.947 (0.916 – 0.969)		0.966 (0.939 – 0.983)		0.978 (0.955 – 0.991)	
Histology						
Serous	0.910 (0.873 – 0.939)		0.910 (0.873 – 0.939)		0.944 (0.912 – 0.967)	
Endometrioid	0.826 (0.771 – 0.872)	NA	0.878 (0.830 – 0.917)	NA	0.939 (0.900 – 0.966)	NA
Clear cell	0.824 (0.769 – 0.870)		0.900 (0.854 – 0.935)		0.846 (0.793 – 0.890)	
Mucinous	0.748 (0.689 – 0.800)		0.794 (0.738 – 0.842)		0.817 (0.763 – 0.863)	
Histology						
Serous	0.910 (0.873 – 0.939)	0.0324	0.910 (0.873 – 0.939)	0.0855	0.944 (0.912 – 0.967)	0.0028
Non-serous	0.777 (0.725 – 0.823)		0.840 (0.794 – 0.880)		0.843 (0.797 – 0.882)	
Histology						
Mucinous	0.748 (0.689 – 0.800)	0.0072	0.794 (0.738 – 0.842)	0.0123	0.817 (0.763 – 0.863)	0.0299
Non-mucinous	0.903 (0.866 – 0.933)		0.911 (0.875 – 0.939)		0.938 (0.906 – 0.961)	

Tumour grade						
Grade 1	0.883 (0.836 – 0.921)		0.903 (0.858 – 0.937)		0.954 (0.920 – 0.977)	
Grade 2	0.929 (0.889 – 0.958)	NA	0.956 (0.921 – 0.978)	NA	0.957 (0.923 – 0.979)	NA
Grade 3	0.914 (0.876 – 0.943)		0.938 (0.904 – 0.962)		0.958 (0.928 – 0.978)	
Tumour grade						
Grade 1-3	0.912 (0.876 – 0.940)	0.0004	0.936 (0.903 – 0.960)	0.0001	0.957 (0.929 – 0.976)	0.0004
Borderline	0.737 (0.679 – 0.790)		0.726 (0.667 – 0.779)		0.775 (0.719 – 0.825)	
Family history						
Yes	0.832 (0.733 – 0.905)	0.4938	0.878 (0.788 – 0.940)	0.8951	0.853 (0.757 – 0.922)	0.2202
No	0.867 (0.823 – 0.904)		0.872 (0.827 – 0.908)		0.913 (0.875 – 0.943)	
Smoking						
Yes	0.825 (0.727 – 0.899)	0.3629	0.871 (0.780 – 0.934)	0.8144	0.890 (0.802 – 0.948)	0.6866
No	0.873 (0.829 – 0.908)		0.882 (0.840 – 0.917)		0.906 (0.867 – 0.937)	
Oral contraceptives						
Yes	0.820 (0.694 – 0.910)	0.6328	0.843 (0.721 – 0.926)	0.9149	0.839 (0.716 – 0.924)	0.7291
No	0.868 (0.785 – 0.927)		0.853 (0.768 – 0.916)		0.873 (0.792 – 0.931)	
Hormone replacement therapy						
Yes	0.683 (0.458 – 0.859)	0.2102	0.810 (0.593 – 0.941)	0.3618	0.778 (0.558 – 0.922)	0.2681
No	0.844 (0.779 – 0.896)		0.908 (0.852 – 0.947)		0.910 (0.854 – 0.949)	
Haemolysis						
Yes	0.800 (0.663 – 0.900)	0.3416	0.907 (0.790 – 0.970)	0.4716	0.853 (0.725 – 0.937)	0.4031
No	0.864 (0.823 – 0.899)		0.874 (0.834 – 0.908)		0.903 (0.866 – 0.933)	
Freezing within 1-4h						
Yes	0.860 (0.819 – 0.894)	0.9053	0.878 (0.839 – 0.910)	0.9625	0.896 (0.859 – 0.926)	0.8247
No	0.852 (0.700 – 0.946)		0.875 (0.727 – 0.960)		0.908 (0.766 – 0.978)	

Abbreviations: EOC: epithelial ovarian cancer; OC: oral contraceptives; HRT: hormone replacement therapy [*] Including EOC, non-epithelial ovarian cancers and metastatic cancers to the ovary. [†] Including borderline or invasive EOC. [‡] Including only invasive EOC.

Discussion:

This study aimed to investigate the performance of serum tumour markers CA125 and HE4, and the risk stratification tool ROMA in a prospective collection of serum samples from patients with an ovarian mass. We found that there was a significant difference between benign and malignant disease with respect to serum CA125, HE4 and ROMA levels. When the AUCs of the different tumour markers were compared, HE4 and CA125 performed similarly, except for the postmenopausal patients, where CA125 performed better. This similar performance of HE4 and CA125 was also noted in other studies [12;14-17]. Combining HE4 and CA125 in the ROMA algorithm improved HE4 but not CA125 performance, regardless of menopausal status. Since CA125 is the current standard for comparison, this means that neither HE4 nor the ROMA algorithm improved the diagnosis of ovarian cancer. This is in contrast with the results of Moore *et al* [4], who found that a combination of CA125 and HE4 performed better than CA125 alone. However, Moore et al excluded all borderline tumours, non-epithelial ovarian cancers and metastatic tumours to calculate the performance of the tumour markers they tested [4]. We decided not to exclude these tumours in our initial analysis since we wanted to study a patient population that reflected a normal clinical setting. When borderline tumours, non-epithelial ovarian cancers and metastatic cancers were excluded, the AUC for CA125 was 0.937 *vs* 0.836 in the study by Moore *et al* [4]. By contrast, the AUCs of HE4 were similar: 0.914 (our data) *vs* 0.908 (Moore et al) [4]. In a more recent study, Moore *et al* included also borderline tumours in their analysis [18]. Within this study, the examination of benign cases *vs* all stages of epithelial ovarian cancers and borderline tumours revealed a AUC of 0.913. Within a setting of a multicenter prospective trial with central review and monitoring it seems plausible that a diagnostic test performs slightly better.

Compared to CA125, HE4 is inversely influenced by age; while CA125 is higher in healthy premenopausal patients [19;20], HE4 tends to be higher in postmenopausal patients [4;16;21]. These slightly higher normal values influence the performance of the tumour markers concerned. Although not significant, this can also be seen in our study population: the AUC of CA125 was higher in the postmenopausal group. Of particular interest, HE4 seems to have a slightly higher AUC in the premenopausal group than in the postmenopausal group. While this difference is not significant, it causes the ROC curves of CA125 and HE4 to come together in the premenopausal group and diverge in the postmenopausal group (Figure 3). In other words,

the performance of HE4 is similar to that of CA125 in the premenopausal group, but significantly worse in the postmenopausal group. This increased performance of HE4 in the premenopausal group is in agreement with previous studies [4;16], and confirms that CA125 and HE4 act independently from each other.

Due to the fact that ROC curves are not used in clinical practice, we aimed to find the cut-off points for the different tumour markers. The cut-off values corresponding to the highest accuracy (minimal false negative and false positive results) for all patients were 62.5 kU/l for CA125, 72.2 pM for HE4 and 22.2% for ROMA. In the product insert, it is suggested that 94.4% of the healthy female subjects (n = 179) that were studied had a HE4 value of 150 pM or below. If we define the reference value as the value that includes 95% of healthy controls, and we use this as a cut-off point to minimize the false positive rate, we obtain a sensitivity of 50.3% and a specificity of 96.5%. In clinical practice, this means that 3.5% of patients with a benign tumour will be treated as if they had a malignant tumour (over-treatment), and 49.7% of patients with a malignant tumour will be treated as if they had a benign tumour (under-treatment). Therefore, in our study, this cut-off point is not useful for differentiating benign from malignant cysts. Andersen *et al* also determined their cut-off at the 95th percentile in a healthy control group [16]. Based on this cut-off they obtained a sensitivity of 77.0% and a specificity of 94.9%. Unfortunately, they failed to mention what their cut-off value was. Using the cut-off point of 70 pM, as previously suggested by Moore *et al* [4], we reached a sensitivity of 74.5% and a specificity of 83.3%. This is therefore comparable to our ideal cut-off point of 72.2 pM, and is thus a reasonable cut-off point for HE4. Concerning ROMA, different cut-off points are used in premenopausal and postmenopausal patients. Both cut-off points are determined to provide a specificity level of 75% for the CA125 + HE4 assay combination. Our ideal cut-off points of 16.6% for the premenopausal patients and 35.9% for the postmenopausal patients were somewhat different from those suggested previously. However, these were not established at 75% specificity, but at the point on the ROC curve where we had minimal false negative and false positive results. Whether we analyse only invasive epithelial ovarian cancer or not, our ideal cut-off point in the premenopausal and postmenopausal category is higher than the suggested cut-off points of 12.5% and 14.4%, respectively.

As expected, histological subtypes seem to be important for the performance of the different tumour markers. Concerning benign tumours,

it was interesting to see that the fibromas/thecomas group and the endometriomas had the highest levels of CA125, whereas for HE4, the endometriomas had the lowest level. As already mentioned by Huhtinen *et al* [22], measuring both CA125 and HE4 together could be of particular interest in differentiating endometriosis from ovarian cancer, since ovarian cancer will cause a raised CA125 and HE4, whereas endometriosis will only cause a raised CA125. This could explain why HE4 performs better in premenopausal patients compared to postmenopausal patients, and vice versa for CA125. However, even for the premenopausal patients, HE4 and ROMA did not perform better than CA125. All malignant tumours expressed high levels of CA125 and HE4, but the highest levels were noted for the serous subtype. High expression levels of HE4 for the different epithelial subtypes, with the exception of the mucinous subtype, were already noticed in previous studies [11;23-25].

Although CA125, HE4, and ROMA are not currently recommended as a screening tool, it is interesting to see how well a tumour marker performs in early stage disease. A definite trend could be seen from stage I to stage IV disease, and CA125 and HE4 performed significantly worse when early and late stages of disease were compared. As a consequence, the ROMA algorithm also performed worse. With these AUCs, the chances that HE4 or ROMA will be successful as a screening marker are low, since very high specificities are required in screening for low prevalent disease.

In summary, this large independent validation study was able to demonstrate similar performance indices as those recently published in the literature. However, in our study, neither HE4 nor the ROMA algorithm increased the detection of malignant disease. HE4, or its combination with CA125, could be useful in diagnosing certain benign or malignant subtypes; however, this needs to be explored in more detail.

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Chapter 1b: Letter to the editor (comment and reply on Chapter 1)

Letter: The performance of the risk of ovarian malignancy algorithm

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Reply: The performance of the risk of ovarian malignancy algorithm

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Letter to the editor: The performance of the risk of ovarian malignancy algorithm

Z Langmár, M Németh, B Székely and G Borgulya

Sir,

In a recent manuscript, Van Gorp *et al* [1] compared the diagnostic performance of serum tumour markers CA125 and HE4 and the ROMA clinical risk stratification tool in a prospective collection of serum samples from patients with ovarian mass. They reported statistically insignificant differences between these three diagnostic tests. However, the non-significance of the results may be due to lack of power. A weakness of the study is that the authors have not explained how they calculated the sample size and what the power of the study was. Van Gorp *et al* started enrolling patients in August 2005 and ended in March 2009. The first publication on the diagnostic performance of ROMA became available in 2008 [2], and there were no published papers on the diagnostic performance of either ROMA or HE4 in 2005. Therefore, it is unlikely that the authors had prior knowledge on the performance of these tests that could have been used for sample size and power calculations. Since enrolment and design of this prospective study preceded the publications of the tests that it intended to validate, this raises the question if the study originally was started as a generic pelvic mass diagnostic study and was re-interpreted as a validation trial after the first ROMA and HE4 publications came out. It is unclear from the publication if all enrolled patients were tested for HE4, CA125 and ROMA or only subsamples. Publication of the selection criteria, if any, is critically important, as it could lead to bias in the results. We think that the incomplete description of the study objectives and study population substantially weakens the conclusions. The non-significant differences in test performance were interpreted by van Gorp *et al* as equivalence in test performance. The authors stated that 'HE4 and ROMA did not increase the detection of malignant disease compared to CA125 alone and neither HE4 nor ROMA increased the detection of malignant disease'. However, the AUC (95%CI) values for ROMA, CA125 and HE4 were 0.898 (0.863 – 0.926), 0.877 (0.840 – 0.908) and 0.857 (0.819 – 0.891), respectively, which shows a trend for better performance for ROMA. Approximate 95%CIs can be calculated from the data presented in the paper: AUC(ROMA) - AUC(CA125) = 0.021 (-0.009 – 0.051), AUC(ROMA) - AUC(HE4) = 0.041 (0.017 – 0.065), AUC(CA125) - AUC(HE4) = 0.020 (-0.018 – 0.058). Therefore, about 5.1 – 6.5% AUC gain when using ROMA in place

of CA125 or HE4 cannot be excluded with statistical certainty. Van Gorp *et al* concluded that measurement of HE4 serum levels does not contribute to the diagnosis of ovarian cancer. This conclusion could have relied on comparisons between AUC(CA125) and AUC(CA125 and HE4 combined) or AUC(CA125 and menopausal status combined) *vs* AUC(ROMA); however, such comparisons were not described in the paper. The AUC(ROMA) - AUC(CA125) comparison may be considered here, and according to the approximate 95%CI the gain may be as large as 5.1%, less the gain attributable to considering menopausal status. Van Gorp *et al* stated that even for the pre-menopausal patients, HE4 and ROMA did not perform better than CA125. Indeed, in this study among pre-menopausal patients, CA125 had a higher AUC than either HE4 or ROMA. These were, however, not significant, and the wide CIs did not even exclude the case that ROMA was 6.1% higher than CA125 in terms of AUC. Based on these considerations, a more appropriate conclusion would be the following: In this study ROMA had a higher AUC value discriminating between malignant and benign tumours than CA125 alone by 2.1%; this difference was, however, not significant. A rough approximation of the 95%CI of the AUC gain (-0.009 – 0.051) does not exclude a large 5.1% AUC gain when using ROMA in place of CA125 alone, but an AUC loss of 0.9% is also compatible with the data. In the pre-menopausal subgroup ROMA performed worse than CA125 alone, AUC difference -0.010 (rough CI: -0.081 – 0.061); the wide CI allows for both a substantial AUC gain and a substantial AUC loss.

A further important consideration is that the Van Gorp's study had a high cancer incidence (41.4%) rate in comparison with Moore's study (24.3%) [3] and a much higher proportion of postmenopausal women were included (~74% in Van Gorp *vs* 53% in the Moore study). There were also differences in the distribution of histological types of tumours. Important differences that could account for the weaker performance of HE4 in the Van Gorp's study include higher proportion of mucinous tumours, tumours of Low Malignant Potential (LMP) and metastatic tumours of extra-ovarian origin, and lower number of serous tumours. There was also a small difference in Stage I/II *vs* Stage III/IV distribution, with more early-stage tumours in Van Gorp's study. Moore's study had a higher proportion of serous epithelial ovarian cancer (64.3 *vs* 52.2%) and more of the endometrioid type (12.4% *vs* only 4.3% in Van Gorp's study). These histological types tend to over-express HE4 and contribute to favourable HE4 performance. Van Gorp's study had more mucinous tumours (13 *vs* 4.7% in Moore's study). This tumour type tends not to express HE4 and contributes to the lower performance of HE4. Van Gorp's study had a higher proportion of LMP

tumours (23.7% *vs* 12.3% in Moore's study) and HE4 tends to lack the sensitivity to detect non-invasive LMP tumours.

Van Gorp *et al* stated that combining HE4 and CA125 in ROMA improved HE4 but not CA125 performance. As CA125 is the current standard for comparison, this means neither HE4 nor the ROMA improved the diagnosis of ovarian cancer. This statement is also debatable, as it is not shown what percentage of patients had increased levels of CA125 in this study. If the majority of patients had increased CA125, in the first place no marker alone or marker combination could improve on it. The interpretation of the HE4 thresholds is also arguable: the optimal threshold depends on the characteristics of the population and the consequences of the true and false test outcomes; but in any case HE4 is not intended to be used for screening in a group comparable to the healthy controls, rather among patients with adnexal mass.

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Authors' Reply: The performance of the risk of ovarian malignancy algorithm

T Van Gorp, D Timmerman and I Vergote

Sir,

We appreciate the data and comments provided by Langmár *et al* [1] regarding our recent report 'HE4 and CA125 as a diagnostic test in ovarian cancer: prospective validation of the Risk of Ovarian Malignancy Algorithm'[2].

In their letter Langmár *et al* suggest that we did not start this prospective study as a generic pelvic mass diagnostic study. They draw this conclusion by the fact that we started this study in 2005, and yet the studies from Moore *et al* were only published in 2008 and 2009 [3;4]. However, we must point out that several publications on HE4 were already published between 2003 and 2005 [5-8]. Between August 2005 and March 2009, 432 consecutive patients with a pelvic mass, who planned to undergo an operation, were eligible to participate in our study. Five patients withdrew consent during the study. Seventeen patients did not have the serum sample taken, or the volume of the retrieved serum was insufficient to perform the marker assays. Pathology reports were missing in 21 patients because the patients were eventually not operated upon due to poor prognosis or patients' decline, or because there was no biopsy taken and therefore no proven histological diagnosis. The remaining 389 patients participated in this study. In 2008 we were confronted with the results from Moore and colleagues. Since they published a significant difference between the performance of CA125 and CA125+HE4 in a study with only 233 patients, one could expect that we should have been able to show a significantly different result with our 389 patients, which is the largest monocentre cohort study on HE4. In fact, if one uses the AUCs of CA125 (0.836) and the combination of CA125 and HE4 (0.914) from the article of Moore *et al*, with an alpha level of 0.05 and a beta level of 0.20, one can obtain a required minimum sample size of 86 in each group. With a sample size of 389 patients, we broadly exceeded the required number of samples.

The authors also suggest that there was a selection bias, with an increased amount of cancers, postmenopausal patients, mucinous tumours, borderline tumours and metastatic tumours of extra-ovarian origin. Indeed, the bigger proportion of these specific categories is a reflection of a patient

population with adnexal masses in a tertiary centre as ours. Recruitment was also largely done in collaboration with our ultrasound department, which is an expert centre in the diagnosis of pelvic masses [9]. Hence, there was a relatively large proportion of stage I and borderline tumours in our study, which are more difficult to differentiate from their benign counterparts compared with stage III or IV ovarian cancers. The fact that we were not able to show a statistical difference between CA125 and ROMA illustrates that the ROMA algorithm does not seem to be of any benefit in this particular setting, which in our opinion is an important finding for many centres. Moreover, in this setting, with more difficult tumours to diagnose, the addition of a good diagnostic tumour marker could have been of great importance, and yet, the addition of HE4 to CA125 does not seem to fulfil these expectations.

Langmár *et al* also claim that the non-significant differences between the AUCs of the different tests cannot exclude a gain in diagnostic accuracy with statistical certainty. This strikes us as a *contradictio in terminis*. As mentioned in the methods section, we used the non-parametric method as described by DeLong *et al* [10] to calculate differences in AUCs. The method of DeLong *et al* is the most widely used method to perform these comparisons. We do not dispute the calculated differences in AUCs and 95% confidence intervals by Langmár *et al*, but as the *p*-level was 0.17 we were not able to reject the null hypothesis, and thus in our setting this means that the results were not statistically different. All comparisons and corresponding *P*-values are clearly mentioned in our results section and are illustrated in the tables and figures.

Finally, Langmár *et al* question our interpretation of the HE4 threshold. They state that the optimal threshold depends on the characteristics of the population and the consequences of the true and false test outcomes. Again, we fully agree with this last remark. In the diagnosis of ovarian cancer one should try to detect as many of the ovarian cancers as possible, and yet avoid unnecessary surgeries for benign cysts. This implies that sensitivity is very important; however, increasing morbidity, costs, pain and fear by performing midline laparotomies for benign cysts are also unacceptable. Therefore we have looked at the optimal cut-off rather than a cut-off at a fixed sensitivity or specificity. As mentioned in our article, the optimal cut-off corresponds to the point on the ROC curve with the highest accuracy. As we did not use healthy controls, we did not intend to find a cut-off for HE4 that could be used in screening. Moreover, we clearly stated that using a cut-off of 150 pM, as suggested in the product insert, would

result in high specificity (96.5%), but in very low sensitivity (50.3%). Therefore, a cut-off of 150 pM cannot be used in a diagnostic situation in a population as ours. A cut-off of 70 pM is more appropriate and very close to our calculated optimal cut-off of 72.2 pM.

In conclusion, we understand that these negative results for this promising HE4 marker and ROMA algorithm are disappointing. We can only conclude that neither HE4 nor the combination of HE4 and CA125 in the ROMA algorithm seems to be an improvement in the diagnosis of ovarian cancer in patients with an adnexal mass as referred to a tertiary centre as ours.

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Chapter 2: Subjective assessment by ultrasound is superior to the risk of malignancy index (RMI) or the risk of ovarian malignancy algorithm (ROMA) in discriminating benign from malignant adnexal masses

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Abstract:

Purpose: The combination of two tumour markers, CA125 and HE4, in the ROMA has been shown to be successful in classifying patients into those who have a high or low risk of epithelial ovarian cancer. In the present study, the diagnostic accuracy of ROMA was assessed and compared to the diagnostic accuracy of the two most widely used ultrasound methods, namely the risk of malignancy index (RMI) and subjective assessment by ultrasound.

Methods: From August, 2005 to March, 2009, 432 women with a pelvic mass who were scheduled to have surgery were enrolled in a single-centre prospective cohort study. A preoperative ultrasound was performed and preoperative CA125 and HE4 serum levels were measured. Once the final surgical pathology reports were obtained, the diagnostic accuracy and performance indices of ROMA, RMI and subjective assessment were calculated.

Results: Of the 432 eligible patients, 374 could be analyzed. Subjective assessment had the highest AUC under the ROC curve (0.968, 95%CI: 0.945 – 0.984), followed by the RMI (0.931, 95%CI: 0.901 – 0.955). The subjective assessment and RMI both had significantly higher AUCs than the ROMA (0.893, 95%CI: 0.857 – 0.922; $p < 0.0001$ and $p = 0.0030$, respectively). The pre- and postmenopausal populations generated similar results.

Conclusion: Although new tumour markers models are promising, they do not contribute significantly to the diagnosis of ovarian cancer. Ultrasound, especially subjective assessment by ultrasound, remains superior in discriminating malignant from benign ovarian masses.

Introduction:

Ovarian cancer is the sixth most common cause of cancer-related death among women in Europe [2]. Differentiating between benign and malignant pelvic masses is difficult due to the anatomical localization of the ovaries. Generally, women are evaluated on the basis of their personal history, a clinical examination, ultrasound, and tumour marker levels. CA125 is the most widely used tumour marker in ovarian cancer [3]. A significant problem associated with CA125 is that it can be expressed in numerous benign and malignant conditions, which leads to false positive results; moreover, it is only expressed by about 50% of early stage ovarian cancers, which leads to false negative results [4]. Another tumour marker which gained attention is the HE4. HE4 is overexpressed by ovarian and endometrial cancer [5-7]. Moore *et al* developed an algorithm, the ROMA, which is based on both CA125 and HE4. The ROMA was suggested to be superior to CA125 alone [5]. Some validation studies confirmed the superiority of the ROMA to CA125 alone [7], while others did not [8;9].

Sonography by greyscale and colour Doppler imaging is also used widely to classify ovarian masses. While many scoring systems and models have been described, the risk of malignancy index (RMI) is probably the most widely used model at present [10;11]. The RMI is calculated by an algorithm based on several ultrasound variables, the menopausal status, and the CA125 level. Its relative simplicity makes it easy to use. Another ultrasound method used to evaluate ovarian masses is the subjective impression of a sonographer, the so-called subjective assessment or pattern recognition. Subjective assessment is a highly accurate method for discriminating benign from malignant ovarian masses [12-16].

Previous studies have compared the ROMA to RMI [9;17], but comparison between subjective assessment and the ROMA has never been made. In 2005, we initiated a prospective cohort study to validate newly discovered biomarkers such as HE4. In a previous study, this cohort was used to compare the accuracy of ROMA to that of CA125 [8]. The aim of this study was to determine whether ultrasound models are similar or superior to the ROMA.

Materials and methods:

Patients:

From August, 2005 until March, 2009, 432 consecutive women were found to be eligible to participate in a prospective single-centre cohort study conducted at the University Hospitals Leuven. Patients were considered to be eligible if they were diagnosed with a pelvic mass that was suspected to be of ovarian origin and they were to undergo surgery. Prior to surgery, imaging by pelvic ultrasound was performed and a serum sample was taken for tumour marker analysis. Patients with a prior bilateral oophorectomy were not eligible. Patients who were diagnosed with ovarian cancer were completely surgically staged. Prior to enrolment in the study, all patients were required to give fully informed consent. The protocol was approved by the Local Ethics Committee (reference: OG032/ML3132). Patient participation in the study was concluded once the final surgical pathology reports were obtained.

Ultrasound:

All ultrasound examinations were performed in the same department by a standardized examination technique that employed standardized terms and definitions and high-quality ultrasound equipment [18]. The examiner was an experienced sonographer or a trainee supervised by an experienced sonographer. Transvaginal sonography was performed in all cases. Transabdominal sonography was added to examine large masses that could not be seen in their entirety by using a transvaginal probe.

RMI: Defined as U × M × CA125, where U = the ultrasound score, M = menopausal status, and CA125 = the level of this marker [10]. U was calculated as follows: multilocularity, solid areas, bilaterality, ascites and intra-abdominal metastases each scored one point and total scores of 0, 1, and ≥2 points yielded U values of 0, 1, and 3, respectively. Postmenopausal status, which was associated with an M score of 3, and was defined as more than 1 year of amenorrhea, or an age of 50 years or older if the woman had had a prior hysterectomy. A premenopausal status yielded an M score of 1. Since the CA125 level (U/mL) was not disclosed to the sonographer at the time of the ultrasound examination, this was entered into the equation after the ultrasound report was finished. A cut-off of 200 was used to differentiate between benign and malignant, as suggested in the literature [10].

Subjective assessment: On the basis of greyscale and colour Doppler findings, the ultrasound examiner was obliged to give his/her subjective impression in two ways: (a) classification of each mass as benign or malignant, and (b) expressing his/her level of confidence as follows: benign, probably benign, uncertain, probably malignant, or malignant. The category ''uncertain'' was split into two subcategories: uncertain but initially classified as benign, and uncertain but initially classified as malignant.

Serum samples and marker assays:

Immediately before surgery, blood samples were obtained in 10 ml clotting tubes (BD Vacutainer® Serum Tube, ref. 369033). Serum tubes were centrifuged at 800 *x g* for 10 minutes. The serum was collected, dispensed into cryotubes, and frozen at -80°C. CA125 and HE4 concentrations were measured by using the CanAg CA125 EIA assay and HE4 EIA assay (Fujirebio Diagnostics, Göteborg, Sweden), according to the manufacturer's instructions. Each ELISA was performed manually in duplicate. CA125 and HE4 were combined in the ROMA, as described previously [8]. For premenopausal and postmenopausal patients, cut-offs of 12.5% and 14.4%, respectively, were used [19].

Histology

The histology of the tumours was classified according the World Health Organization classification of tumours [20]. Borderline tumours were not excluded from the present analysis and were classified as malignant tumours.

Statistical analysis:

Statistical analysis was performed with MedCalc v11.5.1.0 (MedCalc Software, Mariakerke, Belgium). Mean patient ages were compared by using the independent Student's t-test (Welch-test) and menopausal status was compared by using the Chi-square test. ROC curves were constructed and the AUC with binomial exact 95%CIs were calculated [21]. Using the six levels of diagnostic confidence as different cut-offs, a ROC curve could be constructed for the subjective assessment as well. The method described by DeLong *et al* [1] was used to calculate the difference between two AUCs.

The diagnostic performance of the models was also expressed as sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios when using the recommended cut-off values for the ROMA and the RMI. Since the sonographer had to distinguish between benign and malignant, this was also used as a cut-off. However, the

sensitivity and specificity of a model depend on the chosen cut-off, whereas the AUC reflects overall test performance. Therefore, the AUC was considered to be the most important measure of diagnostic performance.

Since both the RMI and the ROMA include the menopausal status in their algorithm, the statistical analysis was performed on the whole population and after stratification for menopausal status. Other exploratory subset analyses are provided in a Supplementary File. For all statistical comparisons, a level of $p < 0.05$ was accepted as being statistically significant.

Results:

Patient and tumour characteristics:

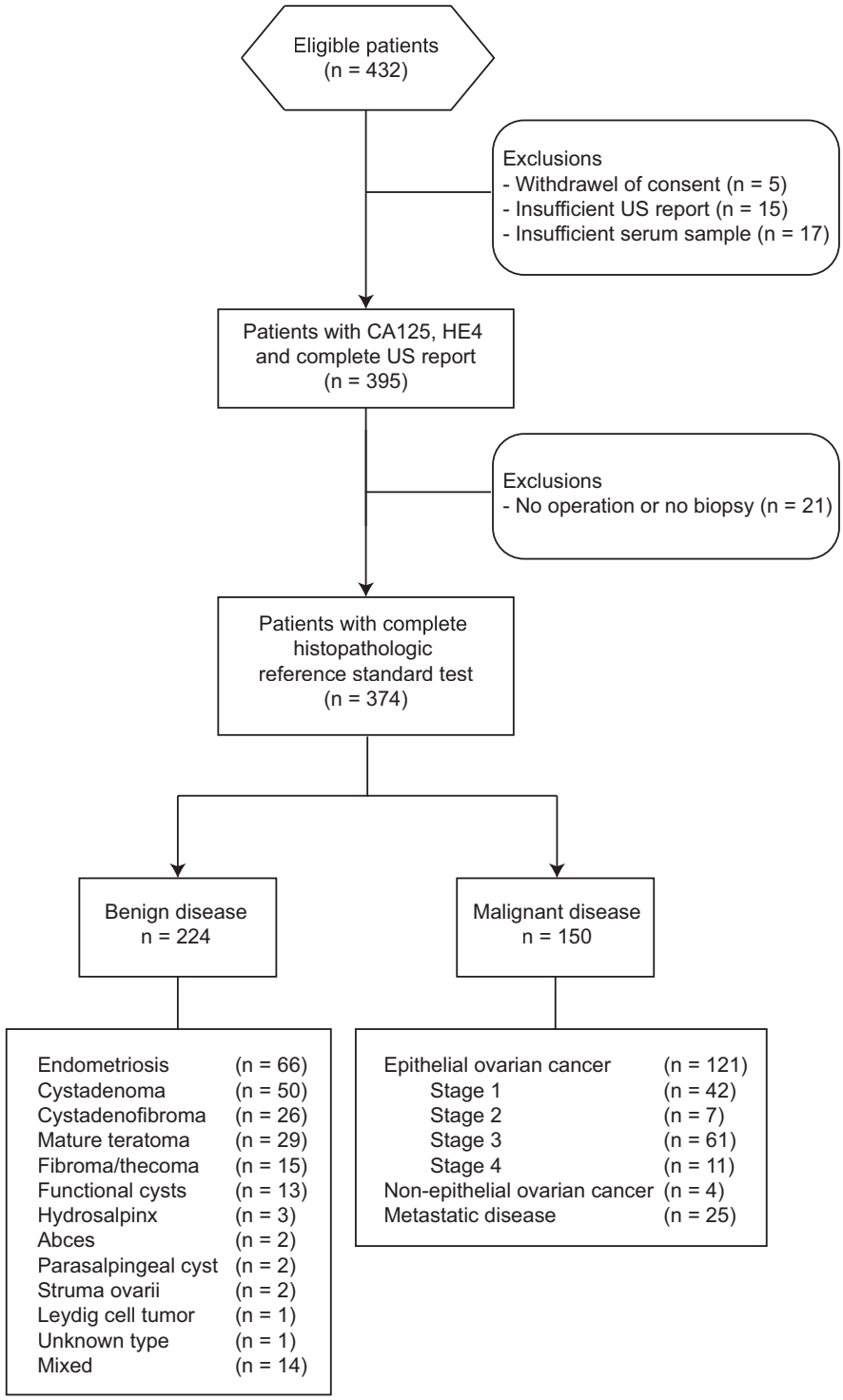
Of the 432 eligible patients, 374 could be analyzed (Figure 1). The reasons for patient exclusion are detailed in Table 1.

Table 1: Reasons for patient exclusion or non-eligibility and their final histological diagnosis.

	n	Final diagnosis		
		Benign	Malignant	Unknown
Withdrawal/refusal of consent	5	-	-	5
Insufficient ultrasound report				
No ultrasound performed	13	3	10	0
Data missing	2	1	1	0
Insufficient serum sample				
No sample taken	5	2	3	0
Insufficient volume of sample	11	3	8	0
Problem processing sample in lab	1	1	0	0
No operation or no biopsy				
Conservative management due to poor prognosis	4	0	4	0
Conservative management for a presumed benign cyst	6	6	0	0
Operated in other hospital: no pathology report obtained	5	0	0	5
No cyst at the time of operation	6	6	0	0
Total	58	22	26	10

For patients without a proven histological diagnosis (no operation or biopsy), the presumed diagnosis was based on the patient's clinical course.

Figure 1: Flow diagram indicating the inclusion and exclusion of eligible patients.



Of the 374 analyzed patients, 224 (59.9%) and 150 (40.1%) patients had benign and malignant disease, respectively. Patients with benign disease were younger (mean age = 46.2 [95%CI: 44.1 – 48.3] *vs* 57.7 [95%CI: 55.7 – 59.8] years; $p < 0.0001$). Of the patients with benign disease, 37.9% (95%CI: 31.6 – 44.3) were postmenopausal, while 74.0% (95%CI: 67.0 – 81.0) of the patients with malignant disease were postmenopausal ($p < 0.0001$).

Table 2: Tumour characteristics of malignant disease.

	n	%
Histological type		
Epithelial	121	80.7
Serous	76	50.7
Mucinous	21	14.0
Endometrioid	6	4.0
Clear cell	6	4.0
Mixed	5	3.3
Carcinosarcoma	4	2.7
Undifferentiated	3	2.0
Granulosa cell	2	1.3
Sarcoma	2	1.3
Metastatic	25	16.7
Endometrium	11	7.3
Colon	5	3.3
Appendix	3	2.0
Mesothelioma	1	0.7
Breast	1	0.7
Lung	1	0.7
Lymphoma	1	0.7
Pancreas	1	0.7
Stomach	1	0.7
Total	*150*	*100.0*
Differentiation grade*		
Borderline	31	25.6
Well differentiated	12	9.9
Moderately differentiated	14	11.6
Poorly differentiated	64	52.9
Total	*121*	*100.0*

*For epithelial ovarian cancer only

The most common benign ovarian tumours were endometriomas, cystadenomas, mature teratomas, cystadenofibromas, fibromas/thecomas and functional cysts (Figure 1). Mixed tumours (n = 14) contain two or more different histological subtypes, making it impossible to categorize these tumours into a specific subtype. The cystadenomas and cystadenofibromas included 46 serous, 25 mucinous, and five other histological types or mixed cystadenomas/cystadenofibromas. The majority of the malignant tumours were epithelial ovarian cancers (Table 2). Most of the epithelial ovarian cancers were high grade and diagnosed at an advanced stage (Table 2 and Figure 1). The non-epithelial primary ovarian tumours were sex cord stromal tumours (n = 2) and sarcomas (n = 2). Along with the primary ovarian tumours, 25 extra-ovarian primary tumours with metastases to the ovary were diagnosed. The metastatic tumours were mainly of an endometrial or gastrointestinal origin.

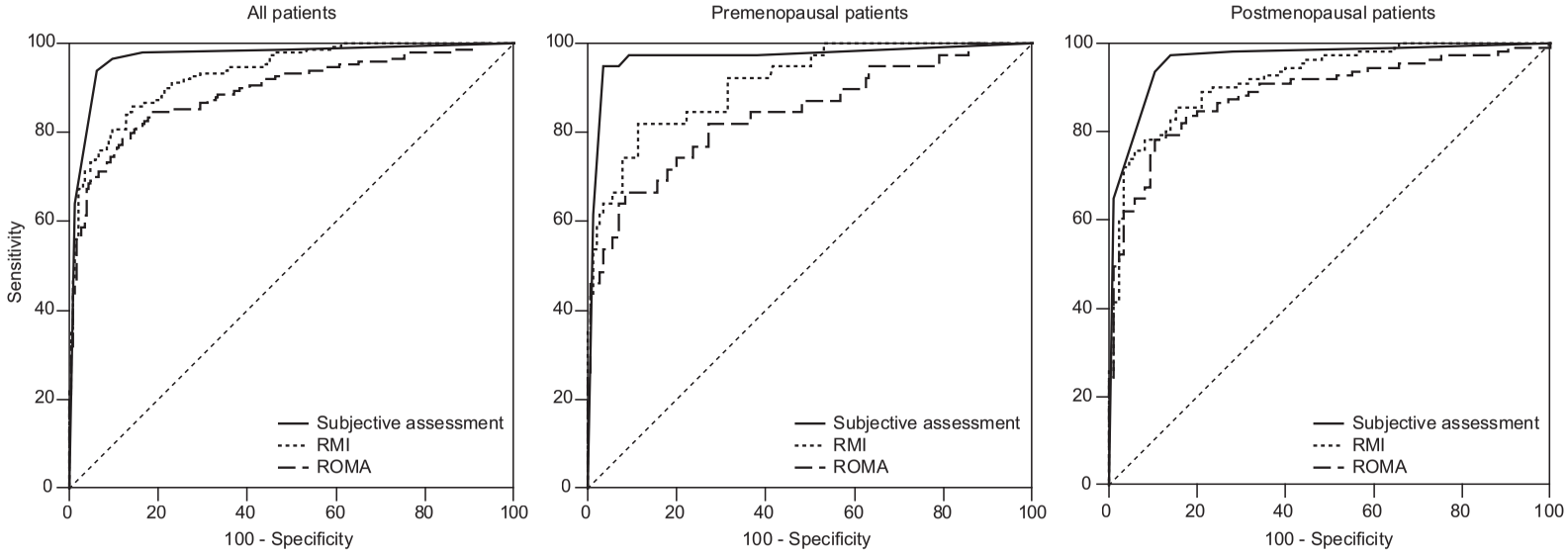
ROC curves:

For the whole study population, subjective assessment was associated with the highest AUC, followed by the RMI, and the ROMA (Figure 2). Pairwise comparison of ROC curves (Table 3) indicated that the AUCs of both ultrasound models (subjective assessment and RMI) were significantly larger than the AUC of the ROMA. Similar results were found after stratification according to menopausal status. When subjective assessment and the RMI were compared to each other, subjective assessment performed significantly better, even after stratification according to menopausal status (Table 3). Exploratory subset analyses (excluding borderline tumours, metastatic tumours, non-epithelial ovarian tumours, and/or advanced stage disease are provided in a Supplementary File. Subjective assessment was consistently associated with the highest AUC.

Performance indices:

The calculated sensitivities and specificities at the recommended cut-off values are shown in Table 4. Subjective assessment scored the highest overall in terms of sensitivity for the whole study population as well as the postmenopausal and premenopausal populations. The RMI had the highest specificity for the whole study population and the postmenopausal population. For the premenopausal population, all diagnostic tests had a high specificity but this was accompanied by a sensitivity below 70% for the RMI, and ROMA.

Figure 2: ROC curves for the detection of malignant disease (including borderline ovarian tumours) for subjective assessment with sonography, risk of malignancy index (RMI), and risk of ovarian malignancy algorithm (ROMA) in the whole population, the pre-menopausal population, and the post-menopausal population. Total AUC values with corresponding 95%CIs are listed below the curves.



	AUC	95%CI
Subjective assessment	0.968	0.945 - 0.984
RMI	0.931	0.901 - 0.955
ROMA	0.893	0.857 - 0.922

	AUC	95%CI
Subjective assessment	0.967	0.929 - 0.988
RMI	0.912	0.860 - 0.949
ROMA	0.842	0.780 - 0.892

	AUC	95%CI
Subjective assessment	0.961	0.924 - 0.984
RMI	0.921	0.873 - 0.954
ROMA	0.886	0.833 - 0.927

Table 3: Differences in the area under the curve (AUC) of the receiver operating characteristic (ROC) curves for the diagnosis of malignant disease (including borderline ovarian tumours) with the corresponding 95% confidence intervals (95%CI) and *p*-values. Pairwise ROC curve comparisons were calculated for the whole study population, for the postmenopausal population, and for the premenopausal population. The method described by DeLong *et al* was used to calculate the difference between two AUCs [1].

Subjective assessment *vs* ROMA						
	All patients		**Premenopausal**		**Postmenopausal**	
Difference	**95%CI**	**P-value**	**Difference**	**95%CI**	**Difference**	**95%CI**
0.076	0.043–0.109	< 0.0001	0.125	0.054–0.197	0.075	0.028–0.121
RMI *vs* ROMA						
	All patients		**Premenopausal**		**Postmenopausal**	
Difference	**95%CI**	**P-value**	**Difference**	**95%CI**	**Difference**	**95%CI**
0.039	0.013–0.064	0.0030	0.070	0.003–0.136	0.034	0.007–0.061
Subjective assessment *vs* RMI						
	All patients		**Premenopausal**		**Postmenopausal**	
Difference	**95%CI**	**P-value**	**Difference**	**95%CI**	**Difference**	**95%CI**
0.037	0.011–0.063	0.0058	0.056	-0.004–0.115	0.041	0.003–0.078

Abbreviations: RMI = risk of malignancy index; ROMA = risk of ovarian malignancy algorithm.

Table 4: Sensitivity, specificity, positive likelihood ratio (+ LR), negative likelihood ratio (- LR), positive predictive value (PPV) and negative predictive value (NPV) of subjective assessment, the risk of malignancy index (RMI), and the risk of ovarian malignancy algorithm (ROMA) for malignant disease (including borderline ovarian tumours). The diagnostic performance indices are calculated for the whole study population, the postmenopausal population, and the premenopausal population. The 95% confidence intervals (95%CI) are indicated between brackets.

		Subjective assessment		RMI		ROMA	
All patients	Sensitivity	96.7%	(92.4 – 98.9%)	76.0%	(68.4 – 82.6%)	84.7%	(77.9 – 90.0%)
	Specificity	90.2%	(85.5 – 93.7%)	92.4%	(88.1 – 95.5%)	76.8%	(70.7 – 82.2%)
	+ LR	9.84	(6.61 – 14.65)	10.01	(6.29 – 15.95)	3.65	(2.85 – 4.67)
	- LR	0.04	(0.02 – 0.09)	0.26	(0.19 – 0.35)	0.20	(0.14 – 0.29)
	PPV	86.8%	(80.7 – 91.6%)	87.0%	(80.0 – 92.3%)	71.0%	(63.7 – 77.5%)
	NPV	97.6%	(94.5 – 99.2%)	85.2%	(80.1 – 89.4%)	88.2%	(82.8 – 92.4%)
Post-menopausal	Sensitivity	97.3%	(92.3 – 99.4%)	80.2%	(71.5 – 87.1%)	91.0%	(84.1 – 95.6%)
	Specificity	85.9%	(76.6 – 92.5%)	87.1%	(78.0 – 93.4%)	58.8%	(47.6 – 69.4%)
	+ LR	6.89	(4.08 – 11.65)	6.20	(3.54 – 10.84)	2.21	(1.70 – 2.87)
	- LR	0.03	(0.01 – 0.10)	0.23	(0.16 – 0.33)	0.15	(0.08 – 0.28)
	PPV	90.0%	(83.2 – 94.7%)	89.0%	(81.2 – 94.4%)	74.3%	(66.1 – 81.4%)
	NPV	96.1%	(88.9 – 99.2%)	77.1%	(67.4 – 85.1%)	83.3%	(71.5 – 91.7%)
Pre-menopausal	Sensitivity	94.9%	(82.7 – 99.4%)	64.1%	(47.2 – 78.8%)	66.7%	(49.8 – 80.9%)
	Specificity	92.8%	(87.2 – 96.5%)	95.7%	(90.8 – 98.4%)	87.8%	(81.1 – 92.7%)
	+ LR	13.19	(7.23 – 24.07)	14.85	(6.56 – 33.62)	5.45	(3.31 – 8.97)
	- LR	0.06	(0.01 – 0.21)	0.38	(0.25 – 0.57)	0.38	(0.24 – 0.59)
	PPV	78.7%	(64.3 – 89.3%)	80.7%	(62.5 – 92.6%)	60.5%	(44.4 – 75.2%)
	NPV	98.5%	(94.6 – 99.8%)	90.5%	(84.5 – 94.7%)	90.4%	(84.1 – 94.8%)

The cut-off values used were 200 for the RMI, and 12.5% and 14.4% for the premenopausal and postmenopausal patients with the ROMA, respectively. The prevalence of malignancy: all patients 40.1% (95%CI: 35.1 – 45.3%), postmenopausal patients 56.6% (95%CI: 49.4 – 63.7%), and premenopausal patients 21.9% (95%CI: 16.1 – 28.7%).

Discussion:

Over the past few years, the performance of HE4 and the ROMA to classify ovarian masses has been studied many times. We showed previously that CA125, HE4 and ROMA perform equally well [8]. In the present study, the ability of the ROMA to diagnose ovarian cancer was compared to that of greyscale and colour Doppler ultrasound. The present data suggest that ultrasound methods are superior to ROMA to classify ovarian masses. Moreover, subjective assessment was superior to the RMI.

The RMI is a very popular because of its simplicity: little experience is needed to detect the different ultrasound features that have to be scored (multilocularity, solid areas, bilaterality, ascites and intra-abdominal metastases), and the algorithm can be memorized readily. It also enables general gynaecologists to refer patients on an objective basis to gynaecological oncologists [22]. With an AUC of 0.931, it seems that the overall performance of the RMI is good. However, due to a high false negative rate at the suggested cut-off of 200, there was a low sensitivity of only 76.0%. This is in accordance with a recent review that calculated a pooled estimate of sensitivity of 78% [11]. This means that in one of four cases, the tumour will be wrongly diagnosed as benign. In the worst case scenario, these patients will not be referred to a gynaecological oncologist and will be operated on by laparoscopy, which is associated with an increased risk of spillage of cyst fluid [23]. Both the non referral and cyst fluid spillage might decrease the overall survival [24;25].

The RMI and ROMA have been compared previously in two studies [9;17]. One was a multicentre study by Moore *et al* that determined the RMI and ROMA values for 457 patients [17]. At a fixed specificity of 75%, the ROMA had a sensitivity of 94.3% while the RMI had a sensitivity of 84.6% (p = 0.0029). However, despite the fact that there was an important quality control for the ROMA (central lab measurements and quality assurance), there was no central radiology review or standardization of the imaging reports. Moreover, the RMI was calculated by using information from a variety of imaging techniques (ultrasound, CT scan and MRI), with only 85.3% of patients having received a standard ultrasound scan. The RMI was developed to be used with ultrasound and was not validated for other imaging techniques. CT scans are usually not indicated in the evaluation of adnexal masses because of poor soft tissue discrimination [26]. In a second study that compared the ROMA to the RMI, the results of both algorithms

in 127 patients with benign or malignant ovarian disease were compared [9]. Although all kinds of subgroups were analyzed in the article, the authors did not describe the overall performance of ROMA and RMI in the comparison of benign *vs* malignant disease, even though this is the most important comparison to be made in a diagnostic setting.

With an AUC of 0.968, subjective assessment appears to be an excellent method to discriminate between benign and malignant adnexal masses. Moreover, its sensitivity is 96.7%, which means that only one of 30 cases will be misdiagnosed as a benign mass. Subjective assessment has been validated by numerous studies [12-15]. Ultrasound examiners take demographic, clinical and ultrasound information into account when they evaluate an adnexal mass, and they subconsciously apply their experience from previous examinations during subsequent evaluations of the adnexal masses. The level of ultrasound expertise therefore has a marked influence on the quality of an ultrasound examination [27]. The International Ovarian Tumour Analysis (IOTA) study group has sought to tackle this problem by developing a two-tiered diagnostic setup. Ten simple ultrasound rules were developed for discriminating between benign and malignant adnexal masses [28]. These rules were applicable in three-quarters of patients with an ovarian mass with a sensitivity of 92% and a specificity of 96% [29]. When these simple rules do not apply the patient should be referred to an expert ultrasound centre. This two-tiered diagnostic setup yielded a sensitivity of 91% and a specificity of 93%.

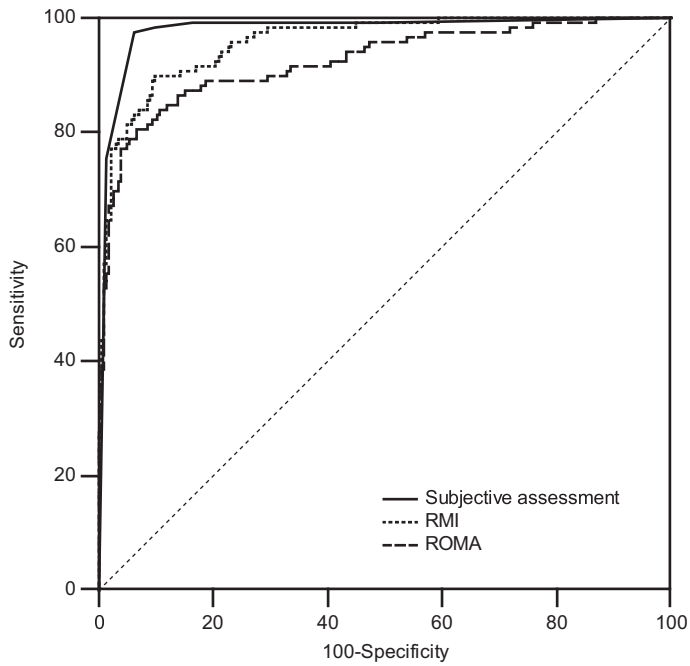
The present study had certain limitations. One was that 13.4% of all eligible patients were excluded; the majority had malignant adnexal masses. We presume that in these cases, ultrasound was not performed due to time constraints. The serum volume was also more frequently insufficient due to a larger number of blood tubes taken for other analyses. Second, the data were obtained in a tertiary referral centre with a specialized gynaecological ultrasound unit. The high prevalence of malignant disease in this centre will have influenced the predictive values in this study. A lower prevalence of ovarian cancer would have decreased the positive predictive value and increased the negative predictive value. However, this would also have been true for all of the diagnostic tests examined in this study. Moreover, in smaller hospitals with a lower prevalence of ovarian cancer, a test with the same sensitivity and specificity would yield an even higher negative predictive value. Third, the fact that these results were obtained within a tertiary referral centre will also have increased the experience of the sonographers and thereby improved the performance of the ultrasound

methods, in particular the subjective assessment. Nevertheless, we hope that the present study will encourage sonographers to increase their knowledge and experience by training in gynaecological sonography.

In summary, although much energy has been put into the discovery and validation of new tumour marker algorithms, such as the ROMA, the present study suggests that the diagnostic value of these algorithms is limited compared to sonography. In particular, subjective assessment seems to be highly accurate. The fact that subjective assessment is influenced by experience should not discourage sonographers in non-expert centres to increase their knowledge and expertise.

Supplementary File 1: ROC curves of subjective assessment, RMI and ROMA for the diagnosis of invasive malignant disease (excluding all borderline ovarian tumours from the analysis). AUC is given with the corresponding 95%CI.

Tumour	Sample size
Invasive malignant	119
(exclusion of borderline tumours)	
Benign	224
Total	343



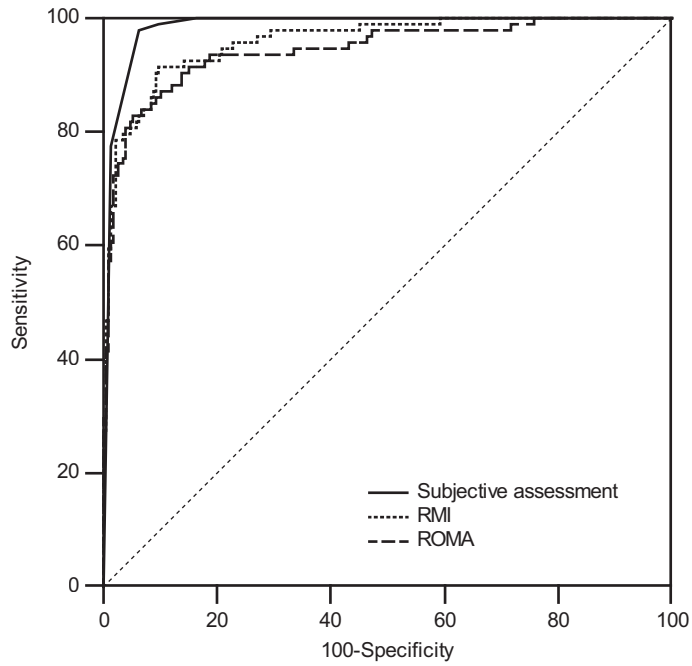
Method	AUC	95%CI
Subjective assessment	0.979	0.957 – 0.991
RMI	0.958	0.931 – 0.977
ROMA	0.924	0.890 – 0.949

Pairwise ROC curve comparisons. The method described by DeLong *et al* was used to calculate the difference between two AUCs [1]. Difference in AUC is given with the corresponding 95%CI.

Comparison			Difference	95%CI
Subjective assessment	*vs*	RMI	0.021	-0.002 – 0.043
Subjective assessment	*vs*	ROMA	0.055	0.025 – 0.086
RMI	*vs*	ROMA	0.034	0.010 – 0.059

Supplementary File 2: ROC curves of subjective assessment, RMI and ROMA for the diagnosis of invasive ovarian cancer (excluding all borderline ovarian tumours and metastatic tumours from the analysis). AUC is given with the corresponding 95%CI.

Tumour	Sample size
Invasive ovarian cancer	94
(exclusion of borderline/metastatic tumours)	
Benign	224
Total	318



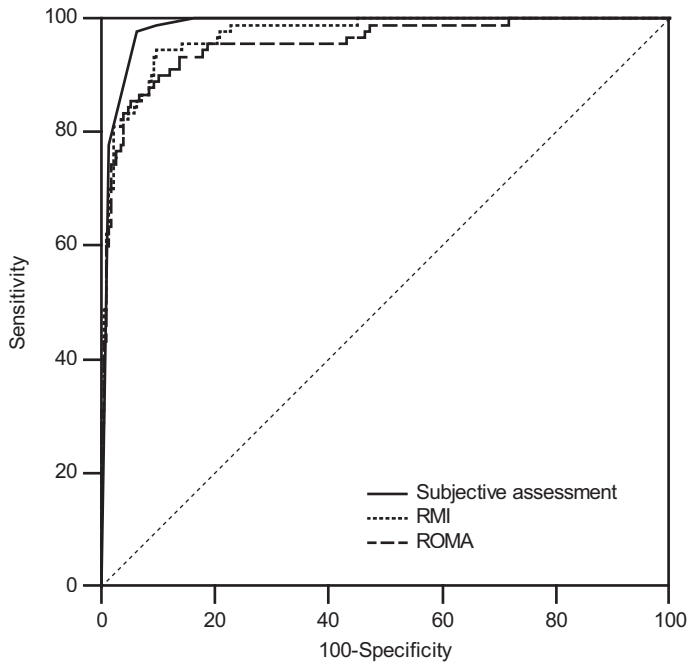
Method	AUC	95%CI
Subjective assessment	0.985	0.965 – 0.995
RMI	0.959	0.931 – 0.978
ROMA	0.944	0.913 – 0.967

Pairwise ROC curve comparisons. The method described by DeLong *et al* was used to calculate the difference between two AUCs [1]. Difference in AUC is given with the corresponding 95%CI.

Comparison			Difference	95%CI
Subjective assessment	*vs*	RMI	0.026	0.003 – 0.048
Subjective assessment	*vs*	ROMA	0.041	0.010 – 0.071
RMI	*vs*	ROMA	0.015	-0.005 – 0.035

Supplementary File 3: ROC curves of subjective assessment, RMI and ROMA for the diagnosis of invasive epithelial ovarian cancer (excluding all borderline ovarian tumours, metastatic tumours and non-epithelial ovarian cancers from the analysis). AUC is given with the corresponding 95%CI.

Tumour	Sample size
Invasive epithelial ovarian cancer *(exclusion of borderline, metastatic and non-epithelial tumours)*	90
Benign	224
Total	314



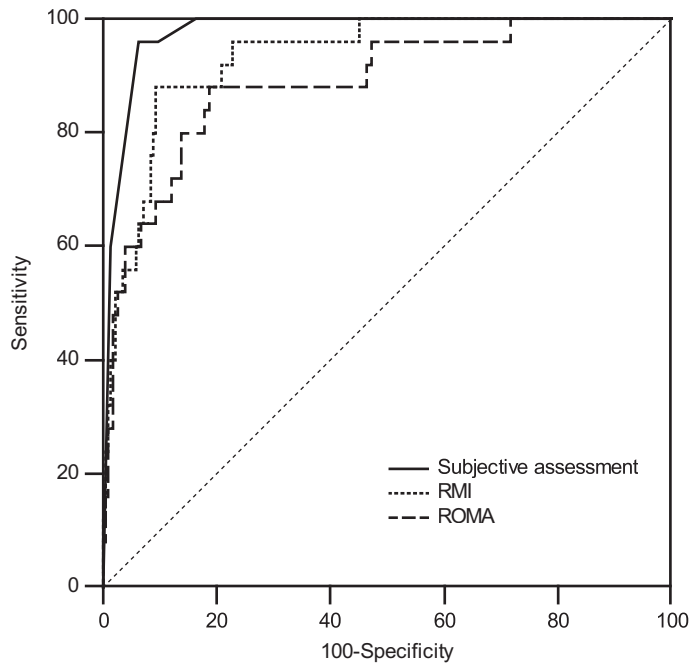
Method	AUC	95%CI
Subjective assessment	0.985	0.964 – 0.995
RMI	0.970	0.945 – 0.986
ROMA	0.956	0.927 – 0.976

Pairwise ROC curve comparisons. The method described by DeLong *et al* was used to calculate the difference between two AUCs [1]. Difference in AUC is given with the corresponding 95%CI.

Comparison			Difference	95%CI
Subjective assessment	*vs*	RMI	0.014	-0.004 – 0.032
Subjective assessment	*vs*	ROMA	0.029	0.003 – 0.056
RMI	*vs*	ROMA	0.015	-0.005 – 0.035

Supplementary File 4: ROC curves of subjective assessment, RMI and ROMA for the diagnosis of invasive epithelial ovarian cancer FIGO I-IIIb (excluding all FIGO stage IIIc-IV epithelial ovarian cancers, invasive borderline ovarian tumours, metastatic tumours and non-epithelial ovarian cancers from the analysis). AUC is given with the corresponding 95%CI.

Tumour	Sample size
Invasive epithelial ovarian cancer FIGO I-IIIb *(exclusion of stage IIIc-IV epithelial ovarian cancer, borderline, metastatic and non-epithelial tumours)*	25
Benign	224
Total	249



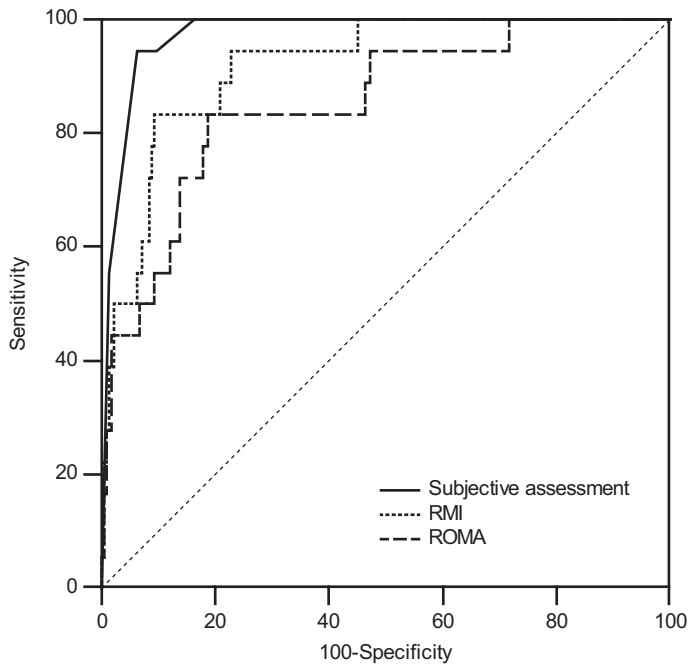
Method	AUC	95%CI
Subjective assessment	0.977	0.950 – 0.992
RMI	0.933	0.894 – 0.960
ROMA	0.887	0.842 – 0.924

Pairwise ROC curve comparisons. The method described by DeLong *et al* was used to calculate the difference between two AUCs [1]. Difference in AUC is given with the corresponding 95%CI.

Comparison			Difference	95%CI
Subjective assessment	*vs*	RMI	0.045	0.001 – 0.088
Subjective assessment	*vs*	ROMA	0.090	0.016 – 0.163
RMI	*vs*	ROMA	0.045	-0.013 – 0.103

Supplementary File 5: ROC curves of subjective assessment, RMI and ROMA for the diagnosis of early stage invasive epithelial ovarian cancer (FIGO I-IIa) (excluding all FIGO stage IIb-IV epithelial ovarian cancers, borderline ovarian tumours, metastatic tumours and non-epithelial ovarian cancers from the analysis). AUC is given with the corresponding 95%CI.

Tumour	Sample size
Early stage invasive epithelial ovarian cancer (FIGO I-IIa) *(exclusion of stage IIb-IV epithelial ovarian cancer, borderline, metastatic and non-epithelial tumours)*	18
Benign	224
Total	242



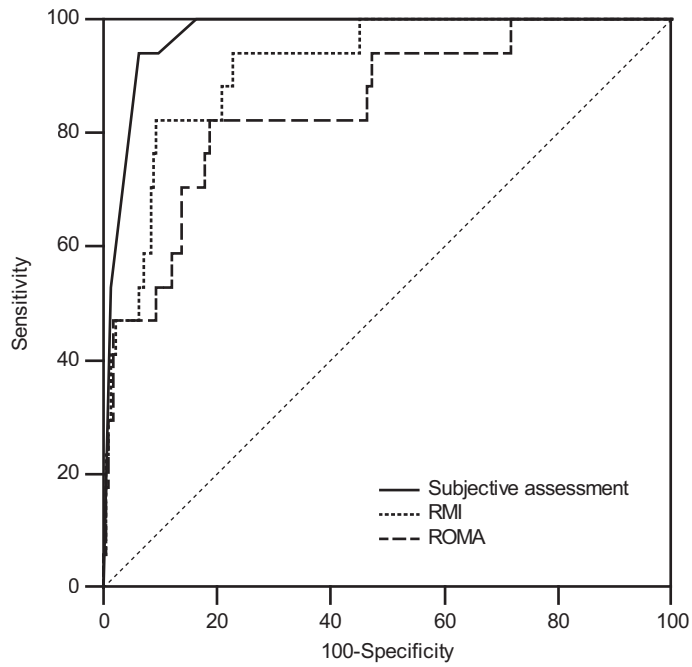
Method	AUC	95%CI
Subjective assessment	0.974	0.945 – 0.990
RMI	0.918	0.876 – 0.950
ROMA	0.852	0.801 – 0.894

Pairwise ROC curve comparisons. The method described by DeLong *et al* was used to calculate the difference between two AUCs [1]. Difference in AUC is given with the corresponding 95%CI.

Comparison			Difference	95%CI
Subjective assessment	*vs*	RMI	0.056	-0.0002 – 0.112
Subjective assessment	*vs*	ROMA	0.122	0.026 – 0.219
RMI	*vs*	ROMA	0.066	-0.011 – 0.143

Supplementary File 6: ROC curves of subjective assessment, RMI and ROMA for the diagnosis of FIGO stage I invasive epithelial ovarian cancer (excluding all FIGO stage II-IV epithelial ovarian cancers, borderline ovarian tumours, metastatic tumours and non-epithelial ovarian cancers from the analysis). AUC is given with the corresponding 95%CI.

Tumour	Sample size
FIGO stage I invasive epithelial ovarian cancer *(exclusion of stage II-IV epithelial ovarian cancer, borderline, metastatic and non-epithelial tumours)*	17
Benign	224
Total	241



Method	AUC	95%CI
Subjective assessment	0.973	0.944 – 0.990
RMI	0.915	0.872 – 0.947
ROMA	0.847	0.796 – 0.890

Pairwise ROC curve comparisons. The method described by DeLong *et al* was used to calculate the difference between two AUCs [1]. Difference in AUC is given with the corresponding 95%CI.

Comparison			Difference	95%CI
Subjective assessment	*vs*	RMI	0.058	-0.001 – 0.117
Subjective assessment	*vs*	ROMA	0.126	0.024 – 0.227
RMI	*vs*	ROMA	0.068	-0.014 – 0.149

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Part II
Discovery studies

Chapter 1: Endorepellin, a novel biomarker for ovarian cancer

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Submission after completing the validation study.

Summary

Background: Angiogenesis is one of the key events in ovarian tumour growth. It is a balance between endogenous stimulators and inhibitors. Inhibitors include various angiostatic peptides from the basement membrane of the vascular endothelium. Endorepellin, the C-terminal fragment of perlecan, is one these angiostatic peptides.

Objective: We conducted a prospective cohort study to investigate whether low levels of plasma endorepellin, as a single marker, or in combination with known markers (CA125 and/or HE4), could differentiate between benign and malignant ovarian masses.

Methods: From October, 2007 until September, 2010, 372 consecutive women with an ovarian mass, planned to undergo surgery, were eligible in a prospective cohort in four different centres in Belgium, Czech Republic, Sweden, and Italy. Preoperative blood samples were tested for CA125, HE4 and endorepellin. Since this was an exploratory study, rare and mixed tumours were excluded from the analysis before logistic regression models were build to be able to perform subset analyses. For each logistic regression model, ROC curves were constructed to calculate the performance in pre- and postmenopausal women.

Results: Three hundred and four patients were analyzed. In the postmenopausal population, all best performing panels included endorepellin with the highest AUC [0.920 (95%CI: 0.863 – 0.959)] for the triple combination of CA125, HE4 and endorepellin. Adding endorepellin to the combination of CA125 and HE4 increased the sensitivity from 72% to 85% at a fixed specificity of 85%, and increased the specificity from 66% to 85% at a fixed sensitivity of 85%. In postmenopausal women, the combination of HE4 and endorepellin was the best performing combination to differentiate FIGO stage I tumours from benign tumours [AUC = 0.820 (95%CI: 0.726 – 0.892)], and endorepellin, as a single marker, was superior to any other marker or combination in differentiating tumours of low malignant potential from benign tumours [AUC = 0.807 (95%CI: 0.699 – 0.890)].

Conclusion: Endorepellin seems to have complementary role to CA125 and HE4, and shows promise as a novel biomarker which contributes significantly in the diagnosis of ovarian cancer in postmenopausal women, especially in stage I and tumours of low malignant potential. However, these findings need to be validated.

Introduction

Due to the location of the ovaries it is very difficult to diagnose ovarian cancer at an early stage. A malignant tumour located in readily accessible organs can be biopsied (e.g. endometrial or cervical cancer). However, this is not the case for ovarian tumours. The decision for planning surgery is purely based on clinical findings, transvaginal sonography, computed tomography and/or magnetic resonance imaging, and serum tumour markers. Moreover, both the quality of clinical judgement and transvaginal sonography are highly dependent on experience [1]. Consequently, a subset of patients with a benign mass will undergo a major laparotomy because they were suspected to have a malignant ovarian mass, and inversely a subset of patients with a malignant mass will undergo a laparoscopy because they were suspected to have a benign ovarian mass. This will cause either over–treatment or under–treatment, with increased costs, morbidity and mortality.

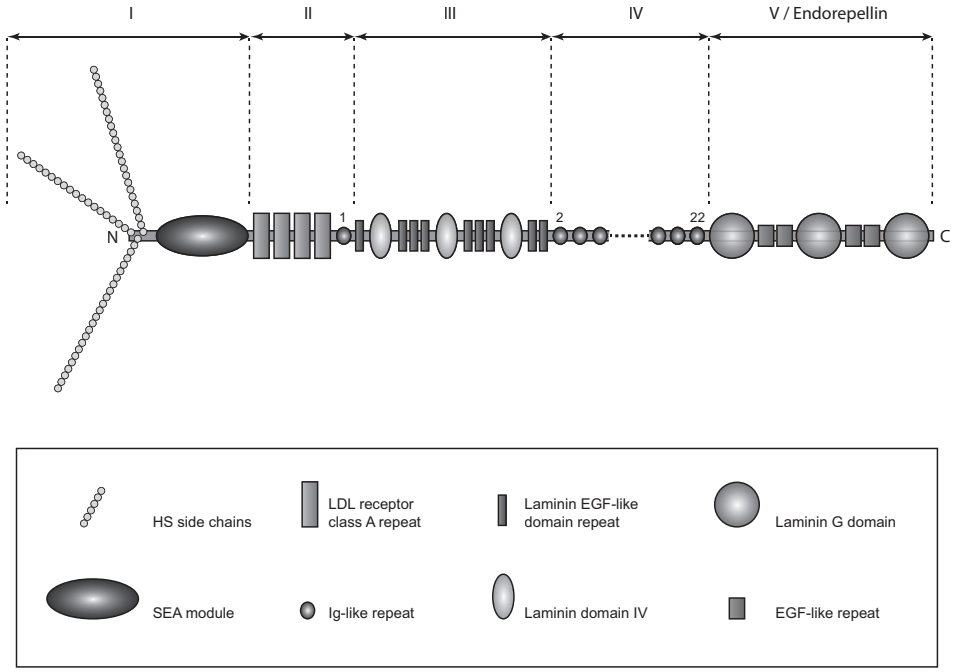
Effective and objective diagnostic tools could aid clinicians in differentiating benign and malignant tumours and would reduce the amount of unnecessary operations while limiting patients' stress. Undoubtedly, tumour markers are the most objective tool that clinicians have to their disposition, however, at present these markers do not have the desired diagnostic performance. CA125, for example, is only raised in approximately 80% of all epithelial ovarian cancers and in only 50% of stage I epithelial ovarian cancers [2]. Moreover, CA125 can be raised in numerous ovarian and non–ovarian, benign and malignant conditions [3]. This also affects the specificity of this tumour marker.

Over the last decade, numerous studies have been published that try to improve the diagnostic performance of CA125 by combining it with other markers. The Human Epididymis protein 4 (HE4) is one of these tumour markers [4;5]. CA125 and HE4 are combined in a logistic regression model called the Risk of Ovarian Malignancy Algorithm (ROMA) [6]. The ROMA has been validated in numerous studies with mixed results: some validation studies confirmed the superiority of the ROMA to CA125 alone [6;7], while others did not [8-10]. Nevertheless, both CA125 and HE4 are probably the best performing tumour markers that are currently commercially available. Strikingly, both markers are tumour associated antigens. Reported markers that are not tumour related, such as inflammatory mediators or plasma carrier proteins, seem to be less successful as tumour markers [11;12].

In our search for new markers, we investigated the process of angiogenesis. Angiogenesis is one of the key events in tumour growth. The expansion of tumours is strictly dependent on the neo–angiogenesis, and the inhibition of angiogenesis can suppress tumour growth [13]. Angiogenesis is a balance between endogenous stimulators and inhibitors. Stimulators include growth factors such as vascular endothelial growth factor (VEGF), placental growth factor (PGF) and angiopoietins. Inhibitors include various angiostatic peptides derived from the vascular basement membrane [14]. Thus, besides providing structural and functional support to the endothelium, the vascular basement membrane can also modulate angiogenesis, and therefore tumour growth. Several of these angiostatic peptides have been shown to inhibit tumour growth and development (e.g. canstatin, endorepellin, endostatin, thrombospondin, and tumstatin) [14]. Some of these peptides have also been investigated in ovarian cancer. Thrombospondin–1, for example, is downregulated in ovarian cancer, and this was more pronounced in advanced stage disease, compared to early stage disease [15]. Endostatin, also an angiostatic peptide, did not only have an anti–angiogenesis effect but also a direct effect on tumour spread and attachment of ovarian cancer cells to the peritoneum [16].

Little is known about the role of endorepellin in ovarian cancer. Endorepellin is a C–terminal angiostatic fragment of perlecan, one of the matrix proteins in the vascular basement membrane (Figure 1). Endorepellin binds members of the fibroblast growth factor (FGF) and VEGF family and their receptors, respectively [17]. Therefore, it plays an important role in several aspects of vascular development. Endorepellin has been found in many body fluids, including blood, urine, and amniotic fluid [18]. Since angiogenesis stimulators are upregulated in ovarian cancer [19-22], one would expect inhibitors, such as endorepellin, to be downregulated. We developed an anti–body free assay to detect endorepellin in body fluids such as serum and plasma. With this assay we conducted a prospective cohort study to investigate whether low levels of endorepellin could add value to CA125 en/or HE4 in differentiating benign and malignant ovarian masses.

Figure 1: Schematic representation of the perlecan protein core. The roman numerals indicate the five domains within perlecan. Endorepellin is the C-terminal domain V. A key for the various modules is presented at the bottom.



Abbreviations: HS: heperan sulfate; SEA: sperm protein, enterokinase and agrin; LDL: low density lipoprotein; Ig: Immunoglobin; EGF: epidermal growth factor.

Materials and methods

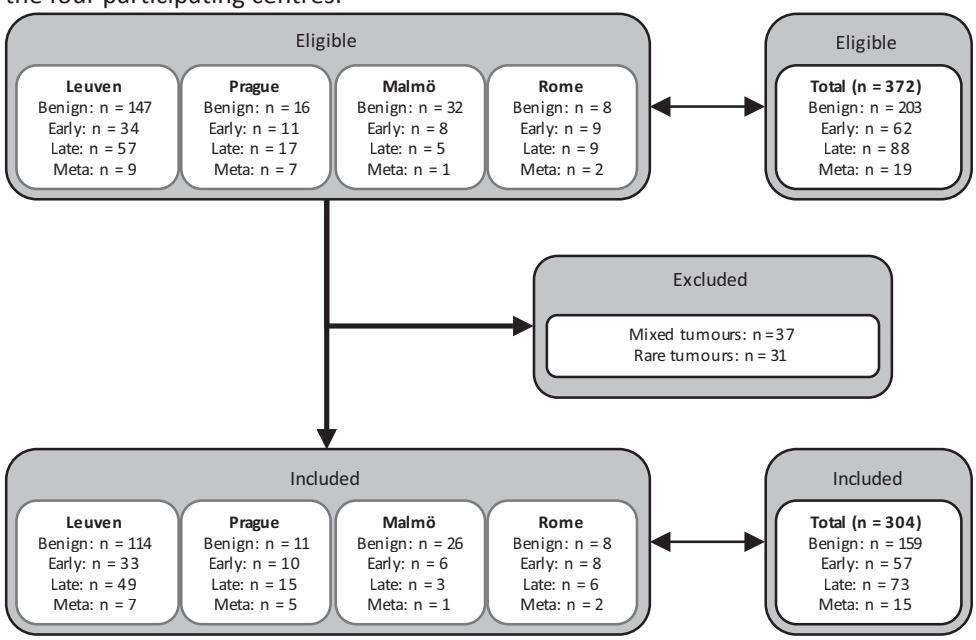
Patients

From October, 2007 until September, 2010, 247 consecutive women were found to be eligible to participate in a prospective cohort study conducted at the University Hospitals Leuven, Belgium. From June, 2009 until September, 2010, the prospective study was extended with a prospective cohort of 125 patients from three additional centres: Prague, Czech Republic (n = 51), Malmö, Sweden (n = 46), and Rome, Italy (n = 28). In these three additional centres, the same inclusion and exclusion criteria applied (Figure 2).

Patients were considered to be eligible if they were diagnosed with a pelvic mass that was suspected to be of ovarian origin and they were to undergo surgery within three months. Patients with a prior bilateral oophorectomy or with a prior diagnosis of a malignant tumour were not eligible. Patients

who were diagnosed with ovarian cancer were completely surgically staged. Prior to enrolment in the study, all patients were required to give fully informed consent. The protocol was approved by the Local Ethics Committee (reference number: OG032/ML3132). Patient participation in the study was concluded once the final surgical pathology reports were obtained.

Figure 2: Flow diagram indicating the inclusion and exclusion of eligible patients in the four participating centres.



Histology

The histology of the tumours was classified according the World Health Organization classification of tumours [23]. Malignant tumours from ovarian origin were classified according to the criteria recommended by the Federation Internationale de Gynécologie et d'Obstétrique (FIGO). Grading was performed with a three–tiered grading system according to Silverberg [24]. Ovarian tumours of low malignant potential (LMP) exhibiting an atypical epithelial proliferation without destructive stromal invasion are so–called borderline and were graded as such. LMP tumours were not excluded from the present analysis and were classified as malignant tumours. Since this was an exploratory study, rare and mixed tumours were excluded from the analysis before logistic regression models were build. This enabled us to perform subset analyses (Figure 2). Tumours of mixed histology (n = 37) included benign or malignant tumours with more than

one histological subtype where each component is present in more than 10% of the total tumour volume. Rare tumours (n = 31) included benign or malignant tumours with only one or two cases per histological subtype.

Blood samples

Within three months before surgery, blood samples were obtained. Blood samples were collected in 10 ml clotting activating tubes (Vacutainer® Serum Tube, ref. 369033, Becton–Dickinson, Erembodegem, Belgium), and standard EDTA plasma tubes (Vacutainer® K2EDTA Tube, ref. 367525, Erembodegem, Belgium) for plasma collection. Blood samples were centrifuged within at 2.000 x *g* for 10 minutes. The serum or plasma was collected, dispensed into a 15 ml Falcon tube and centrifuged a second time at 2.000 x *g* for 15 minutes. Subsequently, the serum and plasma was transferred to multiple cryotubes, and frozen at −80°C. Samples were excluded when they were not frozen within four hours after blood sampling. Serum samples were used to perform CA125 and HE4 assays. Plasma samples were used for the endorepellin quantitation.

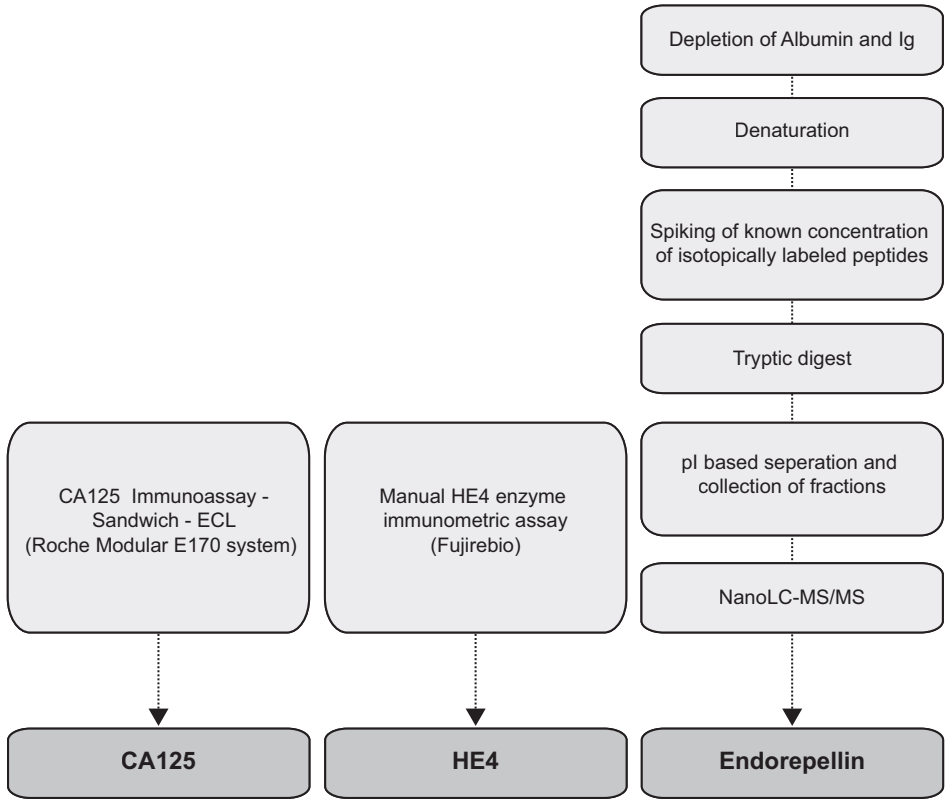
CA125 and HE4 assays

Serum CA125 concentrations were measured by using the E 170 module of the Modular Analytics EVO analyser (Roche Diagnostics, Vilvoorde, Belgium)(Figure 3). The CA125 assay is a sandwich electrochemiluminescence (ECL) immunoassay. Serum HE4 was measured by using the HE4 EIA assay (Fujirebio Diagnostics, Göteborg, Sweden)(Figure 3). The HE4 assay is a solid–phase, non–competitive immunoassays based upon the direct sandwich technique. Both assays were performed according to the manufacturer's instructions. The assays were performed in duplicate for the calibrators, controls and patient samples. The appropriate controls were within the ranges provided by the manufacturer.

Endorepellin quantitation.

Endorepellin levels were measured using targeted mass spectrometry based on a selected reaction monitoring (SRM) peptide quantitation method [25]. This assay includes the following steps (Figure 3):

Figure 3: Methodology for biomarker measurement.



– *Depletion of the plasma sample:* 25 µL of plasma was depleted of albumin and IgG using ProteoPrep Prot–IA immunodepletion spin columns (Sigma–Aldrich, Bornem, Belgium) according to the manufacturer's protocol, except that 20mM NH_4HCO_3 was used as the binding/equilibration buffer.

– *Denaturation of the depleted plasma sample:* the depleted sample (225µL) is denatured for 15min at 95°C and immediately cooled on ice.

– *Spiking of known amounts of isotopically labelled endorepellin peptides:* 2 pmol of the isotopically labelled endorepellin peptides (custom made 'Heavy AQUA' peptide; Thermo Scientific, Erembodegem, Belgium) was spiked in the sample. These peptides had the same amino acid sequence (GSIQVDGEELVSGR and SLPEVPETIELEVR) as the proteotypic peptides of interest, typically with one isotopically labelled amino acid built in to generate a mass difference. During the entire process, the labelled peptides had identical chemical and chromatographic behaviour as the endogenous

peptide, except during the end–stage quantitation step which is based on molecular mass.

– *Tryptic digest of the depleted plasma:* 20µg trypsin was added to the sample and digestion was allowed for 16h at 37°C.

– *Peptide–based fractionation:* the peptides were separated based on their specific isoelectric property (pI). As there was no pI difference between the endogenous peptide and the isotopically labelled variant, they co–eluted. Fractions containing the peptides of interest were pooled together, dried and re–suspended in 0.1% formic acid.

– *Nano–liquid chromatography (NanoLC) – tandem mass spectrometry (MS/MS) quantitation:* 20µL of the resuspended fraction was loaded onto a 25cm x 75µm id PepMap C18 reversed–phase column with 100 Å pore diameter and 5µm size particles (Dionex, Tienen, Belgium). The sample was then separated using a 30 minute mobile phase gradient from 98% solvent A (0.1% formic acid) to 55% Solvent B (80% acetonitrile, 0.1% formic acid). The LC column was online connected to an electrospray needle for the MS/MS. As material elutes from the column, molecules are ionized and enter the mass spectrometer in the gas phase for the quadrupole ion trap mass spectrometry. Multiple reaction monitoring (MRM) was performed by using three–dimensional a 4000 QTRAP system (Applied Biosystems, Gent, Belgium) and single reaction monitoring (SRM) by using the Vantage TSQ system (Thermo Scientific, Erembodegem, Belgium). In the SRM, the monitored peptide was selected to pass the first quadrupole (Q1), based on its mass to charge ratio (*m/z*) (mass window of 0.7 Da). The selected peptide was then fragmented in the second quadrupole (Q2) which was used as a collision cell. The resulting fragments then entered the third quadrupole (Q3). The combination of the *m/z* of the monitored peptide and the *m/z* of the monitored fragment of this peptide is called a transition. Depending on the instrument settings only specific peptide fragments, or transitions, were selected for detection. The used transitions were experimentally determined and selected during protein assay development (Table 1). Both the endogenous peptide (analyte) and its corresponding isotopically labelled synthetic peptide (internal standard) elute at the same retention time, and are measured in the same NanoLC–MS/MS experiment. In the MRM, a series of single reactions (precursor/fragment ion transitions) were measured sequentially for a period starting 2 to 3 minutes before and ending 2 to 3 minutes after the determined NanoLC retention

time of the peptide of interest, making sure that each peak had at least 10 datapoints.

– *Data–analysis:* The raw data was analysed and quantified using the LCQuan software (Thermo Scientific, Erembodegem, Belgium): the area under the analyte peak (= the 'Perlecan' peptides) and under the internal standard peak (the labelled, synthetic 'Perlecan' peptides) peak at the same C18 retention time was determined by automatic peak detection. These were cross–checked manually. The readout was defined by the ratio of the analyte peak area and the internal standard peak area. The measured ratios are differential quantitations of peptides or normalised concentration of a peptide. The concentration of a peptide is proportional to the ratio measured in the mass spectrometer.

Table 1: Peptides used for Endorepellin specific MASSterclass assay. The LG3 peptides of Perlecan domain V were used for the Endorepellin quantitation. The Ig-like repeat peptide of domain IV was used as a negative control.

Amino acid sequence	Location	Protein domain	Peptide domain	SEQ ID NO
GSIQVDGEELVSGR	4305-4318	Domain V	LG3 domain	4
SLPEVPETIELEVR	4222-4235	Domain V	LG3 domain	5
LEGDTLIIPR	3258-3267	Domain IV	Ig-like repeats	6

Abbreviations: LG3: Laminin G3; Ig: immunoglobulin; SEQ ID NO: sequence identification number.

Statistical analysis:

Statistical analysis was performed with R computation language [26] and MedCalc v11.5.1.0 (MedCalc Software, Mariakerke, Belgium). Mean patient ages were compared by using the independent Student's t–test (Welch–test) and menopausal status was compared by using the Chi–square test.

Logistic regression models were computed for all possible combinations of the markers (CA125, HE4 and endorepellin). The analysis were conducted using the log–transformed analyte concentrations, either relative concentrations (endorepellin NanoLC–MS/MS measurements) or absolute levels (CA125 and HE4 immune–assay based measurements). Either the total, the pre– or the postmenopausal population was used as a training set, for the performance of the logistic regression models in the total, the pre– or postmenopausal population, respectively. For each logistic regression model, ROC curves were constructed to calculate the

performance to discriminate malignant from benign disease in pre– and postmenopausal women. AUC with binomial exact 95%CI were calculated [27]. The non–parametric method described by DeLong *et al* [28] was used to calculate the difference between two AUCs. The generalization error on the logistic regression model AUC was estimated using bootstrap cross validation [29]. For all statistical comparisons, a level of $p < 0.05$ was accepted as being statistically significant.

Results

Patient and tumour characteristics:

In total, 372 patients were enrolled in the 4 recruiting centres (Figure 2). After exclusion of rare and mixed tumours we included 304 patients. The patient characteristics of the included patients are shown in Table 2. Of the 304 analyzed patients, 159 (52.3%) had benign disease and 145 (47.7%) patients had malignant disease. Patients with malignant disease were older, more likely to be postmenopausal, and more likely to have a first or second degree relative with ovarian cancer.

Table 2: Patient characteristics.

Patient characteristics	Numerical display	Benign	Malignant	*p* - value
Total	n (%)	159 (52.3)	145 (47.7)	NA
Mean age	Yrs (95%CI)	46.9 (44.2–49.7)	56.9 (54.7 – 59.0)	< 0.0001
Post-menopausal	n (%)	60 (37.7)	100 (68.5)	< 0.0001
Family history*				
Breast cancer	n (%)	24 (15.1)	32 (21.9)	0.1647
Ovarian cancer	n (%)	2 (1.3)	9 (6.2)	0.0468

* First or second degree family members. Yrs: years.

The most common benign an malignant histological subtypes are shown in Table 3. Benign ovarian tumours were endometriomas, mature teratomas, cystadenomas, cystadenofibromas, and fibromas/thecomas. The cystadenomas and cystadenofibromas included 42 serous and 22 mucinous tumours. There were no endometriomas diagnosed in postmenopausal patients. Since all non–epithelial ovarian tumours were excluded, malignant tumours consisted out of epithelial ovarian cancers and metastatic cancers (Table 3). Fifty–seven (43.8%) patients had early stage (FIGO stage I–IIa) and 73 (66.2%) patients had advanced stage (FIGO stage IIb–IV) ovarian cancer (Figure 2). When the LMP tumours were not taken into account, 30

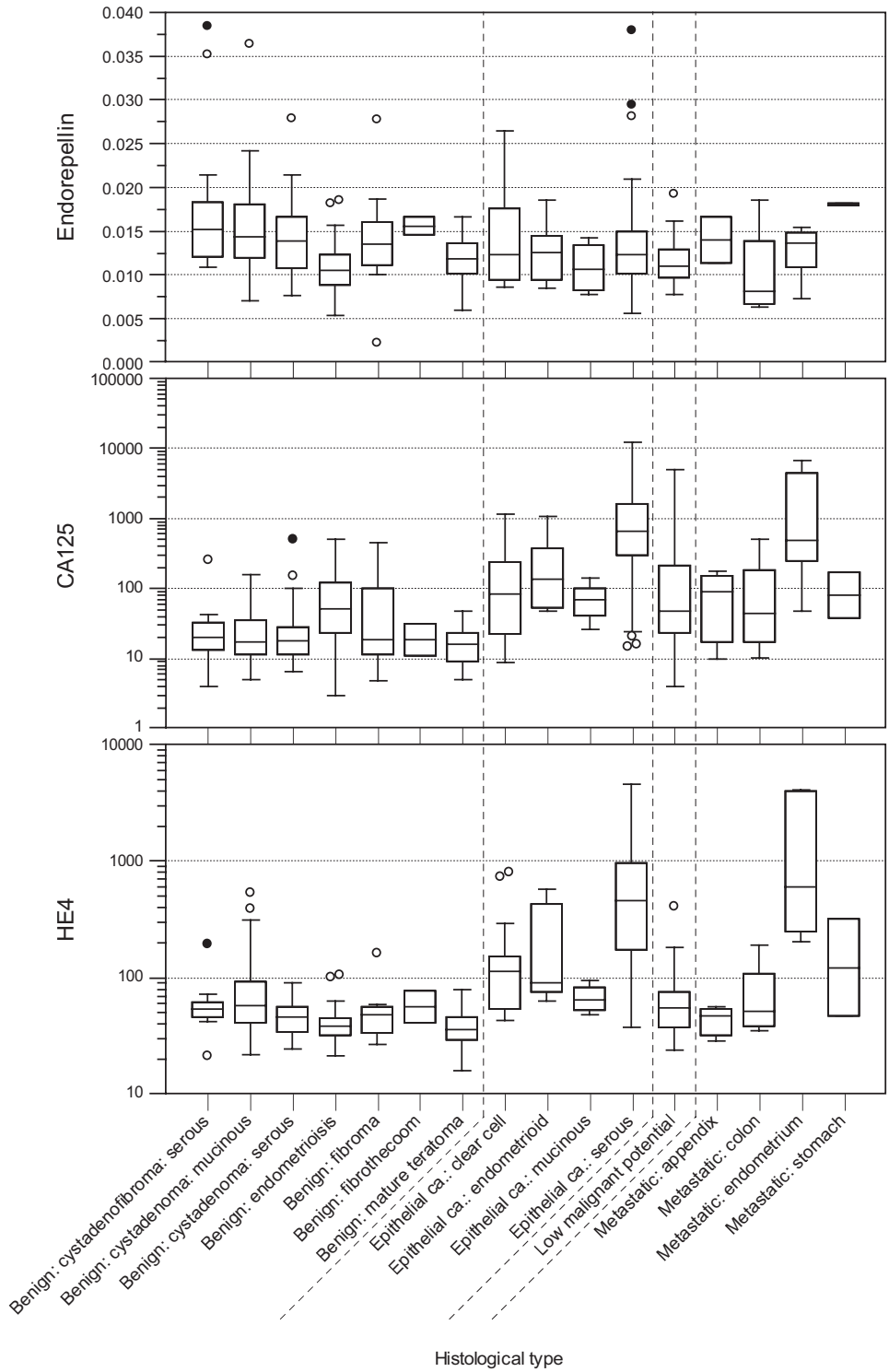
(28.4%) patients had early stage and 68 (71.6%) patients had advanced stage ovarian cancer. The extra–ovarian primary tumours with metastases to the ovarian metastases were mainly located in the endometrium (n = 6), colon (n = 4), appendix (n = 3), stomach (n = 2).

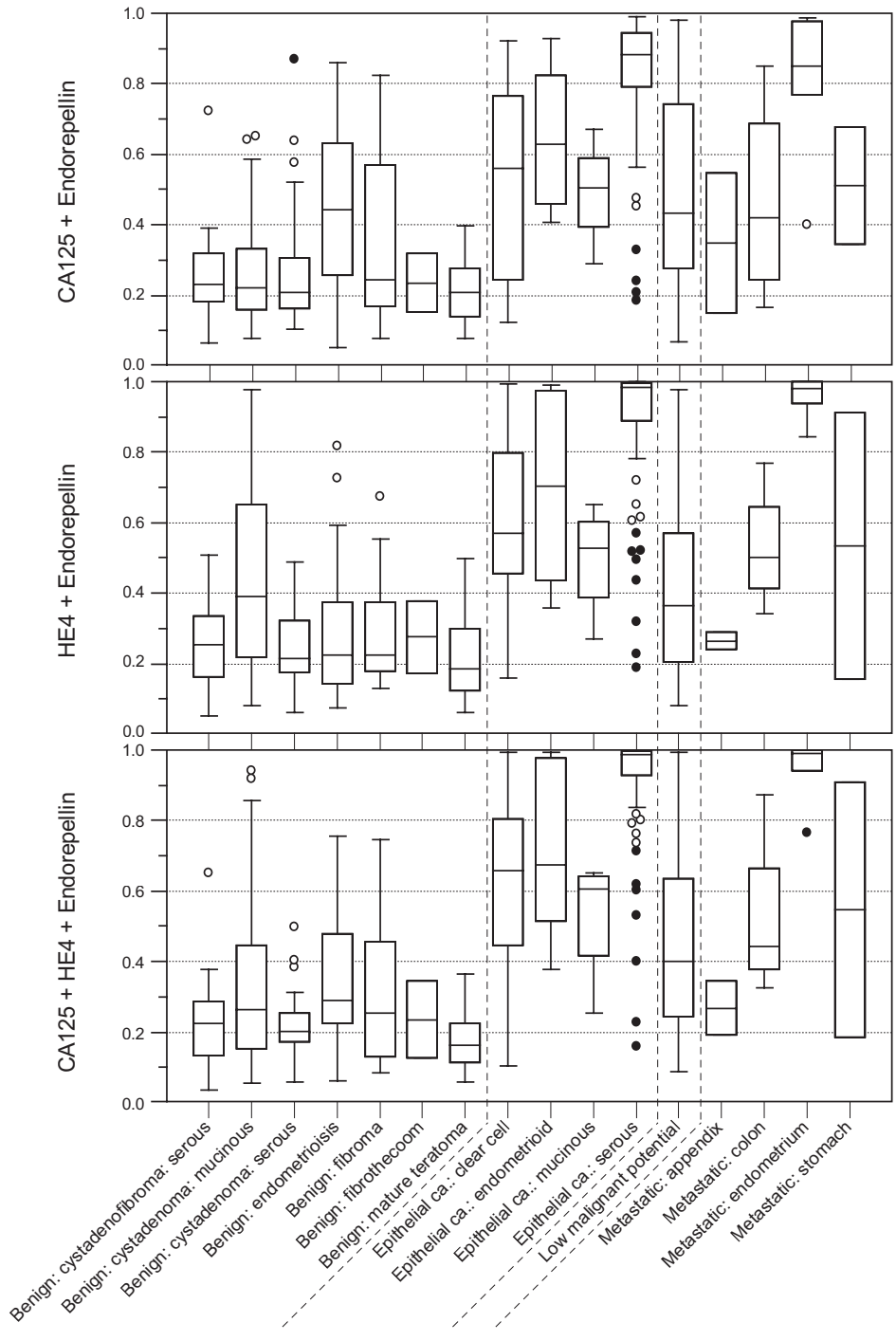
Table 3: Tumour characteristics.

	Pre-menopausal n (%)	**Post-menopausal** n (%)	**All patients** n (%)
Histology			
Benign	99 (68.3)	60 (37.7)	159 (52.3)
Malignant	46 (31.7)	99 (62.3)	145 (47.7)
Total	145 (100.0)	159 (100.0)	304 (100.0)
Benign histology			
Cystadenoma	16 (16.2)	31 (51.7)	47 (29.6)
Cystadenofibroma	3 (3.0)	14 (23.3)	17 (10.7)
Fibroma/Thecoma	3 (3.0)	11 (18.3)	14 (8.8)
Endometriosis	51 (51.5)	0 (0.0)	51 (32.1)
Mature teratoma	26 (26.3)	4 (6.7)	30 (18.9)
Total	99 (100.0)	60 (100.0)	159 (100.0)
Malignant histology			
EOC	42 (91.3)	88 (88.9)	130 (89.7)
Metastatic	4 (8.7)	11 (11.1)	15 (10.3)
Total	46 (100.0)	99 (100.0)	145 (100.0)
Histological subtypes of EOC			
Serous	28 (66.7)	61 (69.3)	89 (68.5)
Mucinous	8 (19.0)	10 (11.4)	18 (13.8)
Endometrioid	5 (11.9)	5 (5.7)	13 (10.0)
Clear cell	1 (2.4)	12 (13.6)	10 (7.7)
Total	42 (100.0)	88 (100.0)	130 (100.0)
FIGO Stage of EOC			
I	21 (50.0)	35 (39.8)	56 (63.6)
II	3 (7.1)	2 (2.3)	5 (5.7)
III	16 (38.1)	38 (43.2)	54 (61.4)
IV	2 (4.8)	13 (14.8)	15 (17.0)
Total	42 (100.0)	88 (100.0)	130 (147.7)
Grade of EOC			
Borderline	19 (45.2)	16 (18.2)	35 (26.9)
Grade 1	3 (7.1)	7 (8.0)	10 (7.7)
Grade 2	5 (11.9)	6 (6.8)	11 (8.5)
Grade 3	14 (33.3)	57 (64.8)	71 (54.6)
Unknown	1 (2.4)	2 (2.3)	3 (2.3)
Total	42 (100.0)	88 (100.0)	130 (100.0)

Abbreviations: EOC: Epithelial Ovarian Cancer; FIGO: International Federation of Gynecology and Obstetrics.

Figure 4: Box-and-whisker plots, according to histopathological subtype, for the different markers and marker combinations.




CA125 + Endorepellin
HE4 + Endorepellin
CA125 + HE4 + Endorepellin
0.0
0.2
0.4
0.6
0.8
1.0
Benign: cystadenofibroma: serous
Benign: cystadenoma: mucinous
Benign: cystadenoma: serous
Benign: endometrioisis
Benign: fibroma
Benign: fibrothecoom
Benign: mature teratoma
Epithelial ca.: clear cell
Epithelial ca.: endometrioid
Epithelial ca.: mucinous
Epithelial ca.: serous
Low malignant potential
Metastatic: appendix
Metastatic: colon
Metastatic: endometrium
Metastatic: stomach
Histological type

Tumour marker levels

Median tumour marker and logistic regression model values and the corresponding box–and–whisker plots, according to histopathological subtype, are provided in Figure 4.

ROC curves

Diagnosis of invasive malignant tumours

The results of the ROC curve analyses are summarized in Table 4. In the whole population as in the premenopausal population separately, endorepellin had an AUC of 0.502 and 0.538, respectively. Both ROC curves coincided with the diagonal. In the postmenopausal population, endorepellin had a significant AUC of 0.619. However, in the postmenopausal population the AUC of endorepellin was significantly lower than any other marker or combinations of markers. This is also reflected in the performance indices: when specificity and sensitivity were fixed at 85%, the sensitivity and specificity was 32.9 % and 23.0 %, respectively.

In the postmenopausal population all best performing panels included endorepellin with the highest AUC (0.920) for the triple combination of CA125, HE4 and endorepellin (Table 4 and Figure 5). Adding endorepellin to the combination of CA125 and HE4 increased the sensitivity from 75.9% to 89% at a fixed specificity of 85%, and increased the specificity from 84.8% to 86.4% at a fixed sensitivity of 85%. Table 5 shows the difference in AUC when endorepellin was added to CA125 and/or HE4 in the postmenopausal population. In the premenopausal population adding endorepellin to CA125 and/or HE4 did not improve the AUC (Table 4).

Diagnosis of FIGO stage I tumours (invasive + LMP tumours) in postmenopausal women.

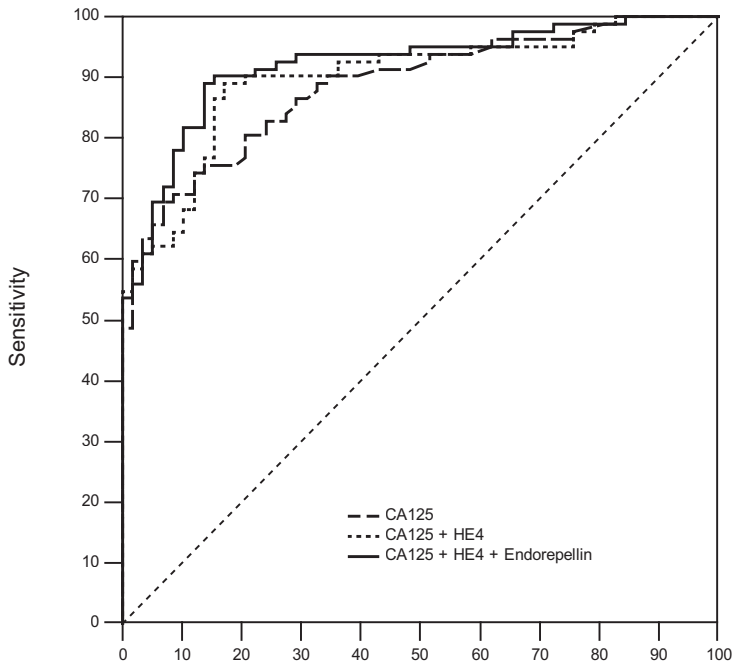
Subset analysis was performed to determine the added value of endorepellin as a biomarker for discriminating FIGO stage I tumours from benign controls in the postmenopausal population. Table 6 summarizes the performance of different marker combinations (with and without endorepellin). Endorepellin as a single marker had an equal performance to detect FIGO stage I tumours as CA125. The triple combination of CA125, HE4 and endorepellin and the combination of HE4 and endorepellin had the highest performance.

Diagnosis of LMP tumours (all stages) in postmenopausal women

Subset analysis was performed to determine whether endorepellin was able to differentiate between benign and LMP tumours. Table 7

summarizes the performance of different marker combinations (with and without endorepellin) to discriminate LMP tumours from benign controls, in the postmenopausal population. Endorepellin as a single marker had the highest AUC of all markers or marker combinations. Endorepellin improved the AUC when added to CA125 and/or HE4, but when the doublets or triplets with endorepellin were compared to endorepellin as a single marker there was no significant difference.

Figure 5: ROC curves for the discrimination of invasive malignant tumours from benign tumours in postmenopausal population for CA125, CA125 + HE4, and CA125 + HE4 + endorepellin. Total AUC values with corresponding 95%CIs are listed below the curves.



	AUC	(95%CI)
CA125	0.887	(0.822 - 0.934)
CA125 + HE4	0.901	(0.839 - 0.945)
CA125 + HE4 + Endorepellin	0.920	(0.863 - 0.959)

Table 4: Performance of the individual markers and the multimarker panels to discriminate invasive malignant tumours from benign tumours in pre- and postmenopausal populations. Low malignant tumours were not included in this analysis. Sensitivity and specificity are calculated at 85% specificity and 85% sensitivity, respectively.

			Sensitivity at 85% specificity	Specificity at 85% sensitivity
	Marker combination	**AUC (95%CI)**	**Sens (95%CI)**	**Spec (95%CI)**
All	Endorepellin	0.502 (0.439 – 0.566)	16.7 (10.2 – 25.1)	13.2 (8.4 – 19.5)
	CA125	0.883 (0.837 – 0.920)	72.7 (63.4 – 80.8)	74.0 (66.1 – 80.9)
	CA125 + Endorepellin	0.882 (0.836 – 0.919)	74.1 (64.8 – 82.0)	73.3 (65.3 – 80.3)
	HE4	0.922 (0.882 – 0.952)	83.6 (75.4 – 90.0)	84.3 (77.7 – 89.6)
	HE4 + Endorepellin	0.931 (0.892 – 0.959)	86.1 (78.1 – 92.0)	86.8 (80.5 – 91.6)
	CA125 + HE4	0.930 (0.891 – 0.958)	89.1 (81.7 – 94.2)	89.0 (82.8 – 93.6)
	CA125 + HE4 + Endorepellin	0.935 (0.897 – 0.962)	88.0 (80.3 – 93.4)	89.0 (82.8 – 93.6)
Premenopausal	Endorepellin	0.538 (0.441 – 0.632)	11.5 (2.4 – 30.2)	22.5 (14.6 – 32.0)
	CA125	0.909 (0.840 – 0.955)	70.4 (49.8 – 86.2)	79.3 (69.3 – 87.3)
	CA125 + Endorepellin	0.909 (0.841 – 0.955)	69.2 (48.2 – 85.7)	79.3 (69.3 – 87.3)
	HE4	0.947 (0.889 – 0.981)	85.2 (66.3 – 95.8)	85.7 (77.2 – 92.0)
	HE4 + Endorepellin	0.943 (0.882 – 0.978)	84.6 (65.1 – 95.6)	86.7 (78.4 – 92.7)
	CA125 + HE4	0.964 (0.911 – 0.990)	88.9 (70.8 – 97.6)	93.1 (85.6 – 97.4)
	CA125 + HE4 + Endorepellin	0.961 (0.906 – 0.988)	88.5 (69.8 – 97.6)	94.3 (87.1 – 98.1)
Postmenopausal	Endorepellin	0.619 (0.534 – 0.700)	32.9 (22.9 – 44.2)	23.0 (13.2 – 35.5)
	CA125	0.887 (0.822 – 0.934)	74.7 (64.0 – 83.6)	71.2 (57.9 – 82.2)
	CA125 + Endorepellin	0.890 (0.827 – 0.937)	75.6 (64.9 – 84.4)	72.9 (59.7 – 83.6)
	HE4	0.875 (0.809 – 0.925)	78.3 (67.9 – 86.6)	75.4 (62.7 – 85.5)
	HE4 + Endorepellin	0.914 (0.855 – 0.955)	89.0 (80.2 – 94.9)	88.5 (77.8 – 95.3)
	CA125 + HE4	0.901 (0.839 – 0.945)	75.9 (65.3 – 84.6)	84.8 (73.0 – 92.8)
	CA125 + HE4 + Endorepellin	0.920 (0.863 – 0.959)	89.0 (80.2 – 94.9)	86.4 (75.0 – 94.0)

Abbreviations: AUC: area under the receiver operating characteristic curve; 95%CI: 95% confidential interval.

Table 5: Added value of endorepellin to CA125 and/or HE4 markers to discriminate benign ovarian tumours from invasive malignant ovarian tumours in postmenopausal women. Tumours of low malignant potential were not included in this analysis.

Marker combination	Difference in AUC (95%CI)	*p* value
CA125 CA125 + Endorepellin	0.004 (-0.002 – 0.009)	0.1728
HE4 HE4 + Endorepellin	0.039 (0.011 – 0.067)	0.0059
CA125 + HE4 CA125 + HE4 + Endorepellin	0.020 (-0.0003 – 0.040)	0.0534
CA125 CA125 + HE4 + Endorepellin	0.034 (0.0007 – 0.066)	0.0451
HE4 CA125 + HE4 + Endorepellin	0.045 (0.016 – 0.074)	0.0025
CA125 + Endorepellin HE4 + Endorepellin	0.024 (-0.023 – 0.071)	0.3181
CA125 + Endorepellin CA125 + HE4 + Endorepellin	0.030 (-0.003 – 0.062)	0.0743
HE4 + Endorepellin CA125 + HE4 + Endorepellin	0.006 (-0.013 – 0.025)	0.5497

Abbreviations: AUC: area under the receiver operating characteristic curve; 95%CI: 95% confidential interval.

Table 6: Performance of the individual markers and the multimarker panels to discriminate FIGO stage I epithelial tumours from benign disease in the postmenopausal populations. Both invasive tumours and tumours of low malignant potential were included in this analysis. The added value of Endorepellin to CA125 and/or HE4 markers is presented as the difference in AUC. Sensitivity and specificity are calculated at 85% specificity and 85% sensitivity, respectively.

				Sensitivity at 85% specificity	Specificity at 85% sensitivity
Marker combination	**AUC (95%CI)**	**Difference in AUC (95%CI)**	***p* value**	**Sens (95%CI)**	**Spec (95%CI)**
Endorepellin	0.710 (0.607 – 0.800)			47.1 (29.8 – 64.9)	40.0 (27.6 – 53.5)
CA125	0.716 (0.612 – 0.805)	0.066 (-0.008 – 0.138)	0.0803	40.0 (23.9 – 57.9)	37.9 (25.5 – 51.6)
CA125 + Endorepellin	0.780 (0.682 – 0.860)			58.8 (40.7 – 75.4)	41.4 (28.6 – 55.1)
HE4	0.685 (0.580 – 0.778)	0.135 (0.040 – 0.230)	0.0055	45.7 (28.8 – 63.4)	25.0 (14.7 – 37.9)
HE4 + Endorepellin	0.820 (0.726 – 0.892)			67.7 (49.5 – 82.6)	60.0 (46.5 – 72.4)
CA125 + HE4	0.714 (0.610 – 0.803)	0.103 (0.026 – 0.181)	0.0085	45.7 (31.4 – 66.0)	27.6 (16.7 – 40.9)
CA125 + HE4 + Endorepellin	0.817 (0.723 – 0.890)			64.7 (46.5 – 80.3)	63.8 (50.1 – 76.0)

Abbreviations: FIGO: International Federation of Gynecology and Obstetrics; AUC: area under the receiver operating characteristic curve; 95%CI: 95% confidential interval.

Table 7: Performance of the individual markers and the multimarker panels to discriminate tumours of low malignant potential from benign disease (all stages) in the postmenopausal populations. The added value of Endorepellin to CA125 and/or HE4 markers is presented as the difference in AUC. Sensitivity and specificity are calculated at 85% specificity and 85% sensitivity, respectively.

				Sensitivity at 85% specificity	Specificity at 85% sensitivity
Marker combination	**AUC (95%CI)**	**Difference in AUC (95%CI)**	***p* value**	**Sens (95%CI)**	**Spec (95%CI)**
Endorepellin	0.807 (0.699 – 0.890)			62.5 (35.4 – 84.8)	60.0 (46.5 – 72.4)
CA125	0.673 (0.555 – 0.778)	0.104 (0.011 – 0.196)	0.0283	43.8 (19.8 – 70.1)	25.9 (15.3 – 39.0)
CA125 + Endorepellin	0.777 (0.665 – 0.866)			62.5 (35.4 – 84.8)	25.9 (15.3 – 39.0)
HE4	0.600 (0.479 – 0.712)	0.203 (0.077 – 0.329)	0.0015	25.0 (7.3 – 52.4)	23.3 (13.4 – 36.0)
HE4 + Endorepellin	0.803 (0.694 – 0.886)			75.0 (47.6 – 92.7)	48.3 (35.2 – 61.6)
CA125 + HE4	0.643 (0.523 – 0.751)	0.153 (0.035 – 0.271)	0.0113	50.0 (24.7 – 75.3)	27.6 (16.7 – 40.9)
CA125 + HE4 + Endorepellin	0.796 (0.687 – 0.881)			68.8 (41.3 – 89.0)	20.7 (11.2 – 33.4)

Abbreviations: AUC: area under the receiver operating characteristic curve; 95%CI: 95% confidential interval.

Discussion

In this study we found that the angiostatic peptide endorepellin was downregulated in postmenopausal women with ovarian cancer and that the use of endorepellin as a biomarker improved the diagnosis of ovarian cancer in postmenopausal patients with an adnexal mass. In premenopausal patients there was no difference in performance when endorepellin was added to CA125 and/or HE4 in logistic regression models. Endorepellin has an angiostatic effect on endothelial cells by causing a disassembly of actin stress fibers and by inhibiting VEGF transcription via the integrin mediated signaling pathway [30-32]. Endorepellin has been identified in urine of patients with severe renal disease [33;34], and in amniotic fluid of pregnant women with premature ruptured membranes [35]. Although perlecan has been investigated in several types of cancer, endorepellin itself has only been investigated as a potential marker in breast cancer [36]. Endorepellin was significantly lower in breast cancer patients, compared to healthy controls. Endorepellin concentrations were measured with Western blot and with LC–MS/MS SRM, similarly as in our current study. However, one of the limitations of the study of Chang *et al* was the limited amount of samples (six healthy samples, six breast cancer samples). Our study included 305 samples, and, up to date, this is the largest study that investigated the potential role of endorepellin as a biomarker.

When the dataset was divided according to menopausal status, the performance of endorepellin was better in the postmenopausal population (AUC = 0.619) than in the premenopausal population (AUC = 0.538). This difference in performance can be explained by several reasons. First, there was a difference in distribution of the benign tumours in the pre– and postmenopausal population: all endometriotic lesions and almost 90% of mature teratomas were diagnosed in the premenopausal population. Both histological subtypes had the lowest endorepellin concentration among benign tumours (Figure 5). Since 78% of benign tumours diagnosed in the premenopausal population were either endometriomas or mature teratomas this could explain why no difference was found between endorepellin concentrations of benign and malignant tumours. These low levels of endorepellin in endometriosis can easily be explained. Although endometriosis is a benign disease, it is also dependent on angiogenesis to develop [37;38]. Since endorepellin is downregulated in patients with endometriosis, this means that the inhibition of VEGF and FGF may be reduced and therefore angiogenesis induced. Moreover, as in cancer, a

down regulation of endorepellin may increase spread and attachment to the peritoneum. A second reason why the performance of endorepellin was worse in the premenopausal population, might be that angiogenesis plays an important physiological role during the menstrual cycle. The cycling endometrium undergoes changes of rapid growth and breakdown and angiogenesis plays an important role in developing the required blood vessels in the proliferative endometrium [39]. Moreover, angiogenesis is also an active process in the ovary: VEGF is detected in the developing follicle, with the most intense signal in the mature follicle [40]. In contrast to angiogenesis during tumour growth, this physiological process is limited, however, endogenous angiogenesis inhibitors might be actively downregulated during more active phases of blood vessel development. Hence, this might have lowered the overall endorepellin concentrations in the premenopausal population and thus the performance of endorepellin as a diagnostic marker in this subset of patients.

Endorepellin was combined with existing markers with the aim to improve the performance of CA125 and/or HE4. When endorepellin was added to one or both markers it improved the AUC with 0.4%, 3.9% and 2.0% for CA125, HE4 and CA125 + HE4, respectively. Endorepellin seemed to be complementary to CA125 and HE4. This may be due to the completely different physiological function of endorepellin. CA125 is a mucin, and the extracellular domain of CA125 extends far from the ovarian cancer cell surface in a brush–like pattern. Evidence suggests that CA125 contributes to cell–cell adhesion [41]. HE4, on the other hand, is thought to be involved in multiple functions: it can act as an endogenous protease inhibitor and may be involved in apoptosis, cell proliferation and metastasis [42]. Since the expression of endorepellin is not related to the expression of CA125 and HE4, this marker can have an additive affect to the performance of one or both markers. When looking at the AUCs of the different combinations, it is clear that the AUC of CA125 + HE4 + endorepellin is highest (AUC = 0.920), but the difference with CA125 + endorepellin (AUC = 0.890) and HE4 + endorepellin (AUC = 0.914) is small and not significant. The combination of CA125 and HE4 had an AUC of 0.901. Since the AUC of CA125 + HE4 + endorepellin was higher, this means that this triple combination could be an improvement to the commercially available ROMA test which combines CA125 and HE4. Future and larger cohort studies will need to determine whether this difference is clinically significant and whether it is cost–beneficial to combine three markers instead of two markers.

Endorepellin also performed well in stage I and LMP tumours. Moreover, as a single marker, endorepellin performed as well as CA125 in stage I and LMP tumours, and as well as HE4 in stage I tumours, and better than HE4 in LMP tumours. This can only be explained by the fact that angiogenesis is an early process in tumour development. Indeed, VEGF, angiotensin and their receptors have been found in early stage ovarian cancer and, to a lesser extent, in LMP tumours [19-22;43]. It can be hypothesised that endorepellin could be a permissive factor for angiogenesis. With normal endorepellin levels no angiogenesis will occur, lowering the endorepellin can allow the process to initiate. Since this angiogenesis is an early event, this explains why endorepellin was also downregulated in early stage ovarian cancer or in LMP tumours. Membrane associated proteins can only be shed into the serum when tumour cells come into direct contact with the blood stream (e.g. metastasizing cells), or shedding can be indirectly with an in between step in the peritoneal cavity followed by lymph drainage towards the blood stream. In any way the tumour needs to have developed to a certain extent before these membrane proteins can be detected in serum or plasma. This explains possibly why CA125 is only raised in 50% of stage I tumours [2]. Proteins involved in angiogenesis, and specifically endogenous inhibitors, are located very close to the bloodstream in the basal membrane of the endothelial cells. Small differences in angiogenic activity could therefore very rapidly be translated into differences in serum or plasma concentrations. However, the results in stage I and LMP tumours need to be interpreted with caution. Both ROC curves had a very wide 95%CI due to the limited amount of cases. Future studies with larger cohorts of stage I and/or LMP tumours need to be carried out to confirm these results.

Bearing the angiostatic role in mind, endorepellin may have a role in various biological processes that are related to angiogenesis. However, by combining different – more or less ovarian specific – tumour markers in a logistic regression model, it is quite possible that the combination of markers will act as a ovarian specific tumour marker algorithm. Several histological subtypes, in particular rare and mixed tumours were excluded from this exploratory study. Since the excluded samples consisted of 18% of the total cohort, this may have created a selection bias and have influenced the outcome of the study. However, it must be stressed that this was an exploratory study. Moreover, internal validation with cross validation is also prone to overfitting. This is why we started an external validation study to confirm our results. An independent set of samples will be analyzed for endorepellin, as well as for CA125 and HE4 markers. Future work also

includes a comparison of the performance of the newly developed risk stratification tool using CA125, HE4 and endorepellin with the RMI as previously reported for the ROMA [44]. In addition, the analysis will test whether adding ultrasound data to the stratification tool of will improve its performance.

In conclusion, endorepellin contributes significantly in the diagnosis of ovarian cancer in the postmenopausal population, especially in stage I and LMP tumours. Future studies are needed to confirm these results and to clarify which combination (CA125 and/or HE4) with endorepellin proves to be the best stratification tool for patients with suspicious adnexal masses.

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Chapter 2: Proteomic biomarkers predicting lymph node involvement in serum of cervical cancer patients

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Abstract:

Background: Lymph node status is not part of the staging system for cervical cancer, but provides important information for prognosis and treatment. We investigated whether lymph node status can be predicted with proteomic profiling.

Material & Methods: Serum samples of 60 cervical cancer patients (FIGO stage I/II) were obtained before primary treatment. Samples were run through a HPLC depletion column, eliminating the 14 most abundant proteins ubiquitously present in serum. Unbound fractions were concentrated with spin filters. Fractions were spotted onto CM10 and IMAC30 surfaces and analyzed with SELDI–TOF MS. Unsupervised peak detection and peak clustering was performed using MASDA software. Leave–one–out (LOO) validation for weighted Least Squares Support Vector Machines (LSSVM) was used for prediction of lymph node involvement. Other outcomes were histological type, lymphvascular space involvement (LVSI) and recurrent disease.

Results: LSSVM models were able to determine lymph node status with a LOO area under the receiver operating characteristics curve (AUC) of 0.95, based on peaks with *m/z* values 2,698.9, 3,953.2, and 15,254.8. Furthermore, we were able to predict LVSI (AUC 0.81), to predict recurrence (AUC 0.94), and to differentiate between squamous carcinomas and adenocarcinomas (AUC 0.88), between squamous and adenosquamous carcinomas (AUC 0.85), and between adenocarcinomas and adenosquamous carcinomas (AUC 0.94).

Conclusions: Potential markers related with lymph node involvement were detected, and protein/peptide profiling support differentiation between various subtypes of cervical cancer. However, identification of the potential biomarkers was hampered by the technical limitations of SELDI–TOF MS.

Background:

Cervical cancer is the seventh most common cancer in both sexes combined and the third most common cancer in women. An estimated 530,000 women across the world were diagnosed with cervical cancer in 2008, accounting for nearly one in ten (9%) of all cancers diagnosed in women. The developing countries carry the biggest burden of cervical cancer, with more than 450,000 cases being diagnosed in 2008 [1].

Lymph node status is not part of the FIGO staging system for cervical cancer [2], but it provides important information for prognosis and treatment, in particular in early stage cervical cancer [3;4]. The incidence of pelvic lymph node metastases varies from 0–2% in FIGO stage IA, 17–24% in FIGO stage IB1, 17– 50% in FIGO stage IB2, and 10–50% in FIGO stage IIa [4-10].

In patients with early stage cervical cancer, the treatment of choice is either surgical, including radical hysterectomy and pelvic lymph node dissection, or chemoradiation. Combining both treatments leads to a higher morbidity, such as lymph oedema and urological complications [11]. Specifically for patients with lymph node metastases, chemoradiation is the treatment of choice since it reduces local and distant recurrences [12]. Preoperative diagnostic modalities such as CT scan and MRI have a good specificity, but a low sensitivity [13;14]. This explains why a certain number of patients, in whom the diagnosis of positive lymph nodes is only made after pathological examination, still receive a combined treatment of surgery and pelvic irradiation.

Various proteomics techniques have been used to detect new biomarkers in gynaecological cancers with variable degrees of success [15]. Over the last decade, SELDI–TOF MS has been a popular proteomics technique due to its ease of use and high throughput. Several studies have published comparative studies on new diagnostic proteins [15].

We investigated whether we could improve the prediction of lymph node involvement with SELDI–TOF MS proteomic profiling.

Material and methods:

Patients:

Serum samples of 60 cervical cancer patients were obtained before primary surgery. All patients were diagnosed with FIGO stage I or II cervical cancer. Prior to enrolment in the study, all patients were required to give fully informed consent. The protocol was approved by the Local Ethics Committee (reference: 3M040097/ML2524).

Depletion:

For each of the 60 serum samples, immunodepletion was performed using a high capacity 4.6 x 100 mm multiple affinity removal system (MARS) column (Agilent Technologies, Diegem, Belgium) in an Agilent 1200 high performance liquid chromatography (HPLC) system (Agilent Technologies, Diegem, Belgium). This column eliminates the 14 most abundant proteins ubiquitously present in serum: albumin, alpha1–acid glycoprotein, alpha2–macroglobulin, antitrypsin, apolipoprotein AI, apolipoprotein AII, complement C3, fibrinogen, haptoglobin, IgA, IgG, IgM, transferrin, and transthyretin. In brief, the serum samples were diluted four–fold with Buffer A (Agilent Technologies, Diegem, Belgium), filtered through a 0.22 mm spin filter and 100 µl of the diluted serum was injected into the column in 100% Buffer A at a flow rate of 0.125 mL/min. After collection of the flow–through (i.e. depleted fraction) for 5.5 minutes, the column was washed and the bound (high abundance) proteins were eluted with 100% Buffer B (Agilent Technologies, Diegem, Belgium) at a flow rate of 1 mL/min for 2.5 minutes. The column was re–equilibrated using 100% Buffer A. Protein elution was monitored at a wavelength of 280 nm during the chromatography fractionation process.

Concentration and buffer exchange:

The collected flow–through fraction containing the low–abundant proteins was filtered using a 1,000 Da molecular weight Microsep spin filter (Pall, Zaventem, Belgium) for the low molecular weight analysis and a 5,000 Da molecular weight Agilent spin filter (Agilent, Diegem, Belgium) for the high molecular weight analysis. After a first filtration step at 7500 *x g* for 100 and 30 minutes for the 1,000 and 5,000 Da spin filter, respectively, a fixed amount of the SELDI–TOF MS binding buffer (CM10 and IMAC binding buffers: see below for specifications) was added and the filtration step was repeated. This last step (adding buffer + filtration) was repeated three times to perform a buffer exchange from Buffer A to the SELDI–TOF MS binding buffers. The samples were then stored at –80 C° until further use.

Protein profiling with SELDI–TOF MS:

Fractions were analysed on CM10 (weak cation exchanger) and copper–coated IMAC30 (immobilized metal affinity capture) arrays (Bio–Rad, Nazareth, Belgium). For the CM10 arrays, spots were pre–incubated twice with CM10 binding buffer (0.1 M sodium acetate, pH 4.0) followed by application of 100 µl of the sample in the same binding buffer. For the IMAC30 arrays, spots were pre–incubated twice with 50 µl of 0.1 M copper sulphate for 5 min at room temperature followed by a wash step with 0.1 M sodium acetate buffer pH 4 for 5 min at room temperature. Spots were then pre–incubated twice with IMAC30 binding buffer (0.1 M sodium phosphate, 0.5 M NaCl pH 7) followed by application of 100 µl of the sample in the same binding buffer. Samples were incubated for 60 min at 4°C with shaking on a MicroMix (Siemens Medical Solutions Diagnostics, Brussels, Belgium). After three additional wash steps with the same binding buffer and two final washes with water, 2 x 1 µl of 20% α–cyano–4–hydroxy cinnamic acid (CHCA) or 100% sinapinic acid (SPA) (Bio–Rad, Nazareth, Belgium) dissolved in 1% TFA/100% ACN were applied. CHCA was predominantly used to improve ionization for lower mass peaks (< 10,000 Da) and SPA for the high mass peaks (10,000–100,000 Da).

Mass analysis was performed using SELDI–TOF MS (PCS 4,000 Enterprise, Ciphergen ProteinChip Reader Inc., Fremont, CA) applying automated data collection protocols for a molecular weight of < 10,000 Da (low molecular weight protocol) and for 10,000–100,000 Da (high molecular weight protocol). The following settings were used for the low molecular weight protocol: (a) laser intensities of 2500 nJ; (b) focus mass 5,000 Da; (c) matrix attenuation 500 Da; (d) sampling rate 400 MHz; (e) 2 warming shots (not included in analysis), 10 data shots per point and (f) total number of points evaluated equal to 12.5% of the spot surface. The following settings were used for the high molecular weight protocol: (a) laser intensities of 2,500 nJ; (b) focus mass 19,000 Da; (c) matrix attenuation 5,000 Da; (d) sampling rate 400 MHz; (e) 2 warming shots (not included in analysis), 10 data shots per point and (f) total number of points evaluated equal to 12.5% of the spot surface. Mass accuracy was calibrated externally using the all–in–one peptide and all–in–one protein standard according to the manufacturer's instructions (Bio–Rad) for the low and high molecular weight analysis, respectively.

A quality control sample (pooled serum) was analyzed weekly to validate the output of the system. Using the Ciphergen Express Software, baseline subtraction and noise reduction were completed before peak intensities

were normalized to the total ion current. Outlier spectra were identified and removed from the analyses when the normalisation factor deviated more than 2 standard deviations. Numeric data were exported to csv–files for further biostatistical processing.

Data analysis:

With the aid of MASDA software the following additional preprocessing steps were performed [16;17]: (1) peak detection based on changes in the first derivative of a sample's intensity curve, (2) peak filtering with exclusion of peaks below a local noise threshold defined as the median plus five times the median absolute deviation, and (3) peak matching/alignment across samples using complete linkage hierarchical one–dimensional clustering. The significance of peaks was determined with the non–parametric Wilcoxon rank sum test. A *p*–value of <0.05 was deemed significant.

Weighted Least Squares Support Vector Machine (LSSVM) in combination with leave–one–out (LOO) cross–validation was used to build classifiers [18;19]. For the optimization of number of peaks included in the classifiers, the number of peaks tested within each LOO iteration ranged from 1 to maximum 10, only including significant peaks ($p < 0.05$). For both CM10 and IMAC30, the low mass and high mass peaks were simultaneously included in the models in order of decreasing significance. The optimal model parameter (regularization parameter of the weighted LSSVM) was chosen as the one corresponding to the largest area under the curve (AUC) of the receiver operating characteristic curve. When multiple parameters with the same AUC, the balanced error rate was minimized with an as high as possible sum of sensitivity and specificity. The main outcome was lymph node status (negative *vs* positive). Secondary outcomes were histological subtype, lymph–vascular space involvement (LVSI) and recurrent disease.

Results:

Patients:

Patient and tumour characteristics are represented in Table 1. Twelve patients were diagnosed with positive lymph nodes. The remainder of the patients had a complete lymphadenectomy performed, but no positive lymph nodes were diagnosed. Both groups were well balanced for age, FIGO stage, histological subtype, number of removed lymph nodes, incidence of LVSI, duration of follow–up and incidence of recurrence. LVSI was – as expected – associated with lymph node status.

Table 1: Patient and tumour characteristics

	Numerical display	LN positive (n = 12)	LN negative (n = 48)	Test	*p* value
Age in years	Mean (95%CI)	45.8 (38.5–53.0)	46.7 (43.3–50.0)	T-test	0.732
FIGO stage					
Ia2	n (%)	0 (0.0)	2 (4.2)	χ^2	0.134
Ib1	n (%)	6 (50.0)	37 (77.1)		
Ib2	n (%)	2 (16.7)	2 (4.2)		
IIa	n (%)	4 (33.3)	7 (14.6)		
Histological subtype					
Squamous cell	n (%)	11 (91.7)	29 (60.4)	χ^2	0.119
Adenocarcinoma	n (%)	1 (8.3)	17 (35.4)		
Adenosquamous	n (%)	0 (0.0)	2 (4.2)		
Lymph nodes					
Positive LN	Median (min-max)	1 (1-7)	0 (0-0)	Mann-Whitney	< 0.001
Removed LN	Median (min-max)	28 (4-50)	34 (18-89)	Mann-Whitney	0.241
LVSI					
positive	n (%)	10 (83.3)	16 (33.3)	χ^2	0.005
negative	n (%)	2 (16.7)	32 (66.7)		
Follow-up					
Follow-up (months)	Mean (95%CI)	61.8 (42.0 – 81.5)	61.7 (49.0 – 66.5)	T-test	0.997
Recurrence					
Recurrence	n (%)	3 (25.0)	7 (14.6)	χ^2	0.665
No recurrence	n (%)	9 (75.0)	41 (85.4)		

Abbreviations: LN = lymph node; FIGO = International Federation of Gynecology and Obstetrics; LVSI = lymphvascular space involvement.

Unsupervised peak detection:

In total 597 different peaks were detected in our panel of 60 samples: 284 peaks on CM10 and 313 on IMAC30. In Table 2 the number of peaks that was differentially expressed according to lymph node status, histological subtype, LVSI and recurrence of disease are shown. In general, the number of differentially expressed peaks was higher in the low mass range, except for the difference between squamous carcinomas and adenocarcinomas. The total number of differentially expressed peaks ranged from 11 to 37, depending on the comparison which was made.

Table 2: The total number of identified peaks and the number of peaks that was significantly differentially expressed for the given comparisons.

	CM10		IMAC		
	Low mass	High mass	Low mass	High mass	Total
Total number of peaks	175	109	172	141	**597**
Lymph node status					
Negative *vs* Positive	2	0	5	5	**12**
Histological subtype					
Squamous *vs* Adeno	8	15	3	5	**31**
Squamous *vs* Adenosquamous	4	2	4	1	**11**
Adeno *vs* Adenosquamous	3	0	18	0	**21**
LVSI					
Negative *vs* Positive	18	0	14	5	**37**
Recurrence					
Negative *vs* Positive	4	0	7	3	**14**

Abbreviations: CM10 = weak cation exchanger array; IMAC = immobilized metal affinity capture array; ca.= carcinoma; LVSI = lymph vascular space involvement.

LOO internal validation for weighted LSSVM

The AUC values obtained by LOO internal validation with the optimal median and mean number of peaks across all LOO iterations are represented in Table 3. For the prediction of lymph node status an AUC value of 0.95 was obtained (Figure 1). Three peaks were repeatedly selected in the LOO iterations: *m/z* values 2,698.9, 3,953.2, and 15,254.8 from the IMAC low mass, CM10 low mass, and IMAC high mass spectra, respectively (Table 4).

LVSI was more difficult to predict. Although a median number of one peak was sufficient, the LOO AUC reached only a value of 0.81. A median number of 1 peak was needed to construct a model that was able to differentiate squamous carcinomas with adenocarcinomas (AUC 0.88), 4 peaks to differentiate between squamous and adenosquamous carcinomas (AUC 0.85), 1 peak to differentiate between adenocarcinomas and adenosquamous carcinomas (AUC 0.94), and 4 peaks to predict recurrence (AUC 0.94). The most frequently selected peaks for the different comparisons are represented in Table 4.

Table 3: AUC obtained by leave-one-out internal validation (LOO) with the optimal median and mean number of peaks per iteration.

	LOO AUC	Median number of peaks per LOO iteration	Mean number of peaks per LOO iteration (SD)
Lymph node status			
Negative *vs* Positive	0.95	1	1 (0)
Histological subtype			
Squamous *vs* Adeno	0.88	1	1 (0)
Squamous *vs* Adenosquamous	0.85	4	3.8 (0.9)
Adeno *vs* Adenosquamous	0.94	1	0.9 (0.3)
LVSI			
Negative *vs* Positive	0.81	1	1 (0)
Recurrence			
Negative *vs* Positive	0.94	4	4 (0)

Abbreviations: SD = standard deviation; ca. = carcinoma; LVSI = lymph vascular space involvement.

Figure 1: Receiver operating characteristics (ROC) curve for the prediction of lymph node status. Abbreviations: AUC: area under the curve, SE = standard error.

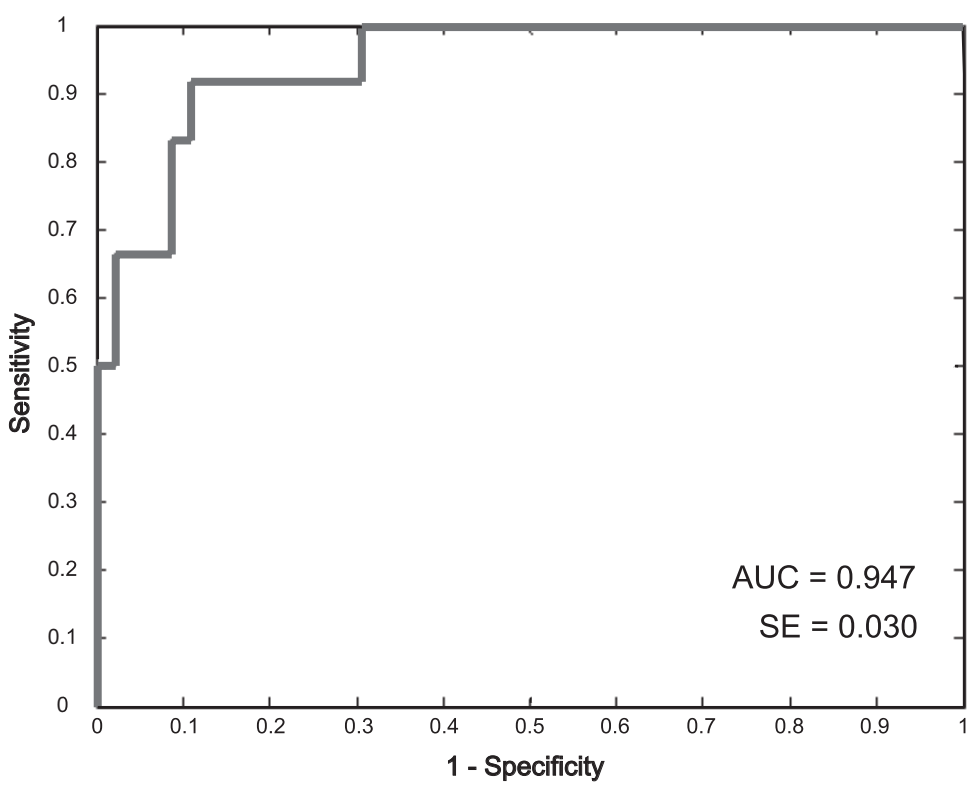

Table 4: Most frequent selected peaks in the leave-one-out internal validation (LOO) iterations, with the corresponding chip surface and mass range.

	Median *m/z* value	Occurrence *	*p*-value	Chip surface	Mass range§
Lymph node status					
Negative *vs* Positive					
	2698.945	15	0.023	IMAC	Low
	15254.808	14	0.022	IMAC	High
	3953.177	13	0.024	CM10	Low
Histological subtype					
Squamous. *vs* Adeno					
	12802.775	28	0.021	CM10	High
	78632.414	11	0.020	IMAC	High
Squamous *vs* Adenosquamous					
	1532.112	39	0.032	CM10	Low
	1532.166	39	0.032	CM10	Low
	1627.269	39	0.032	IMAC	Low
	4783.483	38	0.032	IMAC	Low
Adenoca. *vs* Adenosquamous ca.					
	1531.463	16	0.012	CM10	Low
LVSI					
Negative *vs* Positive					
	1741.204	58	0.008	IMAC	Low
	3224.349	13	0.010	CM10	Low
Recurrence					
Negative *vs* Positive					
	94029.326	53	0.021	IMAC	High
	97177.269	52	0.018	IMAC	High
	78294.986	52	0.027	IMAC	High
	78417.606	34	0.031	IMAC	High
	2044.703	21	0.031	IMAC	Low
	1979.514	15	0.035	IMAC	Low

* The number of times the peak was selected within the different LOO iterations.
§ Low mass range: < 10 kDa; high mass range >10 kDa.
Abbreviations: CM10 = weak cation exchanger array; IMAC = immobilized metal affinity capture array; ca.= carcinoma; LVSI = lymph vascular space involvement.

Discussion

This study investigated whether we could improve the prediction of lymph node involvement with proteomic profiling. We used a combination of

HPLC immunodepletion with SELDI–TOF MS to detect proteins that predict lymph node involvement. Using LSSVM models we were able to predict lymph node involvement with an AUC of 0.95. These findings suggest that serum biomarkers could help us identifying patients with lymph node metastases.

The majority of serum proteins are high–abundance proteins, accounting for almost 99% of the total protein mass [20]. Most of these proteins are true serum or plasma proteins that carry out their functions in the circulation, rather than proteins or peptides that leak into the blood (e.g. tumour tissue proteins) [20]. Removing the high abundant proteins facilitates the discovery and identification of low–abundance proteins that may be biomarkers [21]. The MARS–14 immunodepletion column used in the present study removes 95–99% of the 14 most abundant proteins from serum, thereby increasing the likeliness of finding possible biomarkers [22;23]. This technique has proven to be highly reproducible [23]. However, due to protein–protein or protein–antibody interactions also non–targeted proteins are being removed [23;24] which could hamper the detection of certain proteins. Moreover, some reports mention that the detection of medium abundance proteins improves, but not the detection of the very low abundance proteins (<10 ng/mL) [22]. This is the range in which some of the currently known biomarkers are found (e.g. CEA) [20]. Another problem with immunodepletion in combination with SELDI–TOF MS is that both systems, the HPLC and SELDI–TOF MS are not in–line as other LC–MS techniques. The additional sample handling introduces additional experimental variables, such as additional freezing/thawing cycles, and manually handling of the samples.

Upon establishing the biomarker profiles for lymph node involvement in cervical cancers, it became interesting to identify the proteins behind the differentially expressed peaks. For the 15,254.8 peak detected on the IMAC30 chip, an approach was developed using immunodepletion and SDS–PAGE gel electrophoresis as initial separation steps. Unfortunately, due to the apparently very low concentration of this protein in serum, no Coomassie Blue band could be detected at the level of 15–16 kDa. For the two lower masses (2,698.9 and 3,953.2) an attempt was undertaken for direct identification from the corresponding SELDI target plate. This involved the use of a special SELDI Chip target adapter (Bruker Daltonics, Bremen, Germany) to analyze the spots with a MALDI–TOF/TOF MS (Ultraflex 2, Bruker Daltonics, Bremen, Germany). Indeed, the TOF/TOF MS can induce fragmentation of selected masses, which is essential for their

subsequent identification. However, SELDI–TOF MS is known for having a poor mass accuracy or reproducibility [25]. This made it difficult to determine which peak in the 2,650 – 2,750 and the 3,900 – 4,000 Da range on MALDI–TOF/TOF MS was responsible for the 2,698.9 and 3,953.2 peaks on SELDI–TOF. Moreover, collision induced dissociation (CID) of high mass peaks (>3kDa) is difficult in currently available MALDI–TOF/TOF MS instruments, yielding no or incomplete fragments from this masses. Alternatively, an off–line sample preparation was explored to allow analysis of larger volumes of samples using a MALDI target plate. In this project, SELDI–TOF MS on–chip chromatographic surfaces are used to select proteins with either cationic or metal affinity properties. This gives two advantages to SELDI–TOF MS: (1) the chromatographic surface acts as an additional prefractionation step, selecting only a subset of proteins that will be analyzed (enrichment), and (2) the proteins get separated from salts and other sample contaminants by subsequent on–spot washing with appropriate buffer solutions. As in MALDI–TOF MS analysis, on–chip purification is not possible, sample cleanup procedures must be applied before the sample is put on the target to reduce noise and ion suppression. In our identification experiments we applied an additional desalting step by using revered phase chromatography, either by HPLC, or by C4 or C18 Zip–Tip. These additional steps introduced additional experimental variables making it even more uncertain to identify the correct protein. Taken together, the additional sample preparations resulted in sample loss as well as introducing qualitative and quantitative variances, without leading to the required identification.

When looking at the literature on SELDI–TOF experiments, it can be noticed that in only a minority of papers an identification was performed. Most of the papers mention that identification and validation of the newly discovered biomarkers is ongoing. However, follow–up papers on the identified proteins, or validation studies are rarely published. This is not only the case for biomarker discovery studies for gynaecological cancers [15], but also for various other types of cancer [26;27].

Conclusions

In conclusion, the SELDI–TOF MS approach has allowed to discover a set of proteomic profiles (revealing potential biomarkers) that could help us in the diagnosis of lymph node metastases. However, the proteins/peptides concerned were not identified due to technical limitations of the SELDI–TOF MS technique.

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Part III
Reviews and Comments

Chapter 1: The utility of proteomics in gynaecologic cancers (review)

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Abstract

Purpose of review: For more than a decade, proteomic techniques have been used to unravel the nature and function of human proteins. In 2002 it came to the attention of clinicians that this technique could be used to discover new biomarkers. However, the first reports were hampered by technical and methodological flaws. Since these first reports, proteomics has matured and the technical abilities have grown enormously. An in–depth analysis of fluid or tissue specimens is now possible. We reviewed recent literature to see whether proteomics has changed our clinical practice in the diagnosis and treatment of gynaecological cancers.

Recent findings: In ovarian cancer a great effort has been put into discovering new diagnostic and screening markers. Several proteins have been put forward as possible candidates to fulfil this task. However, none of the proteins turned out to be better than CA125 alone. In endometrial cancer many of the presumed tumour markers are not specific for endometrial cancer but are more tumour markers for cancer in general. The same problem was noticed in cervical cancer. Papers are now focusing more on therapy response and carcinogenesis.

Summary: To date, proteomic studies have not been able to change our clinical practice in gynaecological oncology.

Introduction

Of all gynaecological malignancies, ovarian cancer has been the most frequent cancer investigated in proteomic studies. This is due to problems that still exist in diagnosing and treating patients with ovarian cancer. Most patients consult when the disease is already extended (FIGO stage III–IV disease) and the prognosis is poor with a 5–year overall survival rate of 18–40% [1]. Problematic is that no precursor lesions exist and that the tools for diagnosis of early disease are lacking. Secondly patients with advanced disease are now treated with a combination of extensive debulking surgery and platinum containing chemotherapy without knowing beforehand if the tumour will be sensitive to it or not. Therefore biomarkers predicting early disease and/or response to therapy would be of clinical relevance.

Endometrial cancer usually causes symptoms (e.g. postmenopausal vaginal bleeding) leading to an early diagnosis of the disease: 71% of patients is diagnosed in FIGO stage I with a good prognosis [2]. Thus, screening does not seem useful in these tumours. However, biomarkers can be useful to follow treatment or to understand the tumourigenesis. This could open perspectives for new targeted therapies.

In the developed countries, organized screening with systematic call, recall, follow–up and surveillance systems has been responsible for the significant decline in cervical cancer incidence by as much as 80% [3]. In contrast, in the developing countries cervical cancer is still the major cause of cancer–related death. Every marker that could facilitate the diagnosis or treatment of cervical cancer would aid to the combat against this cancer.

Most proteomic studies used blood samples as biological specimen for protein extraction though ascites and tissue could be very attractive alternatives [4]. As already well known, problems with blood samples are the presence of high abundant proteins and the inter– and intra–individual differences between patient samples. Therefore quite often preprocessing of the samples is used to remove the high abundant proteins and get information of the deeper proteome. Independent of the biological sample used, it is of uttermost importance to work with strict protocols to minimize variation (inherited or caused by handling and processing of the samples) to get reproducible and reliable profiles [5].

In this review we would like to highlight the most recent evolutions in the field of proteomics and gynaecological cancer, in particular ovarian, endometrial and cervical cancer.

Review

1. Ovarian Cancer

Since 2002, when the first manuscript of Petricoin et al [6] was published, a lot of proteomic studies have been performed in ovarian cancer though until now unfortunately none of them was able to change clinical practice.

1.1 Screening for epithelial ovarian cancer: diagnostic markers

Several markers have been investigated and multiple maker sets or protein profiles have been proposed to identify patients with early ovarian cancer [7-13]. Only some of them were confirmed in validation studies and identification of these proteins reveals in the majority of cases acute phase proteins (transferrin, haptoglobin, transthyretin,…) or abundant plasma proteins (apolipoprotein, haemoglobin…). These are less attractive biomarkers as they occur in different conditions and diseases and are therefore not cancer specific.

Visintin et al [14] described a six marker blood test which is commercialized as OvaSure® and determines leptin, prolactin, osteopontin, insulin–like growth factor II, macrophage inhibitory factor and CA125. They claimed that the test has a sensitivity of 95.3% and a specificity of 99.4% for the detection of ovarian cancer though several methodological questions were raised and further confirmation of this test is necessary before it could be used in general practice.

The most promising data were published by Moore et al [15] where they combined human epididymis protein 4 (HE4), CA125 en menopausal status to predict malignancy when an ovarian mass was found. They investigated 9 potential biomarkers (CA125, HE4, soluble mesothelin–related peptide, CA72–4, activin A, inhibin, osteopontin, epidermal growth factor and serum Her2) in 233 patients with a pelvic mass and found that HE4 as a single marker was the best with a sensitivity of 72.9% (at a specificity of 95%) in comparison with a sensitivity of 43.3% (at the same specificity) for CA125 alone. Combining these markers yielded a sensitivity of 76.4% (specificity of 95%). The combination of a third marker (osteopontin or CA72–4) only increased the sensitivity minimally and was not further investigated.

Furthermore the authors stated that HE4 would be of particular interest in patients with stage I disease and premenopausal patients stressing its importance as a diagnostic marker. They then applied this algorithm on a prospective collected database of 531 patients and investigated the diagnostic potential of this dual biomarker set [16]. For the postmenopausal patients the test showed a sensitivity of 92.3% with a specificity of 74.7% and for the premenopausal patients a sensitivity of 76.5% for a specificity of 74.8%. Again this shows its improvement in the premenopausal patients although the numbers of premenopausal patients with epithelial ovarian cancer in this study were rather small.

In a recent study [17] they compared their risk of ovarian malignancy algorithm (ROMA) with the known risk of malignancy index (RMI) based on architectural features of the mass, menopausal status and CA125 described by I Jacobs [18] and found the ROMA test to be superior. In 457 patients, ROMA had a sensitivity of 94.3% and RMI of 84.6% for a fixed specificity of 75% in distinguishing benign disease from epithelial ovarian cancer (p = 0.0029). In patients with stage I and II disease, ROMA achieved a sensitivity of 85.3% compared with 64.7% for RMI ($p < 0.0001$). Consensus on the radiographic images by the different interpreters was though only 78,4% which could reflect the lower RMI.

These results are promising though an independent validation of these results is necessary and needs to be awaited before using this algorithm in general practice. Van Gorp et al [19] performed an independent validation study and found similar performance indices though no improvement of the detection rate of malignant disease was found in favour of HE4 or the combination with CA125 vs CA125 alone.

These results suggest that combining proteomic results with other methods (multiple marker sets, radiographic findings, clinical data,…) could lead to an increase in detection rate of epithelial ovarian cancer. The first results of the UK collaborative trial of ovarian cancer screening (UKCTOCS) suggest this is the case. They compared the use of ultrasound alone versus multimodality screening (MMS) and found that the sensitivity was comparable between the two groups though the specificity was in favour of the MMS group reflecting lower rates of multiple testing and surgery. Furthermore an improved sensitivity in detecting early stage disease above usual clinical detection (48 *vs* 28%) was described though the effects on mortality need to be awaited [20;21].

1.2 Predicting response to current therapy: prognostic markers

In contrary with genomic studies, only few proteomic studies focused on predictive markers of chemotherapy response or optimal debulking surgery.

Some cell culture studies examined differentially expressed proteins between platinum sensitive and resistant ovarian cancer cell lines. They identified proteins which are responsible for (a) an accelerated detoxification of drug substrates, (b) inhibition of apoptotic cell death through e.g. modulation of the actin cytoskeleton and (c) inhibiting pathways leading to a decreased basal metabolism which helps cells to live through the duration of drug therapy [22-26]. This has not been confirmed in *in vivo* studies as manipulation of cells during culture handling itself can cause changes at protein level stressing the importance of validation studies.

We already showed in a small pilot study that with optimized protocols differentially expressed proteins can be found in laser microdissected platinum sensitive vs resistant ovarian cancer cells using SELDI–TOF MS [27]. Currently this is being investigated in a large prospective European trial, the OVCAD study (www.ovcad.eu), were molecular traces at different levels (DNA, RNA and proteins) are investigated signaling the presence of minimal residual disease.

An interesting study was recently published by Carey et al [28] identifying a number of candidate proteins indicating response to primary chemotherapy. Forty–five tumour specimens were analyzed using reverse phase protein arrays. As primary outcome measure they used normalization of CA125 after the third cycle of chemotherapy. Twenty five patients had a normalization of the marker while 20 patients retained abnormal levels. They found EGFR, JNK and Smad 3 to be independent determinants on multivariate logistic regression analysis. Unfortunately, 41 of 45 patients (91%) underwent debulking surgery with macroscopic residual disease. Residual disease after debulking surgery has previously been shown to be one of the most independent predictors of prognosis in advanced ovarian cancer [29;30] and therefore the division of this mixed group of patients into a group with and without normalization of CA125 has no clinical relevance. Furthermore no information is given regarding platinum sensitivity of these patients which would be a better primary outcome measure for response to primary chemotherapy. It would have been more interesting to perform this study in completely debulked

patients (no residual disease) who received standard platinum based chemotherapy and of which platinum sensitivity was known.

Risum et al [31] evaluated the use of CA125 or a 7 marker panel (OvaRI) as a predictor for complete debulking surgery. They used a test set of 75 patients and could predict complete cytoreductive surgery with a sensitivity of 71% or 73% with a specificity of 57% or 70% for CA125 and OvaRI respectively. Again, the relevance of this is limited as no validation was performed.

2. Endometrial cancer

One research group, from the university of Toronto, Ontario, Canada has been particularly active in the field of biomarker discovery for endometrial cancer. They published several discovery and validation studies over the past years [32-38]. With a variety of proteomic techniques they were originally able to identify 2 proteins that were differentially expressed between normal endometrium and endometrial cancer: chaperonin–10 (also known as heat shock protein (HSP) 10) and calgranulin–A (also known as S100A8) [32;33]. They confirmed the presence of these proteins within the malignant epithelium and not in the surrounding stroma [34]. In a later study they used different proteomics techniques and were now able to identify a set of 9 differentially expressed proteins [39]. Based on the existence of antibodies, they selected 5 proteins (chaperonin–10, calgranulin–A, pyruvate kinase M2, polymeric immunoglobulin, and alfa–1–antitrypsin precursor), to be tested in a large tissue microarray validation study on 148 samples [35]. When they combined chaperonin–10, pyruvate kinase M2, and alfa–1–antitrypsin precursor, they were able to achieve a sensitivity of 0.85 and a specificity of 0.93. The authors recognize the fact that alfa–1–antitrypsin is in fact a large serum protein, produced in the liver, and therefore it is unlikely that this protein will be able to act as a tumour marker for endometrial cancer. Chaperonin–10 and pyruvate kinase M2 have been linked to tumourigenesis in general, and may be good candidates for tumour markers. However, both proteins are not unique to endometrial cancer and their role has been confirmed in several malignancies [40;41]. So they may act as ‘cancer’ biomarkers, but not necessarily as ‘endometrial cancer’ biomarkers. Studies were several types of cancer are included, and compared for the presence of these markers are to be awaited.

A second research group, from the Sighuan University, Chengdu, China has also been active over the last few years. They discovered epidermal fatty

acid binding protein, calcyphosine, cyclophilin A as overexpressed proteins in endometrial cancer [42;43]. In their study they also perform a knock–down experiment. In the absence of cyclophilin A the tumour stopped growing, and thus this proved that cyclophilin A is involved in tumourigenesis, making it an attractive target for targeted therapy [43]. Epidermal fatty acid binding protein and calcyphosine were also of prognostic importance [44].

3. Cervical cancer

The causal relationship between the human papilloma virus and cervical cancer is well known and although precursor lesions are easy to detect, the last few years several studies used proteomics to examine cervical cancer. This is partially due to the fact that tissue or fluid samples are easy to obtain.

3.1 Diagnostic markers for cervical cancer

With the aim to identify new diagnostic markers for cervical cancer, Zhu *et al* [45] analyzed 10 pairs of human squamous cervical cancer and matching adjacent normal cervical tissues with two–dimensional gel electrophoresis, matrix–assisted laser desorption/ionization time–of–flight mass spectrometry, and Western blotting. They identified Tyk2, S100A9, and Zinc finger protein 217 as proteins involved in squamous cervical cancer. Interestingly, S100A9 was also found to be upregulated in squamous cervical cancer in a cDNA microarray experiment [46]. Later, the same protein was found to induce apoptosis and inhibit migration in a CaSki cervical cancer cell line [47]. However, it must be pointed out that S100A9 cannot be considered as a unique marker for cervical cancer since members of the S100 protein family have been shown to be raised in multiple cancers (e.g. cancers from the skin, lung, breast, endometrium, ovary, oesophagus, colon, liver, soft tissue and bone) [48]. A more recent study, by Hwang *et al* [49], came to the conclusion that HSP 60 was elevated in cervical cancer. In their experiment they examined 20 tissue samples from cervical cancer patients and 20 from normal controls. HSPs are one of the most frequently identified proteins that are repeatedly differentially expressed in nearly 30% of all experiments on both human and rodent tissues [50]. Therefore the value of this finding is also relatively small.

3.2 Detection of cervical intra–epithelial neoplasia (CIN)

Although CIN lesions are fairly easy to diagnose with cervical smears, some proteomics experiments have focused on the analysis of proteins involved in dysplastic lesions. The aim is not to find new diagnostic markers but to

gain insight in the carcinogenesis and progression from an normal cervix to a CIN lesion and further to cervical cancer. One of these experiments used plasma proteomics to detect differentially expressed proteins between normal controls, CIN lesions and cervical cancer [51]. As always in serum or plasma experiments, several acute phase reactants, transport proteins, and coagulation factors were found to be differentially expressed. Besides these proteins, also cytokeratin 19 and tetranectin were elevated. Their function or role in the carcinogenesis of cervical cancer remains to be clarified. Two studies have focused on the use of cervical–vaginal fluid to characterize the cervical mucus proteome [52;53]. Both studies shortlisted proteins with various functions. Although samples from normal and CIN were used, they did not compare between both conditions. The main goal was to analyze the proteome, not to find differentially expressed proteins. Undoubtedly, some of these shortlisted proteins will play a role in carcinogenesis and may act as markers for progression from normal to CIN lesions.

3.3 Therapy response

Chemoradiotherapy, cisplatinum and paclitaxel are the most frequently used anticancer treatments in advanced or recurrent cervical cancer. Using a cervical cancer cell lines or fresh tissue samples, some groups have performed proteomics to identify the proteins that are upregulated or downregulated in case of one of these treatments. Chemoradiotherapy resistant cells, for example, show an increased expression of S100A9 and galectin–7, but decreased expression of nuclear matrix protein 238 and HSP 70 [54]. These studies could help us in understanding mechanisms of therapy response and resistance.

Conclusion

To date proteomic studies have not been able to change clinical practice. Future directions should focus more on combinations of clinical parameters with genomic, proteomic and metabolomic findings.

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Chapter 2: Comment: Methodology in plasma proteomic pattern experiments

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To the Editor:

With great interest we read the article by Lin *et al* [1] investigating whether it is possible to identify new plasma biomarkers in ovarian cancer patients using mass spectrometry protein profiling and artificial intelligence. The authors claim to have identified six possible markers with a sensitivity of 90–96.3% and specificity of 100%. Four of these markers were only present in plasma of ovarian cancer patients and two only in plasma of controls. The authors used SELDI–TOF MS (Ciphergen Biosystems, Inc., Fremont, CA) in combination with Biomarker Pattern's Software (Ciphergen Biosystems, Inc.).

However, after careful examination of the data analysis strategy of Lin *et al*, we noticed that the software to build the algorithm used all 65 samples that were used in this current study. No independent test was used to validate the model. Thus, the above mentioned sensitivity and specificity are solely based on a training set. Unfortunately, this implies that the reported performance indices are overestimated, and it will probably be impossible to reproduce these sensitivity and specificity on new prospective data. After model selection and in order to obtain a realistic estimate of the true performance, it is therefore imperative to test a new model on completely independent and prospective data. One other solution is to use cross–validation. The authors used a technique called ''internal one out of ten sample exclusion, crossvalidation.'' This technique is not clearly explained in the materials and methods section, and yet this crossvalidation technique is essential in estimating errors in generalization of the results. In tenfold cross–validation, the data is divided in ten subsets of (approximately) equal size. The results of this cross–validation technique are only mentioned very briefly in the discussion section (sensitivity of 84% and specificity of 89%). However, even cross–validation is prone to an underestimation of the error rate, and the authors do not mention whether this cross–validation was performed before or after selection of the possible markers [2]. Note that the generalization performance can only be estimated in a reliable way in case the entire model building process, including the selection of the informative markers, is repeated for each cross–validation training set [3]. Furthermore, since the tumours used to build the predictor may not reflect all characteristics of the population of interest, it is still preferable to use an independent test set to overcome this problem. Secondly, we want to mention that Lin *et al* stated that it is desirable to know the identity of the biomarkers in the pattern. However, no effort was performed to realize this identification. We believe that the identification of the markers in the pattern is not only desirable but

mandatory. Validation of results can be performed in many ways [4]. One way is to perform the same experiment on a different set of patients or cross–validation. However, to our opinion, validation of the results by using other experimental techniques is as important. Identification will allow for using other techniques, including enzyme–linked immunosorbent assay, immunohistochemistry, Western blot, polymerase chain reaction, reverse transcription–polymerase chain reaction, etc. Furthermore, it will give more information on the function of the protein within the organism. Examples in literature show that many of the claimed markers turn out to be trivial proteins, more related to immune response than to cancer biology [5]. In this perspective, the identity of the protein will not only inform about the function of the protein but also on possible sources of bias. To give one example: if a truncated protein is more often found in cases than in controls, one possible explanation could be that the plasma samples were collected differently (e.g., longer interval between drawing and freezing of the samples), leading to ex vivo proteolysis. Although Lin *et al* show a great effort to minimize the experimental variables by careful selection of cases and controls and by a strict collection protocol, we believe that the identification of proteins can add value to proteomic research. In summary, we believe that the magnitude of the performance indices of the six protein model by Lin *et al* are unlikely to be confirmed on an independent test set. In the absence of new prospective data to properly assess the current model, we believe that model training should be repeated using a crossvalidation method that refrains from using information from the training set, i.e., by repeating the entire model–building process in each cross–validation iteration. Only under these circumstances is it possible to correctly estimate the generalization performance of this model. Furthermore, we believe that the identification of the markers together with validation of the results with other experimental techniques is mandatory.

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General discussion and future perspectives

The diagnosis of ovarian cancer remains troublesome. Most of the time the diagnosis is obvious. For example, a small simple cyst is almost always benign, and, on the other end of the spectrum, a pelvic mass with ascites and peritoneal implants is almost always malignant. In these cases all diagnostic tests will practically indicate a correct diagnosis. Whether this test is a single marker, a combination of markers, and/or ultrasound is in these cases not so important. However, in a subset of patients the diagnosis seems to be more difficult. The consequences for the patient, surgeons and health care system are substantial. Planning a major laparotomy (with midline incision) for a presumed malignant cyst which turns out to be benign will cause additional anxiety, pain, and mutilation for the patient, but also additional costs due to an increase use of medical resources, length in hospital stay and the inappropriate and additional use of high specialist care by a gynaecological oncologist. Inversely, planning a laparoscopy for a presumed benign cyst which turns out to be malignant will at least cause additional morbidity and costs because the patient will need at a second staging or debulking procedure, but even worse, may also be deleterious for the overall survival of the patient when this laparoscopy caused spill of cyst fluid [1]. The increased risk of recurrence will also increase the costs for the health care system.

From the start, one of the primary aims of this thesis was to explore the possibility to reduce the number of patients with an incorrect preoperative diagnosis and operation. The project started in 2004, and at that time the gold standard in the diagnosis of ovarian cancer was CA125 together with ultrasound. In 2008 and 2009, Moore *et al* published a series of papers that used a combination of CA125, HE4 and menopausal status to predict the presence of a malignant ovarian tumour [2;3]. They claimed that the combination of CA125 and HE4 had a higher AUC than CA125 alone. An important notice is that Richard Moore owns the intellectual property of the use of HE4 as a diagnostic marker (United States Patent Application US20070286865) and that all papers were financed or co–authored by Fujirebio, the company that markets the HE4 ELISA. This does not necessarily mean that this would have influenced the results of the study, but an independent validation study seemed mandatory. In ***Part I – Chapter 1***, we aimed to independently validate HE4 and the ROMA [4]. In this large study we were able to demonstrate similar performance indices as those recently published in the literature. However, in our study, neither HE4 nor the ROMA increased the detection of malignant disease. Since these results

were disappointing from a medical point of view, this evoked various reactions among the readers of the article. This resulted in a letter to the editor by Langmár et al (***Part I – Chapter 1b***)[5] to which we to which we replied accordingly.

Based on a multicenter study, Moore et al also claimed that the ROMA performed significantly better than the RMI [6]. This would change triage and referral of women with a pelvic mass to appropriate surgeons and institutions dramatically, and would improved care for patients with an ovarian mass. This claim needed to be put to the test and in our next study we used the same prospective cohort that we used before to compare the accuracy of ROMA to that of RMI (***Part I – Chapter 2***)[7]. Moreover, we also tested the so–called subjective assessment or pattern recognition. On the basis of greyscale and colour Doppler findings, the ultrasound examiner was obliged to give his/her subjective impression on the nature of the ovarian mass. Subjective assessment is a highly accurate method for discriminating benign from malignant ovarian masses [8-12]. Subjective assessment had the highest AUC (0.968), followed by the RMI (0.931) and the ROMA (0.893). Based on these results we concluded that ultrasound, especially subjective assessment by ultrasound, remains superior in discriminating malignant from benign ovarian masses. However, one caveat needs to be made: this study was performed in a specialised referral centre. This will have influenced the quality of ultrasound in a positive way. Future studies need to be carried out to examine whether the same results can be achieved in smaller/regional hospitals with less experience in ultrasound. Nevertheless, this also shows that extensive specialisation (and maybe even centralisation) in gynaecological ultrasound is beneficial to the patient.

The fact that the combination of CA125 and HE4 did not improve the diagnosis of ovarian cancer and that ultrasound remains very important in the diagnosis of ovarian cancer did not withhold us from our search for new 'objective' parameters such as tumour markers. In ***Part II – Chapter 1*** we examined whether endorepellin as a single marker, or in combination with known markers (CA125 and/or HE4), could differentiate between benign and malignant ovarian masses. Endorepellin is the C–terminal fragment of perlecan, a protein which is located in the basement membrane of vascular endothelium [13]. Again we conducted a prospective cohort study. This cohort study was conducted in four different centres across Europe: Belgium, Czech Republic, Sweden, and Italy. Preoperative blood samples were tested for CA125, HE4 and endorepellin. Endorepellin levels were measured using targeted mass spectrometry. The results were promising.

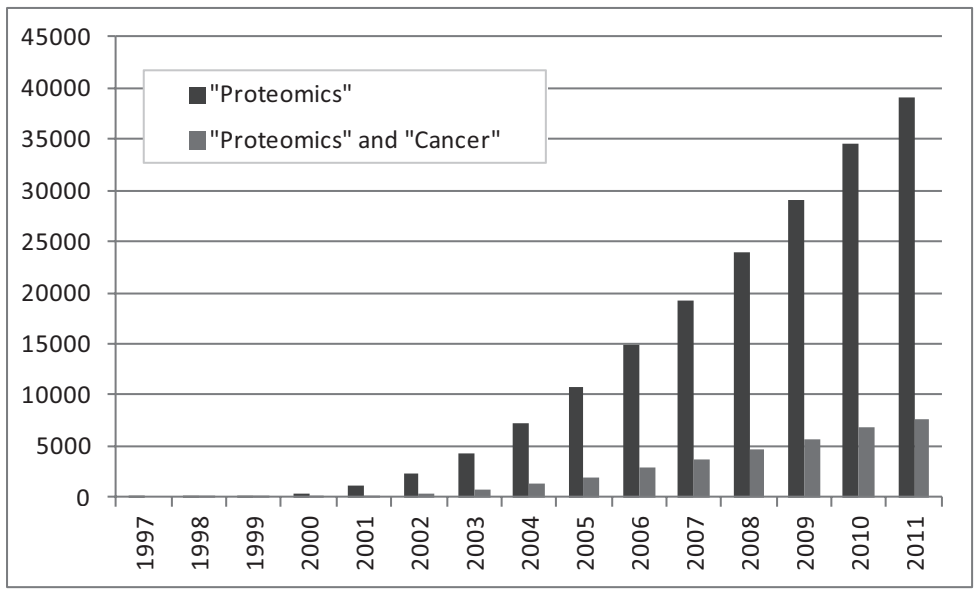
In postmenopausal women, all best performing panels included endorepellin with the highest AUC (0.920) for the triple combination of CA125, HE4 and endorepellin. A downregulated endorepellin seemed to have complementary role to CA125 and HE4, and showed promise as a novel biomarker. Endorepellin also performed well in stage I and LMP tumours. Moreover, as a single marker, endorepellin performed as well as CA125 in stage I and LMP tumours, and as well as HE4 in stage I tumours, and better than HE4 in LMP tumours. This was an exploratory study and the results need to be validated. Currently, the triple combination model (CA125 – HE4 – endorepellin) is being tested in an independent and external validation study with samples from Berlin, Germany. Future work also includes a comparison of the performance of the newly developed risk stratification tool using CA125, HE4 and endorepellin with the Risk of Malignancy Index (RMI) as previously reported for the ROMA [7]. In addition, the analysis will test whether adding ultrasound data to the stratification tool of will improve its performance. Little is also known about the specific role of endorepellin in ovarian carcinogenesis. Endorepellin binds members of the fibroblast growth factor (FGF) and VEGF family and their receptors, respectively [14]. Therefore, it plays an important role in several aspects of vascular development. But the relative role and interaction of endorepellin with other angiostatic peptides, such as canstatin, endostatin, thrombospondin, and tumstatin needs to be clarified.
Proteomics was also used to discover new biomarkers in cervical cancer (***Part II – Chapter 2***). For this we used a global mass spectrometry approach. The aim was to find new serum markers that could predict lymph node metastases. Lymph node involvement is a strong prognostic factor for cervical cancer. Whenever positive lymph nodes are found in patients with cervical cancer, the overall 5–year survival drops dramatically [15]. Patients with a cervical carcinoma with positive lymph nodes usually receive adjuvant radiotherapy. The combination of surgery and radiotherapy can cause serious side effects, such as lymphoedema of the legs, fistulas and wound breakdown. Hitherto, positive lymph node are difficult to detect with radiological examinations [16]. These biomarkers can facilitate the selection of patients requiring pelvic lymphadenectomy or irradiation. We analysed serum samples of 60 cervical cancer patients. After depletion of the most abundant proteins we analysed the samples with SELDI–TOF MS. With the aid of bioinformatics we performed unsupervised peak detection and peak clustering. Using LSSVM models and LOO cross validation we were able to predict lymph node involvement with an AUC of 0.95. These findings suggest that serum biomarkers could help us identifying patients with lymph node metastases. However, the attempts to identify the

proteins behind the differentially expressed peaks were unsuccessful. Neither the low mass peaks, nor the high mass peaks were identified, despite additional experiments with SDS–PAGE gel electrophoresis, MALDI–TOF MS and reversed phase HPLC or C4–C18 zip–tips. This was mainly due to the technical specifications of SELDI–TOF MS. SELDI–TOF MS is able to detect very low concentrations of peptides/proteins without interference of salts or other sample contaminants. This is due to the chromatographic surfaces that enrich the proteins and eliminate contaminants. However, the downside of SELDI–TOF MS is the poor mass accuracy or reproducibility [17]. Since identification on SELDI–TOF MS is difficult we needed to use other techniques. With the other techniques we were not able to detect the same peaks and thus identify the peptides or proteins behind the peaks. This questions also the fact whether a global proteomics analysis on serum should be performed with SELDI–TOF MS. When looking at the literature on SELDI–TOF MS experiments, it can be noticed that in only a minority of papers an identification was performed. Most of the papers mention that identification and validation of the newly discovered biomarkers is ongoing. However, follow–up papers on the identified proteins, or validation studies are rarely published. This is not only the case for biomarker discovery studies for gynaecological cancers [18], but also for various other types of cancer [19;20].

In 2002 it came to the attention of clinicians that global proteomics could be used to discover new biomarkers [21]. In the years that followed there was a boom in the number of publications on proteomics (Figure 1). The literature concerning the use of proteomics in the field of gynaecological oncology was critically reviewed (***Part III – Chapter 1***) [18]. It can be noticed that, although a lot of effort has been put into global proteomics, none of the effort has paid off. In ovarian cancer, for example, several proteins have been put forward as candidate markers, but none of the proteins turned out to be better than CA125 alone. This is mainly due to certain 'growing pains' during the first few years. The initial reports were hampered by technical and methodological flaws (***Part III – Chapter 2***)[22], but the field of proteomics has matured and the technical abilities have grown enormously. Scientist learned that sources of variability and artefacts can be minimized by very strict standard operating procedures and that both identification and validation are mandatory. Over the last decade the field of mass spectrometry has evolved and expanded with new techniques: high–definition MS equipment and new software enables scientists to detect proteins up to the femtogram level. Future developments include

tandem expansions with multiple connections to HPLC equipment. In–depth analyses of fluid or tissue specimens seems now possible.

Figure 1: Cumulative number of papers retrieved from Medline with search terms "Proteomics" and "Proteomics and Cancer". Note the sudden increase after 2002.



In conclusion, in the paper of Petricoin *et al* the following can be read: "*Generation of the mass spectra requires only a small serum sample that could be obtained by fingerpick, and results are obtained in less than 30 min. Cost–effective, high–throughput screening is feasible.*" [21]. This was, to say the least, overoptimistic. This optimism has appealed many clinicians and scientist to start experiments in the field of proteomics. Now, exactly ten years later, Petricoins' premise is not fulfilled. On the contrary, we have learned that proteomics is always in need for strict standard operating procedures, sometimes very laborious, and never cheap. To date proteomic studies have not been able to change clinical practice. We do believe that there is a place for global proteomics, but this should be an in–depth proteomic profiling with high levels of fractionation, separation and identification. Future directions should also focus on combining clinical data with genomic, proteomic and metabolomic findings.

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Summary

English summary

The objective of this research project was to validate newly discovered biomarkers and to discover new biomarkers by using proteomics in serum or plasma of ovarian and cervical tumours.

The first diagnostic problem was the **differentiation between a benign and a malignant ovarian mass**. Even when a pelvic mass is present, it is difficult to discriminate between benign and malignant disease.
In a first study the ROMA algorithm, a combination of CA125 and HE4, was validated and compared to the gold standard CA125. In this large study we were able to demonstrate that neither HE4, nor the ROMA increased the detection of malignant disease.

However, the differentiation of benign and malignant masses is not purely based on tumour markers alone. This is why a second study was performed, in which the diagnostic accuracy of ROMA was assessed and compared to the two most widely used ultrasound methods, the risk of malignancy index (RMI) and subjective assessment by ultrasound. Subjective assessment had the highest AUC (0.968), followed by the RMI (0.931) and the ROMA (0.893). Based on these results we concluded that ultrasound, especially subjective assessment by ultrasound, remains superior in discriminating malignant from benign ovarian masses.

In an exploratory study we investigated whether endorepellin, an angiostatic peptide, could be used as a tumour marker for the differentiation between benign and malignant ovarian masses. This international cohort study was conducted in Belgium, Czech Republic, Sweden, and Italy. In postmenopausal women, all best performing panels included endorepellin with the highest AUC (0.920) for the triple combination of CA125, HE4 and endorepellin. Endorepellin also performed well in stage I and LMP tumours. These results need to be validated. Currently, the triple combination model is being validated in an independent study.

The second diagnostic problem is the **diagnosis of positive lymph nodes in cervical cancer**. Lymph node involvement is a strong prognostic factor for cervical cancer. There is a strong need for biomarkers that predict lymph node involvement in cervical cancer. After depletion and SELDI–TOF MS analysis we were able to predict lymph node involvement with an AUC of

0.95. However, the attempts to identify the proteins behind the differentially expressed peaks were unsuccessful.

Finally, the **literature was critically reviewed and commented**. It can be noticed that, although a lot of efforts have been put into global proteomics, none of the efforts have paid off. To date proteomic studies have not been able to change clinical practice.

Nederlandstalige samenvatting

Het doel van dit project was het valideren en ontdekken van nieuwe tumormerkers in serum of plasma van patiënten met een ovarium– of cervixtumor.

Het eerste diagnostisch probleem was de **differentiatie tussen benigne en maligne ovariumtumoren.** Zelfs wanneer een massa in het bekken aanwezig is, is het moeilijk om een onderscheid te maken tussen een benigne en maligne massa.

In een eerste studie werd het ROMA algoritme, een combinatie tussen CA125 en HE4, gevalideerd en vergeleken met de gouden standaard CA125. In deze grote studie toonden we aan dat noch HE4, noch ROMA de detectie van maligne ovariële tumoren verbeterde.

In de praktijk gebeurt de differentiatie tussen benigne en maligne massa's niet op basis van tumormerkers alleen. Daarom voerden we nog een tweede studie uit waarbij de accuraatheid van ROMA vergeleken werd met de twee vaak gebruikte echografie methodes, de risk of malignancy index (RMI) en subjectieve indruk d.m.v. echografie. De subjectieve indruk had de hoogste AUC (0.968), gevolgd door de RMI (0.931) en ROMA (0.893). Op basis van deze resultaten concludeerden we dat echografie, en in het bijzonder subjectieve indruk d.m.v. echografie, superieur blijft voor de discriminatie tussen benigne en maligne ovariële massa's.

In een exploratieve studie onderzochten we of endorepellin, een angiostatisch peptide, als tumormerker kon gebruikt worden voor de discriminatie tussen benigne en maligne ovariële massa's. Deze internationale studie werd uitgevoerd in België, Tsjechië, Zweden en Italië. Bij postmenopauzale vrouwen, was endorepellin steeds bij de best presterende combinaties, met de hoogste AUC (0.920) voor het trio bestaande uit CA125, HE4 en endorepellin. Endorepellin presteerde ook goed bij stadium I en borderline tumoren. Deze resultaten moeten nog gevalideerd worden en op dit ogenblik loopt een onafhankelijke validatiestudie waarin dit trio gevalideerd wordt.

Het tweede diagnostisch probleem is de **diagnose van positieve lymfeklieren bij het cervixcarcinoom**. Lymfeklieraantasting is een belangrijke prognostische factor voor cervixkanker. Er is een sterke nood aan biomerkers die de aanwezigheid van aangetaste lymfeklieren kunnen voorspellen. Na depletie en SELDI–TOF MS analyses waren we in staat om

lymfklieraantasting te voorspellen met een AUC van 0.95. We zijn er echter niet in geslaagd om de eiwitten achter de differentieel tot expressie gekomen pieken te identificeren.

Tenslotte werd ook de **literatuur kritisch geëvalueerd en becommentarieerd**. Het is opmerkelijk dat, ondanks veel energie gestopt wordt in proteomics, geen van de inspanningen tot resultaat hebben geleid. Op dit ogenblik hebben de nieuw ontdekte merkers de klinische praktijk nog niet veranderd.

Non scientific summary (English)

We are not able to predict the (benign or malignant) nature of an ovarian tumour with certainty before it is removed and examined under the microscope. However, it is of great importance that we try to estimate as good as possible whether a cyst is benign or malignant before we perform this operation, because it will determine who will operate (general gynaecologist or gynaecological oncologist) and how it will be performed (key–hole surgery or open surgery). There is a need for a good predictive test.

Tumour markers are substances that can be measured in blood and that give us information about the nature of a cyst. 'CA125', a currently available tumour marker is not accurate enough and better tumour markers are needed. Recently, a new tumour marker, called 'HE4' was discovered. When used together with CA125 it was able to give more certainty about the nature of the ovarian tumour. The combination of both markers was called 'ROMA' (Risk of Ovarian Malignancy Algorithm). In a first study we tested whether this test was as good as claimed. In total 389 women with an ovarian tumour participated in the study. Disappointingly, the ROMA did in our study not better than CA125.

In 374 patients we also performed an ultrasound and in a subsequent study we investigated whether ultrasound was better than CA125 or ROMA in distinguishing benign and malignant ovarian tumours. We concluded that ultrasound was indeed better. This shows that ultrasound remains the cornerstone in the diagnosis of ovarian cancer. We also tried to find new tumour markers in the hope to improve the diagnosis of ovarian cancer. For the first time, a protein called 'endorepellin' was investigated in a study with 304 patients with an ovarian tumour. Patients were recruited in Belgium, Czech Republic, Sweden and Italy. We discovered that the triple combination (CA125/HE4/endorepellin) had the best performance among postmenopausal patients. These promising results are being validated on a cohort of 400 patients from Germany.

Cervical cancer patients with or without lymph node metastases are treated differently. Lymph node metastases are difficult to diagnose. We investigated whether we could find tumour markers that were able to predict the presence of lymph node metastases. We discovered 3 proteins, but due to technical difficulties we were not able to determine the identity of the proteins.

Finally, we reviewed all published articles on protein research over the last decade to see whether it was able to change clinical practice. Although many proteins have been described, not one of them used as a tumour marker in the diagnosis of gynaecological tumours.

In conclusion, the studies in this project helped us to discover new tumour markers. Endorepellin is a promising marker but needs further validation. Improving the technical abilities in protein research would increase our chances of finding a new tumour marker.

Algemene niet wetenschappelijke samenvatting (Nederlands)

Zekerheid over de aard (goedaardig of kwaadaardig) van een afwijking ter hoogte van de eierstokken kan alleen worden vastgesteld nadat de afwijking is weg genomen en onder de microscoop onderzocht. Nochtans zouden we voor een operatie zoveel mogelijk zekerheid willen hebben, want de aard van de afwijking bepaald ook door wie (algemeen gynaecoloog of gespecialiseerde gynaecoloog) en hoe (open buik operatie of kijkoperatie) een patiënt geopereerd zal worden. Er is dus behoefte aan een goede voorspellende test.

Tumormerkers zijn stoffen die we in bloed kunnen meten en die informatie kunnen geven over de aard van een afwijking. 'CA125', de tumormerker die nu voorhanden is, geeft onvoldoende zekerheid over de diagnose. Er is dus nood aan nieuwe en betere tumormerkers. Recent werd een nieuwe tumormerker gevonden, met de naam 'HE4'. Wanneer deze samen met CA125 bepaald werd kon men met meer zekerheid zeggen of de afwijkingen ter hoogte van de eierstokken goed– of kwaadaardig was. De combinatie van de twee tumormerkers kreeg de naam 'ROMA' (Risk of Ovarian Malignancy Algorithm). In een eerste studie zijn we nagegaan of deze test inderdaad zo goed werkt als beweerd werd. In totaal namen 389 vrouwen met een afwijking ter hoogte van de eierstok(ken) deel aan dit onderzoek. Teleurstellend deed het ROMA het in onze studie niet beter dan CA125.

Bij 374 patiënten was ook een echografie uitgevoerd en in een volgende studie hebben we nagekeken of echografie beter in staat was om een verschil te zien tussen goed– en kwaadaardig in vergelijking met CA125 of ROMA. Uit deze studie blijkt dat echografie inderdaad met de meeste zekerheid een verschil kan zien. Hieruit blijkt dat echografie (nog steeds) de hoeksteen is in de diagnose van ovariumkanker. We zijn zelf ook op zoek gegaan nieuwe tumormerkers, in de hoop de diagnose van ovariumkanker te kunnen verbeteren. We hebben voor de eerste keer het eiwit 'endorepellin' onderzocht. Dit deden we bij 304 patiënten met een afwijking ter hoogte van de eierstokken uit België, Tsjechië, Zweden en Italië. Bij patiënten na de menopauze zagen we dat het trio van merkers (CA125/HE4/endorepellin) met de meeste zekerheid een onderscheid kon maken tussen goed– en kwaadaardig. Om deze veelbelovende resultaten te bevestigen wordt dit trio op dit ogenblik nog verder nagekeken bij 400 patiënten uit Duitsland.

Patiënten met baarmoederhalskanker met uitzaaiingen naar de lymfeklieren worden anders behandeld dan wanneer dat niet het geval is. We zijn we ook bij deze patiënten op zoek gegaan naar tumormerkers die de aanwezigheid van uitzaaiingen naar de lymfklieren konden voorspellen. We hebben 3 eiwitten gevonden die inderdaad een verschil konden maken tussen aan– of afwezigheid van uitzaaiingen naar de lymfklieren maar door technische beperkingen van het toestel waarmee we te werk zijn gegaan zijn we niet in staat geweest om de identiteit van deze eiwitten te achterhalen.

Tenslotte zijn we aan de hand van literatuuronderzoek nagegaan of het eiwitonderzoek dat de laatste 10 jaar uitgevoerd werd een verschil heeft kunnen maken in hoe we nu gynaecologische kankers onderzoeken of behandelen. Dit is niet het geval.

Kortom, deze studies hielpen ons om op zoek te gaan naar nieuwe tumormerkers. Endorepelline is wat dat betreft een veelbelovende tumormerker die verder nagekeken moet worden. Een verbetering in de technieken van het eiwitonderzoek zou ons hier bij kunnen helpen.

Curriculum Vitae

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Professional record and training

08–2009 – present	**Gynaecologist/Gynaecological Oncologist** Maastricht University Medical Center (MUMC+), Maastricht, Netherlands
08–2004 – present	**PhD**, entitled "The evaluation of new biomarkers in gynaecological tumours The emerging role of proteomics."
08–2007 – 07–2009	**Subspecialty fellow in the field of Gynaecological Oncology:** University Hospital Gasthuisberg, Leuven, Belgium Under supervision of Prof. I. Vergote *Official Certificate of Accreditation as European Gynaecologic Oncologist by the ESGO (European Society of Gynaecologic Oncology) on 11–10–2009.*
08–2006 – 07–2007	**Subspecialty fellow in the field of Breast Surgery:** University Hospital Gasthuisberg, Leuven, Belgium Under supervision of Prof. M.R. Christiaens
08–2004 – 07–2006	**Research fellow** University Hospital Gasthuisberg, Leuven, Belgium

08–1999 – 07–2004	**Training in Obstetrics and Gynaecology** (5 year)
5th year	University Hospital Gasthuisberg, Leuven, Belgium
4th year	Watford General Hospital, Watford, U.K
1st – 3rd year	Algemeen Ziekenhuis St.–Jan, Brussels, Belgium
1992 – 1999:	**Medical degree** Katholieke Universiteit Leuven Graduated magna cum laude

Awards:
July 2004: Laureate of the 'Pfizer Educational Prize'

GCP training:
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Memberships

EACR	European Association for Cancer Research
ENYGO	European Network of Young Gynaecologic Oncologists
ESGO	European Society of Gynaecologic Oncology
IGCS	International Gynecologic Cancer Society
ISUOG	International Society of Ultrasound in Obstetrics and Gynecology
NVOG	Dutch Society of Obstetrics and Gynaecology
VVOG	Flemish Society of Obstetrics and Gynaecology
VWOG	Flemish Gynaecological Oncology Group
SERGS	Society of European Robotic Gynaecological Surgery

Peer reviewer

- Acta Obstetricia et Gynecologica Scandinavica
- BMC Cancer
- European Journal of Cancer
- European Journal of Cancer Prevention
- International Journal of Gynaecological Cancer
- Journal of Gynecologic Oncology
- Journal of Proteome Research
- Genes, Chromosomes and Cancer
- Gynecologic and Obstetric Investigation
- Ultrasound in Obstetrics and Gynecology
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- Tumori

Publications

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