Proteomics of endometrial cancer diagnosis, treatment and prognosis

Parul Mittal[1], Manuela Klingler-Hoffmann[1], Georgia Arentz[1], Chao Zhang[1], Gurjeet Kaur[3], Martin K. Oehler[2], Peter Hoffmann[1]*

1. Adelaide Proteomics Centre, School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, South Australia, 5005
2. Department of Gynaecological Oncology, Royal Adelaide Hospital, North Terrace, Adelaide, South Australia, 5000
3. Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia

* To whom all correspondence and requests for reprints should be addressed:

Prof. Peter Hoffmann,

Adelaide Proteomics Centre, University of Adelaide,

Gate 8 Victoria Drive, Adelaide, South Australia, 5005

Phone: +61 (08) 8313 5507; Fax: +61 (08) 0 8313 4362;

Email: peter.hoffmann@adelaide.edu.au

Keywords: Biomarker, Endometrial cancer, Proteomics,

Abbreviations: EC (Endometrial cancer), DDA (Data Dependent Acquisition), DIA (Data Independent Acquisition), FFPE (Formalin Fixed Paraffin Embedded), MALDI-MSI (Matrix Assisted Laser Desorption/Ionisation-Imaging)

Abstract

This review discusses the current status of proteomics technology in endometrial cancer diagnosis, treatment, and prognosis. The first part of this review focuses on recently identified biomarkers for endometrial cancer, their importance in clinical use as well as the proteomic methods used in their discovery. The second part highlights some of the emerging mass spectrometry based proteomic technologies that promise to contribute to a better understanding of endometrial cancer by comparing the abundance of hundreds or thousands of proteins simultaneously.

Introduction

Endometrial cancer (EC) is the most common malignant tumour of the female reproductive tract. According to the American Cancer Society, an estimated 52,630 new EC were diagnosed and 8,590 patients died from the disease in 2014 in the USA. From 2006 to 2010, incidence rates of EC increased by 1.5% per year among women younger than 50 years and by 2.6% per year among women 50 years and older (1). Based on the classification system introduced by Bokhman in 1983, EC is divided into two histological subtypes: endometroid adenocarcinomas (Type I) and non-endometroid (Type II) carcinomas (2). Type I adenocarcinomas account for 90% of all EC. These cancers are usually low grade, diagnosed at an early stage and have a good prognosis. They are associated with oestrogen excess, obesity and atypical hyperplasia. In contrast, type II non-endometroid tumours are high grade tumours with more aggressive biological behaviour compared to type I disease. Clinically, type II cancers are often diagnosed at advanced stage when prognosis is poor. Type II tumours include serous, and clear cell carcinomas as well as carcinosarcomas.

Proteomics, more precisely the comparative quantitation of protein subsets, holds great promise in improving outcome of patients with EC as it provides unique tools for discovery of new biomarkers and therapeutic targets. To date, biomarkers have proven their tremendous clinical value in early diagnosis (3, 4), categorizing different subtypes of malignancies (5, 6) and in monitoring patient's response to therapy (7, 8). Regardless of the numerous proteomic studies that have contributed to the standardization of experimental protocols for digestion (9), separation (10), enrichment (11), identification (12) and quantification (13) of less abundant proteins by highly efficient mass spectrometric techniques, proteomic research is still restricted by both technology and bioinformatics tools. Many protein and peptide peaks have been reported to bear significant diagnostic (14), prognostic (15) or predictive value (16) for EC; however, the candidate biomarkers have not yet been validated for use in clinical patient care (17). Some authors have speculated that this might be due to studies using a single proteomics approach, which is not sufficient to gain an in

depth understanding of the protein function and does not eliminate false-negative and false-positive results (18). Nevertheless, implementing 'omics' integration approaches and the use of the rapidly emerging mass spectrometry based proteomic technologies will aid in the venture to elucidate protein markers and their function, ultimately providing more reliable, sensitive, and specific biomarkers for EC.

In the first part of this review article we will summarize the various mass spectrometry based proteomic approaches that have been used in EC studies. In the second part, we will introduce emerging proteomic technologies which may not only be used as discovery tools but also allow the implementation of mass spectrometry in the diagnosis and prognosis of EC.

1. Clinical considerations of endometrial cancer

The clinical management of EC remains a challenge with patients presenting with a full spectrum of disease ranging from those with excellent prognosis and high curability to aggressive disease with poor outcome (19). EC is staged according to the International Federation of Gynaecology and Obstetrics (FIGO) system (Table 1) (20). The classification of EC into low and high risk disease is dependent on a number of parameters, only two of which are assessable pre-operatively [http://wiki.cancer.org.au/australiawiki/index.php?oldid=86682] after a biopsy has been obtained, namely the histological type and grade. However, Jacques *et al*. have shown a large percentage of cancer will be classified as a higher grade tumour after definitive surgery (21). Therefore, the exact type, grade and stage of EC can only be determined by surgery and subsequent histopathological assessment.

1.1. Diagnosis

EC is frequently diagnosed at an early stage, as it regularly presents with symptoms such as post-menopausal bleeding which usually develops early in the disease process. Nevertheless, the discovery of serum biomarkers for early detection of EC has become a high priority (22). A number of

serum biomarkers has been identified so far, with the most commonly used serum biomarker in gynaecological oncology being Carbohydrate Antigen 125 (CA 125). In EC, an elevated serum CA 125 level have been detected in 11-43% of the cases and is shown to be correlated with advanced stage and with the presence of extrauterine disease (23-25). However, due to lack of specificity and sensitivity, CA 125 has limited significance in the diagnosis of EC. Human Epididymis protein 4 (HE4) has recently emerged as a promising biomarker for EC (26). Brennan *et al*. highlighted the utility of serum HE4 using ELISA for pre-operative risk stratification to identify high-risk patients within low-grade endometrioid EC patients who might benefit from lymphadenectomy (27). For stage I EC, HE4 showed a 17% improvement in sensitivity when compared to CA 125 (28).

Hareyama *et al.* investigated the immunohistochemical CA72-4 expression in EC. The authors reported an elevated level of serum CA72-4 in 22-32% of the cases, which is associated with depth of myometrial invasion, adnexal metastasis, lymphovascular space invasion, and pelvic and para-aortic lymph node metastasis (29). Konno *et al.* demonstrated the level of serum soluble Fas (sFas) is significantly higher in EC patients with advanced cancer when compared to localized cancer ($p < 0.0001$) (30). A study by Sawada *et al*. showed the raised serum level of immunosuppressive acidic protein (IAP) in 55-76% of EC cases and the level increases with the stage of the disease (31). Hakala *et al*. reported the serum level of macrophage colony-stimulating factor 1 (mcsf 1), correlates significantly with tumour grade and poor prognosis in 25-73% of EC cases (32) (33). A recent study by Kang-Wai Mu *et al.* reported differential levels of zinc alpha-2 glycoprotein, alpha 1-acid glycoprotein, and CD59 in the urine of EC patients when compared to urine from healthy controls using Two Dimensional Gel Electrophoresis (2-DIGE) and *O*-Glycan binging lectin (34).

1.2. Treatment

EC is usually treated by surgery consisting of a hysterectomy, bilateral salpingo-oophorectomy and pelvic lymph node dissection and has a very good prognosis when it is confined to the uterus (28). In contrast, EC with metastasis to the lymph nodes has a high mortality rate. Lymph node metastasis is

104 therefore a crucial factor in the prognosis and choice of treatment of EC patients. Clinicians
105 presently face the challenge that conventional surgical-pathological variables (e.g. tumour size,
106 depth of invasion and grade of disease) and radiological imaging are unreliable in determining if a
107 gynaecological cancer has spread. Consequently, although only 5% of patients suffer from
108 metastasis, the majority undergo radical treatment including removal of the lymph nodes. Lymph
109 node dissection, however, is associated with significant complications such as lower extremity
110 lymphoedema (35). Predictive tissue markers for lymph node metastasis are therefore warranted to
111 determine the optimal treatment strategy and to avoid morbidity in gynaecological cancer.

112 **1.3. Prognosis**

113 The prognosis of EC depends on various factors including the histological subtype, grade and stage of
114 the disease (which is determined by depths of tumour invasion, lymph node metastasis and spread
115 of the disease to other organs) (36). The depth of myometrial invasion and histological grade
116 strongly correlate with the prevalence of lymph node metastasis and patient survival (37).

117 Various protein biomarkers have been described in EC that could be of benefit for the prediction of
118 disease outcome. According to Cocco *et al*. serum amyloid A (SAA) may represent a novel biomarker
119 for EC to monitor disease recurrence and response to therapy (38). Lo SS *et al*. shown the increased
120 levels of CA125, CA15.3 and CA19.9 were significantly associated with poor prognostic clinical
121 parameters (39). Lambropoulou *et al*. evaluated the prognostic significance of survivin, c-erbB2, and
122 COX-2 levels in EC and stated that in a 10-year follow-up, patients with tumors expressing more of
123 these three antigens had significantly lower survival rate that those with smaller expression score
124 (40). Yilmaz *et al*. have also shown higher nuclear expression of survivin in type I when compared to
125 type II EC (p=0.040); but no difference for cytoplasmic survivin and matrix metalloproteinase-2
126 expressions between type I and type II EC (41). Zeimet *et al*., identified L1 cell adhesion molecule
127 (L1CAM) as the best variable for predicting recurrence (sensitivity = 0.74; specificity = 0.91) and
128 death (sensitivity = 0.77; specificity = 0.89) (42). Although, the above mentioned biomarkers are able

to identify high risk patients with low grade EC, for clinical application additional biomarkers are required. New prognostic EC biomarkers will help distinguish patients who are at a low-risk of developing metastasis as compared to those who are at a high risk. Ideally, novel biomarkers will also be able to distinguish patients who have already developed metastasis. This would consequently allow patients to selectively undergo radical surgery while avoiding those who will not benefit from it and subsequently decreasing the risk of post-surgical morbidity.

2. Molecular Genetics of endometrial cancer

Aside from the morphologic and clinical features, EC can be further distinguished into type I and type II EC on the basis of genetic alterations (43). Type I and type II ECs are associated with mutations of independent gene sets (44). Type I endometrial adenocarcinomas are characterized by mutations in the PTEN, K-ras and β catenin, as well as DNA mismatch repair genes (35) while type II non-endometrioid EC frequently shows aneuploidy and p53 mutations (35).

PTEN has been reported to be the most frequent genetic alteration of type I EC. PTEN, a tumour suppressor gene has been altered in up to 83% of endometrial adenocarcinoma and 55% in pre-cancerous lesions (45). Microsatellite instability (MSI) has been demonstrated in 20% of type I EC (46). According to Bilbao *et al*. both PTEN mutations and MSI represent early events in endometrial carcinogenesis (47). Other genetic alterations that occur in type I EC include mutations in K-ras (48) and β catenin (49). PTEN, MSIS and K-Ras mutations often coexist with each other, whereas mutations in β-catenin are usually observed alone (50).

The most common genetic alteration in type II EC is in p53, the tumour suppressor gene with an occurrence of 93% mutations in p53 in type II EC (48, 51). Other frequent genetic alteration in type II ECs are inactivation of the tumour suppressor gene p16 and over-expression of the oncogene, HER-2/neu (52).

3. Metastasis and protein biomarker

Metastatic cancer cells proliferate, lose contact with neighbouring cells, migrate through interstitial matrix, invade blood and lymph vessels and grow out again into lymph nodes or distant organs (53). Most cancer cells fail to undergo metastasis due to deficiency in one of the required steps like invasion, detachment, and survival (54). Discovery of EC biomarkers would significantly aid gynaecological oncologists who currently face the challenge that radiological imaging and conventional surgical-pathological variables such as tumour size, depth of invasion and grade of disease are unreliable in determining if an EC has metastasized.

The working model of metastasis implies that primary tumour cells acquire genetic alterations over time, which enables these cells to metastasize and form new solid tumours at distant sites (55). It has been proposed that the gene expression program of metastasis is actually present in the bulk of primary tumour cells (56). Ramaswamy *et al*. shown some primary tumours are preconfigured to metastasize, and their susceptibility is detectable at the time of diagnosis. They defined a 17-gene signature pattern associated with metastasis, in which eight genes are up-regulated, while nine genes are down-regulated. They also concluded that metastasis is not dependent on only a single gene but on the complete 17 gene sequence.

Yi *et al*., shown the expression of cyclooxygenase (COX-2) plays an important role in metastasis of EC (57). They identified the COX-2 inhibitor NS-398 inhibits proliferation, viability and invasion of the EC cell line RL95–2 (57). Maxwell *et al*. confirmed annexin A2 (ANXA2) and peroxiredoxin (PRDX1) were both being overexpressed in stage 1 EC when compared to normal endometrium (58).

4. Mass Spectrometry based proteomic approaches in endometrial cancer

The use of proteomic technologies are now enabling the identification and relative quantification of multiple proteins simultaneously from a single experiment to identify disease related and specific biomarkers. Proteomics in general deals with the large-scale determination of gene and cellular

function directly at the protein level (59). The proteome has also been defined as the protein complement expressed by a genome (60) but in reality, due to post-translational modifications and alternative splicing, it is estimated that about 22,000 protein coding genes (61) code for more than 500,000 proteins (62) in the human proteome.

In serum it is estimated that cancer derived proteins are 10 million times less abundant than common high abundant proteins secreted by normal cells. Therefore, it is challenging to correctly identify and quantify tumor derived proteins from the whole serum proteome (63). Alternatively, it is obvious that cancer derived proteins will be present in higher concentration in native tissue, organs and their proximal fluids from where the tumor has originated as compared to the distant sites in which the tumor derived proteins may be secreted or leaked. Therefore, targeting those regional sites will dramatically increase the possibility of isolating and identifying tumor specific biomarkers (64).

Proteomic analysis principally relies on mass spectrometry (MS) for protein identification. The uses of MS based techniques have made it possible to build comprehensive profiles of near complete proteomes, comparing the expressions of individual proteins that may serve as biomarkers. Generally, MS based proteomic techniques can be classified as 'gel based' and 'gel free' (Figure 1). In gel based approaches such as One Dimensional Polyacrylamide Gel Electrophoresis (1D-PAGE), Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) and Two-Dimensional Differential Gel Electrophoresis (2D-DIGE), proteins are separated on polyacrylamide gels via electrophoresis in one or two dimensions, creating unique proteomic patterns based on the mass and/or charge/pH of the proteins.

Gel free MS approaches can be either label based or label free. Label based proteomic techniques are common for mass spectrometry approaches where proteins are tagged either with isotopes such as isotope coded affinity tags (ICAT), isobaric tags for relative and absolute quantification (iTRAQ) or labelled chemically such as stable isotope labelling by amino acids in cell culture (SiLAC). Labelling

facilitates the quantification of proteins during MS analysis and is especially useful when acquiring data in the standard form of data dependent acquisition (DDA), as labelling allows for the mixing and analysis of multiple samples at one. This alleviates the stochastic nature of DDA which can otherwise lead to sampling biases and poor reproducibility. Label free techniques in the form of data independent acquisition (DIA) help resolve the issue of sampling biases and eliminates any problems encountered due to artifacts from labelling which can interfere with the identity of the protein, leading to false conclusions (65, 66). DIA is addressed in more depth later in this article.

MS based proteomics techniques have developed substantially over the past several years. Previously, protein analysis was reliant on 1D and 2D gel electrophoresis followed by sequencing; finally resulting in the identification of tens to hundreds of proteins. This limitation has been overcome with novel mass spectrometric approaches, which can identify many more proteins at once. Identification and quantification of proteins/peptides by MS can be done either by 'bottom-up' or 'top-down' approaches. The term 'bottom-up' refers to the reconstruction of the protein sequence after proteolytic digestion, and thereby, identification and quantification of the peptide fragments using mass spectrometry and appropriate databases (67). The second approach 'top-down', refers to the identification of the whole proteins directly without enzymatic digestion (68).

To date, the most widely used proteomic technologies applied to the identification of the EC biomarkers includes liquid chromatography coupled online/offline with MS (LC-MS). This approach is generally used with 'bottom-up' methods. LC-MS has been used to identify and quantify differential change in protein abundance in healthy versus disease state. Identification of proteins by LC-MS/MS can be broken down into a few essential components. First, complex protein samples are digested with an enzyme (protease) into peptides, which are separated by a high resolution chromatographic technique coupled directly to a MS. Following chromatographic separation samples are sprayed into the MS whilst being ionised, a process called Electrospray ionisation (ESI). Here the intact peptide mass is measured, followed by collision with an inert gas to induce fragmentation, upon which the

mass of the fragment ions are measured. The acquired data is then processed to gain peptide sequences and protein identifications, and examined using mathematical algorithms, functions to detect and identify differences within the sample and/or between the samples. In traditional label free approaches, peptide separation is compared across chromatographic profiles, and quantitation is determined either by spectral counting or by peak intensity during MS (69).

A recent study by Alconada *et al*. has identified and validated annexin (ANXA2) as a reliable biomarker of recurrent disease in EC by 2D DIGE followed by traditional LC-MS/MS. They demonstrated *in vitro* and with an *in vivo* mouse model that the increased expression of ANXA2 is associated with an improved ability of the cells to metastasise (70). Monge *et al*. analysed the role of the transcription factor ERM/ ETV5 in myometrial invasion by 2D-DIGE to evaluate the differential expression of proteins in EC cell lines overexpressing ERM/ETV5 (71). The authors have demonstrated that ERM/ETV5 acts by involving matrix metalloproteinase-2 to provide the migratory and invasive capabilities associated with the switch to myometrial infiltration in Hec-1A EC cell line (71). They further characterized a role for ETV5 in a modulated response to oxidative stress associated with the promotion of invasion in EC (71). Ihata *et al*. analysed the amino acid concentrations in plasma samples by LC-MS and reported that the amino acid profile index (API) is a potential new modality that could eventually play a significant role in the preoperative evaluation of EC and its screening (72).

The matrix assisted laser desorption/ionisation (MALDI) technique has also been widely used for the discovery of gynaecological cancer biomarkers but in a more limited context for EC. Qiu *et al*. have reported that MALDI-Time of flight (TOF) MS is a high-throughput, sensitive, highly predictive and rapid method for the early detection, diagnosis and prognosis of EC and will be widely used in future clinical screening work (73). In this technique, molecules including proteins, peptides, lipid and metabolites, are first co- crystallized with large excess of suitable matrix (usually a weak organic acid) and then spotted on to a MALDI target plate. MALDI TOF MS is then performed, a process in

which the laser transfers the energy to the aromatic matrix molecules and the matrix transfer a proton to the analyte, resulting in soft energy transfer without fragmentation. According to the mass to charge (*m/z)* ratios, the ionised analytes are then separated in the time-of flight mass analyser and the detector transforms the incoming ions into an electric current which is proportional to their abundance (74). In the case of protein analysis, the generated protein/peptide mass list can then be compared to protein databases that allow for the matching of peptide masses and identification of proteins. Casado-Vela *et al*. used an integrated approach combining three different but complementary strategies (in-solution digest followed by reverse phase HPLC-MS/MS, protein separation by SDS-PAGE followed by HPLC-MS/MS analysis, 2D-PAGE followed by MALDI-TOF/TOF analysis) to describe comprehensive proteomic analysis of EC fluid aspirate which led to the successful identification of 803 different proteins in the International Protein Index (IPI) human database (v3.48) (75). Therefore, EC biomarkers can be used for screening, estimating risk of disease, distinguishing benign from malignant, determining prognosis, and monitoring status of the disease, either to detect recurrence or response to therapy. Importantly, some biomarkers are only used in a specific setting, whereas others can serve more than one purpose (76).

5. Emerging techniques for the proteomic analysis of endometrial cancer

Traditional proteomic approaches as described above, continue to be the most widely applied technologies for protein identification; nonetheless, a major drawback of these proteomics techniques is that not every peptide from a complex biological sample can be detected. As a result, the proteomes will be under sampled. New techniques are emerging that offer innovative solutions to the analysis of low abundant proteins from complex biological samples (77). Those proteomics technologies have the potential for direct clinical applications including the identification of novel biomarkers for the monitoring of therapy response, and disease progression, delivering on the great promise of personalized cancer medicine (78).

5.1. Data Independent Acquisition (DIA)

Protein identification by LC-MS is a straightforward technique, as the detection of only two unique peptides from a protein is considered sufficient. However, relative quantification by LC-MS is a more complex process and the failure to identify or detect a peptide does not necessarily confirm absence, as the peptides may simply be below the threshold of detection (79). Traditional LC-MS or shotgun approaches are based on Data dependent acquisition (DDA), where peptides are selected for isolation and fragmentation based on their relative abundance within the sample. This makes the technique stochastic and can result in under sampling of the injected analyte (80). Abundance measurements can be inaccurate as the quantification of a peptide is highly influenced by co-eluting peptides and their signal to noise ratios, meaning peptides of a higher abundance are quantified more accurately and more often than peptides of low abundance. We do however; know that many cancer-relevant proteins including signalling and regulatory proteins are typically expressed in low concentrations. As a result, the DDA approach tends to miss out on acquiring the most-valuable information (81). Data independent acquisition (DIA) aims to solve the problem of DDA by measuring all precursor ions within a defined mass range of ~25 Da, fragmenting and measuring all of the ions within that mass range (82). Typically a 25 Da mass window is stepped across the entire mass range covered in traditional LC-MS/MS (~150-2200 Da) in cycle times compatible with the coupled chromatographic separation (82). Vowinckel *et al*. shown the number of precisely quantifiable peptides can be increased up to 5 fold when using DIA compared to DDA, with a coefficient of variation (CV) less than 5% (83).

5.2. MALDI Imaging (MALDI-MSI)

Another innovative technology that can be used for both biomarker discovery and validation is MALDI imaging (MALDI-MSI), which facilitates the direct analysis of protein distribution and abundance in thin tissue sections (84). This technique has the potential to detect and characterize tumour margins and their environment in a spatial context (56). MALDI-MSI is a valuable method for the identification of biomarkers and can complement histology, immunohistochemistry (85) and

molecular pathology in various fields of histopathological diagnostics, especially with regard to identification and grading of tumors (86). Whereas, MALDI-MSI is optimally suited to detect a large number of molecular features in a given tissue sample, the tissue microarray (TMA) method enables expansion of this analysis to hundreds of tissue samples in a single experiment (87, 88). In MALDI-MSI, proteins are directly ionised from the surface of the tissue sample and a characteristic histological pattern is derived for hundreds or thousands of individual peptides simultaneously, which may be further investigated and identified in the same tissue section by *in-situ* MS/MS (89). During MS/MS, a desired peptide is isolated based on its *m/z* ratio and fragmented (i.e. breaking peptide bonds within the peptides), producing a series of fragment ions that are detected as a MS/MS spectrum. The fragmentation pattern is compared to the theoretical fragmentation pattern for every peptide in the proteome to find the closest match. In this way the sequence of the peptide ion is inferred from its fragmentation pattern (90). Realistically in the case of MALDI-MSI, this form of tandem mass spectrometry is limited to highly abundant ionisable peptides whereas the identifications of lower abundance peptides can be made by matching back to fragmentation spectra acquired using LC-MS/MS.

MALDI-MSI has been successfully used to classify HER2 receptor expression in breast cancer (91). Meding *et al*. have shown the power of MALDI-MSI in classification of metastasis for different tumour entities (92). In addition, their results indicated that even closely related entities such as primary tumour of the colon and its liver metastasis could be classified efficiently (92). Quaas *et al*. detected several molecular signals associated with phenotypic features of oesophageal cancer cells, highlighting the potential of MALDI-MSI to identify new molecular markers with relevance to oesophageal cancer (87). Another strength of MALDI-MSI is that it can be used in the discovery phase for the identification of tumor derived proteins and then the verification of the presence of those particular proteins can be carried out in body fluid (blood, urine or saliva), aiming to develop non-invasive diagnostic tests. Addona *et al*. developed a pipeline integrating proteomic technologies from the discovery to the verification stages of plasma biomarker identification and applied it to

identify early biomarkers of cardiac injury (93). Thus, combining the histopathology reports with MALDI-MSI data will help surgeons to make right medical decisions.

In the similar approach, we performed MALDI imaging on EC formalin fixed paraffin embedded (FFPE) tissue samples collected from the Royal Adelaide Hospital (RAH), South Australia. Ethics approval was granted by RAH Human ethics committee.

In this study 6µm FFPE tissue sections were sliced and mounted onto indium tin oxide (ITO) coated conductive glass slides (Bruker Daltonics, Bremen, Germany) and samples were prepared as described by Gustafsson *et al*. (12). Briefly upon Citric acid antigen retrieval (CAAR), *in-situ* tryptic digestion was performed using an Image prep station (Bruker Daltonics, Bremen, Germany) at 37°C for 2 hours. α-Cyano-4-hydroxycinnamic acid (CHCA) matrix solution was prepared at 7mg/ml in 50% ACN/ 0.2% TFA and was overlayed onto the tissue section using an Image Prep station. Following matrix deposition, analyses was performed on an UltrafleXtreme MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany) in positive reflectron mode with FlexControl V3.0.1 and FlexImaging V4.0.1 (Bruker Daltonics) software package. The data was acquired at 100µm lateral resolution with a laser frequency of 2000 Hz over *m/z* 800–4500. Following MS acquisition, an overlapped MALDI spectral profile of tumour and healthy regions was generated by FlexAnalysis software (V4.0.1, Bruker Daltonics, Bremen, Germany) and as expected, a number of *m/z* values have been identified which are differentially expressed between healthy and tumour regions (Figure 2).

In order to determine data dependent visualization of tissue morphological regions, the raw data was loaded into SCiLS lab software (V2014b, Bruker Daltonics, Bremen, Germany). Data was processed by baseline removal and total ion count (TIC) normalization. Healthy and tumour regions were then grouped based on annotations done by a pathologist and discriminatory *m/z* values were calculated by ROC (Receiver operating characteristic) curve. ROC curve compares sensitivity verses specificity across a range of *m/z* values by plotting a curve called as Area under the ROC curve (AUC). The AUC assumes values between 0 and 1 and expresses the discrimination power of the *m/z* signal.

A perfect discrimination would yield an AUC value equal to 1 (abundant in group 1) or 0 (abundant in group 2) (94). The AUC closer to 0.5 indicates that there is no discrimination between two sample groups. An example of a discriminatory *m/z* value (*m/z* 1111.553Da ± 0.250Da) is shown in Figure 3. Here the spatial intensity of *m/ z* 1111.553Da ± 0.250Da is clearly higher in the healthy tissue regions as compared to the tumour tissue region, shown also in the ROC curve (Figure 4). The results from this study will provide crucial new tools to assist in the diagnosis and prognosis of EC, with the ultimate aim to prevent overtreatment of patients whose primary tumours do not have metastatic potential.

5.3. Targeted Approach

Sensitive detection of low abundant proteins in complex samples has been typically achieved by enzyme-linked immunosorbent assay (ELISA) which require antibodies specific to the protein(s) of interest. However, this poses a problem as development of antibodies is associated with high cost, long development lead times, and high failure rates (95). To overcome this problem, for the past few decades intensive research has been carried out aiming to develop more targeted strategies that have been designed specifically to analyse preselected peptides/proteins of interest within complex samples.

Unlike untargeted approach, which aims to detect all proteins within a complex sample in an unbiased manner, Multiple reaction monitoring (MRM) is a targeted approach, in which the protein(s) of interest are preselected for quantification and analysed using highly sensitive triple quadrupole MS (96). MRM-MS provides high sensitivity and accuracy needed in the discovery phase and offers high reproducibility necessary for clinical validation (97). Due to the high specificity, sensitivity and throughput of MRM, it can be used for the validation of a single protein of interest or a subset of proteins (98). To ensure good quantification by MRM, three of the most intense ion transitions (precursor ion fragmentation to product ions) per protein are monitored. DeSouza *et al*. shown the MRM of iTRAQ labelled peptides enables absolute quantification of pyruvate kinase in

cancerous and normal endometrial tissues (99). Elizabeth *et al*. pioneered a new technique that couples the ability to perform MRM on a triple quadrupole mass spectrometer with a MALDI source (100). The authors have shown that this approach has the feasibility for the precise and accurate quantitation of tissue protein concentrations over 2 orders of magnitude, while maintaining the spatial localization information for the proteins (101). The greatest advantage of the MALDI MRM-based imaging technique is improved sensitivity and selectivity of the analysis, enabling measurement of the accurate protein concentration with the addition of stable isotope labelled internal standards. This technique combines the spatial information gained by traditional MALDI MSI with the accurate quantification achievable by MRM and may develop to be an optimal strategy for the analysis of abundant peptide biomarkers.

6. Conclusion

Above mentioned studies have discovered a number of candidate EC biomarkers, for example CA-125, CA 19-9, CA 15-3, HE4 for the diagnosis and L1CAM, COX-2, Survivin, c-erb B2 for the prognosis of EC. Unfortunately due to their lack in sensitivity and specificity none of those protein biomarkers are currently used in clinical practice. Recently, the major focus of biomarker research has shifted from single biomarker discovery to the multiparametric analysis of proteins, as the proteomic pattern analysis can obtain exceptional results. As part of this review we have introduced two emerging techniques with exceptional sensitivity and dynamic range. These techniques are refining our capability to identify and quantify relative changes in protein expression and will ultimately result in the discovery of new EC biomarkers panels.

Moreover, the integration of the data obtained from various 'omics' approaches such as genomics, transcriptomics and proteomics will help to reduce the false positives and false negatives obtained from single 'omic' approaches and provide us with a number of potential protein targets. Taken together, this is an exciting time for EC biomarker research and we are hopeful that novel EC biomarker panels will perform well and will be used in the clinic in the future.

7. Conflict of interest statement

The authors have declared no conflict of interest.

8. References

1. American Cancer Society, Cancer Facts and Figures 2014. Atlanta: American Cancer Society; 2014.

2. Bokhman JV. Two pathogenetic types of endometrial carcinoma. Gynecologic oncology. 1983;15(1):10-7. Epub 1983/02/01.

3. Toiyama Y, Tanaka K, Kitajima T, Shimura T, Kawamura M, Kawamoto A, et al. Elevated Serum Angiopoietin-like Protein 2 Correlates with the Metastatic Properties of Colorectal Cancer: A Serum Biomarker for Early Diagnosis and Recurrence. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014;20(23):6175-86. Epub 2014/10/09.

4. Bie Y, Zhang Z. Diagnostic value of serum HE4 in endometrial cancer: a meta-analysis. World journal of surgical oncology. 2014;12:169. Epub 2014/06/03.

5. Qendro V, Lundgren DH, Rezaul K, Mahony F, Ferrell N, Bi A, et al. Large-scale proteomic characterization of melanoma expressed proteins reveals nestin and vimentin as biomarkers that can potentially distinguish melanoma subtypes. J Proteome Res. 2014;13(11):5031-40. Epub 2014/10/17.

6. Le Faouder J, Laouirem S, Alexandrov T, Ben-Harzallah S, Leger T, Albuquerque M, et al. Tumoral heterogeneity of hepatic cholangiocarcinomas revealed by MALDI imaging mass spectrometry. Proteomics. 2014;14(7-8):965-72. Epub 2014/03/29.

7. Kralj E, Žakelj S, Trontelj J, Pajič T, Preložnik Zupan I, Černelč P, et al. Monitoring of imatinib targeted delivery in human leukocytes. European Journal of Pharmaceutical Sciences. 2013;50(1):123-9.

8. Ubhi BK, Cheng KK, Dong J, Janowitz T, Jodrell D, Tal-Singer R, et al. Targeted metabolomics identifies perturbations in amino acid metabolism that sub-classify patients with COPD. Molecular bioSystems. 2012;8(12):3125-33. Epub 2012/10/12.

9. Meding S, Martin K, Gustafsson OJ, Eddes JS, Hack S, Oehler MK, et al. Tryptic peptide reference data sets for MALDI imaging mass spectrometry on formalin-fixed ovarian cancer tissues. Journal of proteome research. 2013;12(1):308-15. Epub 2012/12/12.

10. Weiland F, Zammit CM, Reith F, Hoffmann P. High resolution two-dimensional electrophoresis of native proteins. Electrophoresis. 2014;35(12-13):1893-902. Epub 2014/03/20.

11. Danihlik J, Sebela M, Petrivalsky M, Lenobel R. A sensitive quantification of the peptide apidaecin 1 isoforms in single bee tissues using a weak cation exchange pre-separation and nanocapillary liquid chromatography coupled with mass spectrometry. Journal of chromatography A. 2014;1374:134-44. Epub 2014/12/02.

12. Gustafsson OJ, Eddes JS, Meding S, McColl SR, Oehler MK, Hoffmann P. Matrix-assisted laser desorption/ionization imaging protocol for in situ characterization of tryptic peptide identity and distribution in formalin-fixed tissue. Rapid communications in mass spectrometry : RCM. 2013;27(6):655-70. Epub 2013/02/19.

13. Li J, Zhou L, Wang H, Yan H, Li N, Zhai R, et al. A new sample preparation method for the absolute quantitation of a target proteome using O labeling combined with multiple reaction monitoring mass spectrometry. The Analyst. 2015. Epub 2015/01/09.

14. Ueda Y, Enomoto T, Kimura T, Miyatake T, Yoshino K, Fujita M, et al. Serum biomarkers for early detection of gynecologic cancers. Cancers. 2010;2(2):1312-27. Epub 2010/01/01.

15. Engelsen IB, Akslen LA, Salvesen HB. Biologic markers in endometrial cancer treatment. APMIS. 2009;117(10):693-707.

16. Dong P, Kaneuchi M, Konno Y, Watari H, Sudo S, Sakuragi N. Emerging Therapeutic Biomarkers in Endometrial Cancer. BioMed Research International. 2013;2013:11.

17. Galvao ER, Martins LM, Ibiapina JO, Andrade HM, Monte SJ. Breast cancer proteomics: a review for clinicians. Journal of cancer research and clinical oncology. 2011;137(6):915-25. Epub 2011/04/06.

18. Ge H, Walhout AJ, Vidal M. Integrating 'omic' information: a bridge between genomics and systems biology. Trends in genetics : TIG. 2003;19(10):551-60. Epub 2003/10/11.

19. Zhang Y, Wang J. Controversies in the management of endometrial carcinoma. Obstetrics and gynecology international. 2010;2010:862908. Epub 2010/07/09.

20. Haltia UM, Butzow R, Leminen A, Loukovaara M. FIGO 1988 versus 2009 staging for endometrial carcinoma: a comparative study on prediction of survival and stage distribution according to histologic subtype. Journal of gynecologic oncology. 2014;25(1):30-5. Epub 2014/01/25.

21. Jacques SM, Qureshi F, Munkarah A, Lawrence WD. Interinstitutional surgical pathology review in gynecologic oncology: I. Cancer in endometrial curettings and biopsies. International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists. 1998;17(1):36-41. Epub 1998/02/25.

22. Ueda Y, Enomoto T, Kimura T, Miyatake T, Yoshino K, Fujita M, et al. Serum Biomarkers for Early Detection of Gynecologic Cancers. Cancers. 2010;2(2):1312-27.

23. Powell JL, Hill KA, Shiro BC, Diehl SJ, Gajewski WH. Preoperative serum CA-125 levels in treating endometrial cancer. The Journal of reproductive medicine. 2005;50(8):585-90. Epub 2005/10/14.

24. Jhang H, Chuang L, Visintainer P, Ramaswamy G. CA 125 levels in the preoperative assessment of advanced-stage uterine cancer. American journal of obstetrics and gynecology. 2003;188(5):1195-7. Epub 2003/05/16.

25. Scambia G, Gadducci A, Panici PB, Foti E, Ferdeghini M, Ferrandina G, et al. Combined use of CA 125 and CA 15-3 in patients with endometrial carcinoma. Gynecologic oncology. 1994;54(3):292-7. Epub 1994/09/01.

26. Brennan DJ, Hackethal A, Metcalf AM, Coward J, Ferguson K, Oehler MK, et al. Serum HE4 as a prognostic marker in endometrial cancer — A population based study. Gynecologic oncology. 2014;132(1):159-65.

27. Brennan DJ, Hackethal A, Metcalf AM, Coward J, Ferguson K, Oehler MK, et al. Serum HE4 as a prognostic marker in endometrial cancer--a population based study. Gynecologic oncology. 2014;132(1):159-65. Epub 2013/11/12.

28. Moore RG, Brown AK, Miller MC, Badgwell D, Lu Z, Allard WJ, et al. Utility of a novel serum tumor biomarker HE4 in patients with endometrioid adenocarcinoma of the uterus. Gynecologic oncology. 2008;110(2):196-201. Epub 2008/05/23.

29. Hareyama H, Sakuragi N, Makinoda S, Fujimoto S. Serum and tissue measurements of CA72-4 in patients with endometrial carcinoma. Journal of clinical pathology. 1996;49(12):967-70. Epub 1996/12/01.

30. Konno R, Takano T, Sato S, Yajima A. Serum soluble fas level as a prognostic factor in patients with gynecological malignancies. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000;6(9):3576-80. Epub 2000/09/22.

31. Sawada M, Okudaira Y, Matsui Y, Shimizu Y. Immunosuppressive acidic protein in patients with gynecologic cancer. Cancer. 1984;54(4):652-6. Epub 1984/08/15.

32. Hakala A, Kacinski BM, Stanley ER, Kohorn EI, Puistola U, Risteli J, et al. Macrophage colony-stimulating factor 1, a clinically useful tumor marker in endometrial adenocarcinoma: comparison with CA 125 and the aminoterminal propeptide of type III procollagen. American journal of obstetrics and gynecology. 1995;173(1):112-9. Epub 1995/07/01.

33. Suzuki M, Ohwada M, Sato I, Nagatomo M. Serum Level of Macrophage Colony–Stimulating Factor as a Marker for Gynecologic Malignancies. Oncology. 1995;52(2):128-33.

34. Mu AK, Lim BK, Hashim OH, Shuib AS. Detection of differential levels of proteins in the urine of patients with endometrial cancer: analysis using two-dimensional gel electrophoresis and o-glycan binding lectin. International journal of molecular sciences. 2012;13(8):9489-501. Epub 2012/09/06.

35. Frederick PJ, Straughn JM, Jr. The role of comprehensive surgical staging in patients with endometrial cancer. Cancer control : journal of the Moffitt Cancer Center. 2009;16(1):23-9. Epub 2008/12/17.

504 36. Larson DM, Connor GP, Broste SK, Krawisz BR, Johnson KK. Prognostic significance of gross
505 myometrial invasion with endometrial cancer. Obstetrics and gynecology. 1996;88(3):394-8. Epub
506 1996/09/01.
507 37. Boronow RC, Morrow CP, Creasman WT, Disaia PJ, Silverberg SG, Miller A, et al. Surgical
508 staging in endometrial cancer: clinical-pathologic findings of a prospective study. Obstetrics and
509 gynecology. 1984;63(6):825-32. Epub 1984/06/01.
510 38. Cocco E, Bellone S, El-Sahwi K, Cargnelutti M, Buza N, Tavassoli FA, et al. Serum amyloid A: a
511 novel biomarker for endometrial cancer. Cancer. 2010;116(4):843-51. Epub 2009/12/31.
512 39. Lo SS, Cheng DK, Ng TY, Wong LC, Ngan HY. Prognostic significance of tumour markers in
513 endometrial cancer. Tumour biology : the journal of the International Society for
514 Oncodevelopmental Biology and Medicine. 1997;18(4):241-9. Epub 1997/01/01.
515 40. Lambropoulou M, Papadopoulos N, Tripsianis G, Alexiadis G, Pagonopoulou O, Kiziridou A, et
516 al. Co-expression of survivin, c-erbB2, and cyclooxygenase-2 (COX-2): prognostic value and survival
517 of endometrial cancer patients. Journal of cancer research and clinical oncology. 2010;136(3):427-
518 35. Epub 2009/09/17.
519 41. Yilmaz E, Koyuncuoglu M, Gorken IB, Okyay E, Saatli B, Ulukus EC, et al. Expression of matrix
520 metalloproteinase-2 and survivin in endometrioid and nonendometrioid endometrial cancers and
521 clinicopathologic significance. Journal of gynecologic oncology. 2011;22(2):89-96. Epub 2011/08/24.
522 42. Zeimet AG, Reimer D, Huszar M, Winterhoff B, Puistola U, Azim SA, et al. L1CAM in early-
523 stage type I endometrial cancer: results of a large multicenter evaluation. J Natl Cancer Inst.
524 2013;105(15):1142-50. Epub 2013/06/20.
525 43. Bansal N, Yendluri V, Wenham RM. The molecular biology of endometrial cancers and the
526 implications for pathogenesis, classification, and targeted therapies. Cancer control : journal of the
527 Moffitt Cancer Center. 2009;16(1):8-13. Epub 2008/12/17.

528 44. Hecht JL, Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis.
529 Journal of clinical oncology : official journal of the American Society of Clinical Oncology.
530 2006;24(29):4783-91. Epub 2006/10/10.
531 45. Arango HA, Hoffman MS, Roberts WS, DeCesare SL, Fiorica JV, Drake J. Accuracy of lymph
532 node palpation to determine need for lymphadenectomy in gynecologic malignancies. Obstetrics
533 and gynecology. 2000;95(4):553-6. Epub 2000/03/22.
534 46. Bilbao-Sieyro C, Ramirez R, Rodriguez-Gonzalez G, Falcon O, Leon L, Torres S, et al.
535 Microsatellite instability and ploidy status define three categories with distinctive prognostic impact
536 in endometrioid endometrial cancer. Oncotarget. 2014;5(15):6206-17. Epub 2014/07/16.
537 47. Bilbao C, Rodriguez G, Ramirez R, Falcon O, Leon L, Chirino R, et al. The relationship between
538 microsatellite instability and PTEN gene mutations in endometrial cancer. International journal of
539 cancer Journal international du cancer. 2006;119(3):563-70. Epub 2006/03/01.
540 48. Lax SF, Kendall B, Tashiro H, Slebos RJ, Hedrick L. The frequency of p53, K-ras mutations, and
541 microsatellite instability differs in uterine endometrioid and serous carcinoma: evidence of distinct
542 molecular genetic pathways. Cancer. 2000;88(4):814-24. Epub 2000/02/19.
543 49. Moreno-Bueno1 G, DH, CSn, DS, 1, RlC, et al. Abnormalities of the APC/b-catenin pathway
544 in endometrial cancer. Oncogene (2002) 2002;21, :7981 – 90.
545 50. Saegusa M, Hashimura M, Yoshida T, Okayasu I. β-Catenin mutations and aberrant nuclear
546 expression during endometrial tumorigenesis. British Journal of Cancer. 2001;84(2):209-17.
547 51. Lluis Catasus AG, Miriam Cuatrecasas and Jaime Prat. Concomitant PI3K–AKT and p53
548 alterations in endometrial carcinomas are associated with poor prognosis. Modern Pathology (2009).
549 2009;22:522–9.
550 52. Doll A, Abal M, Rigau M, Monge M, Gonzalez M, Demajo S, et al. Novel molecular profiles of
551 endometrial cancer-new light through old windows. The Journal of steroid biochemistry and
552 molecular biology. 2008;108(3-5):221-9. Epub 2007/12/07.

53. Takes RP, Baatenburg de Jong RJ, Wijffels K, Schuuring E, Litvinov SV, Hermans J, et al. Expression of genetic markers in lymph node metastases compared with their primary tumours in head and neck cancer. The Journal of Pathology. 2001;194(3):298-302.

54. René Bernards, Weinberg RA. A progression puzzle. 2002(22 August 2002). Epub volume 418.

55. Fidler IJ, Kripke ML. Metastasis results from preexisting variant cells within a malignant tumor. Science (New York, NY). 1977;197(4306):893-5. Epub 1977/08/26.

56. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. Nature genetics. 2003;33(1):49-54. Epub 2002/12/07.

57. Yi Z, Jingting C, Yu Z. Proteomics reveals protein profile changes in cyclooxygenase-2 inhibitor-treated endometrial cancer cells. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society. 2009;19(3):326-33. Epub 2009/05/02.

58. Maxwell GL, Hood BL, Day R, Chandran U, Kirchner D, Kolli VS, et al. Proteomic analysis of stage I endometrial cancer tissue: identification of proteins associated with oxidative processes and inflammation. Gynecologic oncology. 2011;121(3):586-94. Epub 2011/04/05.

59. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422(6928):198-207.

60. Hochstrasser DF. Proteome in perspective. Clinical chemistry and laboratory medicine : CCLM / FESCC. 1998;36(11):825-36. Epub 1999/01/07.

61. Pertea M, Salzberg SL. Between a chicken and a grape: estimating the number of human genes. Genome biology. 2010;11(5):206. Epub 2010/05/06.

62. Ewing B, Green P. Analysis of expressed sequence tags indicates 35,000 human genes. Nat Genet. 2000;25(2):232-4. Epub 2000/06/03.

63. Ong S-E. Whole proteomes as internal standards in quantitative proteomics. Genome Medicine. 2010;2(7):49-.

64. Kulasingam V, Diamandis EP. Tissue culture-based breast cancer biomarker discovery platform. International journal of cancer Journal international du cancer. 2008;123(9):2007-12. Epub 2008/08/21.

65. Grossmann J, Roschitzki B, Panse C, Fortes C, Barkow-Oesterreicher S, Rutishauser D, et al. Implementation and evaluation of relative and absolute quantification in shotgun proteomics with label-free methods. J Proteomics. 2010;73(9):1740-6. Epub 2010/06/26.

66. Arentz G, Weiland F, Oehler MK, Hoffmann P. State of the art of 2D DIGE. PROTEOMICS – Clinical Applications. 2015;9(3-4):277-88.

67. Tiss A, Timms J, Menon U, Gammerman A, Cramer R. Proteomics approaches towards early detection and diagnosis of ovarian cancer. Journal for Immunotherapy of Cancer. 2014;2(Suppl 1):O5-O.

68. Tran JC, Zamdborg L, Ahlf DR, Lee JE, Catherman AD, Durbin KR, et al. Mapping intact protein isoforms in discovery mode using top-down proteomics. Nature. 2011;480(7376):254-8.

69. Liu H, Sadygov RG, Yates JR. A Model for Random Sampling and Estimation of Relative Protein Abundance in Shotgun Proteomics. Analytical Chemistry. 2004;76(14):4193-201.

70. Alonso-Alconada L, Santacana M, Garcia-Sanz P, Muinelo-Romay L, Colas E, Mirantes C, et al. Annexin-A2 as predictor biomarker of recurrent disease in endometrial cancer. International Journal of Cancer. 2014:n/a-n/a.

71. Monge M, Colas E, Doll A, Gil-Moreno A, Castellvi J, Diaz B, et al. Proteomic approach to ETV5 during endometrial carcinoma invasion reveals a link to oxidative stress. Carcinogenesis. 2009;30(8):1288-97. Epub 2009/05/16.

72. Ihata Y, Miyagi E, Numazaki R, Muramatsu T, Imaizumi A, Yamamoto H, et al. Amino acid profile index for early detection of endometrial cancer: verification as a novel diagnostic marker. International journal of clinical oncology. 2014;19(2):364-72. Epub 2013/05/24.

73. Qiu F, Gao YH, Jiang CG, Tian YP, Zhang XJ. Serum proteomic profile analysis for endometrial carcinoma detection with MALDI-TOF MS. Archives of Medical Science : AMS. 2010;6(2):245-52.

604 74. Hillenkamp F, Karas M, Beavis RC, Chait BT. Matrix-assisted laser desorption/ionization mass
605 spectrometry of biopolymers. Anal Chem. 1991;63(24):1193A-203A. Epub 1991/12/15.
606 75. Casado-Vela J, Rodriguez-Suarez E, Iloro I, Ametzazurra A, Alkorta N, Garcia-Velasco JA, et al.
607 Comprehensive proteomic analysis of human endometrial fluid aspirate. J Proteome Res.
608 2009;8(10):4622-32. Epub 2009/08/13.
609 76. Henry NL, Hayes DF. Cancer biomarkers. Molecular Oncology. 2012;6(2):140-6.
610 77. Meehan KL, Rainczuk A, Salamonsen LA, Stephens AN. Proteomics and the search for
611 biomarkers of female reproductive diseases. Reproduction (Cambridge, England). 2010;140(4):505-
612 19.
613 78. Meehan KL, Rainczuk A, Salamonsen LA, Stephens AN. Proteomics and the search for
614 biomarkers of female reproductive diseases. Reproduction (Cambridge, England). 2010;140(4):505-
615 19. Epub 2010/07/16.
616 79. Ong S-E, Mann M. Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol.
617 2005;1(5):252-62.
618 80. Arentz G, Weiland F, Oehler MK, Hoffmann P. State of the art of 2D DIGE. Proteomics Clinical
619 applications. 2014. Epub 2014/11/18.
620 81. Wang N, Li L. Exploring the Precursor Ion Exclusion Feature of Liquid
621 Chromatography–Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry for
622 Improving Protein Identification in Shotgun Proteome Analysis. Analytical Chemistry.
623 2008;80(12):4696-710.
624 82. Tate S, Larsen B, Bonner R, Gingras A-C. Label-free quantitative proteomics trends for
625 protein–protein interactions. Journal of Proteomics. 2013;81(0):91-101.
626 83. Vowinckel J, Capuano F, Campbell K, Deery MJ, Lilley KS, Ralser M. The beauty of being
627 (label)-free: sample preparation methods for SWATH-MS and next-generation targeted proteomics.
628 F1000Research. 2013;2:272.

84. Cornett DS, Reyzer ML, Chaurand P, Caprioli RM. MALDI imaging mass spectrometry: molecular snapshots of biochemical systems. Nat Meth. 2007;4(10):828-33.

85. Bateson H, Saleem S, Loadman PM, Sutton CW. Use of matrix-assisted laser desorption/ionisation mass spectrometry in cancer research. Journal of pharmacological and toxicological methods. 2011;64(3):197-206. Epub 2011/05/10.

86. Kriegsmann J, Kriegsmann M, Casadonte R. MALDI TOF imaging mass spectrometry in clinical pathology: A valuable tool for cancer diagnostics (Review). International journal of oncology. 2014. Epub 2014/12/09.

87. Quaas A, Bahar AS, von Loga K, Seddiqi AS, Singer JM, Omidi M, et al. MALDI imaging on large-scale tissue microarrays identifies molecular features associated with tumour phenotype in oesophageal cancer. Histopathology. 2013;63(4):455-62.

88. Steurer S, Borkowski C, Odinga S, Buchholz M, Koop C, Huland H, et al. MALDI mass spectrometric imaging based identification of clinically relevant signals in prostate cancer using large-scale tissue microarrays. International Journal of Cancer. 2013;133(4):920-8.

89. Breuer EK, Murph MM. The Role of Proteomics in the Diagnosis and Treatment of Women's Cancers: Current Trends in Technology and Future Opportunities. International journal of proteomics. 2011;2011. Epub 2011/09/03.

90. Wang P, Whiteaker JR, Paulovich AG. The evolving role of mass spectrometry in cancer biomarker discovery. Cancer biology & therapy. 2009;8(12):1083-94. Epub 2009/06/09.

91. Rauser S, Marquardt C, Balluff B, Deininger Sr-O, Albers C, Belau E, et al. Classification of HER2 Receptor Status in Breast Cancer Tissues by MALDI Imaging Mass Spectrometry. Journal of Proteome Research. 2010;9(4):1854-63.

92. Meding S, Nitsche U, Balluff B, Elsner M, Rauser S, Schone C, et al. Tumor classification of six common cancer types based on proteomic profiling by MALDI imaging. J Proteome Res. 2012;11(3):1996-2003. Epub 2012/01/10.

654 93. Addona TA, Shi X, Keshishian H, Mani DR, Burgess M, Gillette MA, et al. A pipeline that
655 integrates the discovery and verification of plasma protein biomarkers reveals candidate markers for
656 cardiovascular disease. Nature biotechnology. 2011;29(7):635-43. Epub 2011/06/21.
657 94. Klein1 O, GN, DT, MB, TA, 4,5, , et al. SCiLS Lab 2D: Comparative Analysis for Uncovering
658 Discriminative M/z-markers,2015.
659 95. Shi T, Fillmore TL, Sun X, Zhao R, Schepmoes AA, Hossain M, et al. Antibody-free, targeted
660 mass-spectrometric approach for quantification of proteins at low picogram per milliliter levels in
661 human plasma/serum. Proceedings of the National Academy of Sciences of the United States of
662 America. 2012;109(38):15395-400. Epub 2012/09/06.
663 96. Huttenhain R, Malmstrom J, Picotti P, Aebersold R. Perspectives of targeted mass
664 spectrometry for protein biomarker verification. Current opinion in chemical biology. 2009;13(5-
665 6):518-25. Epub 2009/10/13.
666 97. Cohen Freue GV, Borchers CH. Multiple reaction monitoring (MRM): principles and
667 application to coronary artery disease. Circulation Cardiovascular genetics. 2012;5(3):378. Epub
668 2012/06/21.
669 98. Kitteringham NR, Jenkins RE, Lane CS, Elliott VL, Park BK. Multiple reaction monitoring for
670 quantitative biomarker analysis in proteomics and metabolomics. Journal of chromatography B,
671 Analytical technologies in the biomedical and life sciences. 2009;877(13):1229-39. Epub 2008/12/02.
672 99. DeSouza LV, Taylor AM, Li W, Minkoff MS, Romaschin AD, Colgan TJ, et al. Multiple reaction
673 monitoring of mTRAQ-labeled peptides enables absolute quantification of endogenous levels of a
674 potential cancer marker in cancerous and normal endometrial tissues. J Proteome Res.
675 2008;7(8):3525-34. Epub 2008/07/18.
676 100. Christoph W. Sensen BH. Advanced Imaging in Biology and Medicine.
677 101. Clemis EJ, Smith DS, Camenzind AG, Danell RM, Parker CE, Borchers CH. Quantitation of
678 spatially-localized proteins in tissue samples using MALDI-MRM imaging. Analytical chemistry.
679 2012;84(8):3514-22. Epub 2012/02/24.

680 102. Creasman W. Revised FIGO staging for carcinoma of the endometrium. International journal
681 of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and
682 Obstetrics. 2009;105(2):109. Epub 2009/04/07.

683

684

685

Figure 1: Proteomic approaches used in the EC biomarker discovery: Gel based and Gel free. In Gel based proteomic approaches, proteins are separated on the basis of their charge/pH or mass by isoelectric focussing and electrophoresis. Gel free techniques include MS based technologies.

Figure 2: In this analysis *in-situ* tryptic digestion was performed on 6µm thick FFPE tissue. The matrix used was α-cyano-4-hydroxycinnamic acid and data was collected in positive reflectron mode using an UltrafleXtreme MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany). An overlapped MALDI spectral profiles of tumour and healthy regions was generated by FlexAnalysis software (V4.0.1, Bruker Daltonics, Bremen, Germany). (a) The overlapped spectral profile show a multitude of differentially expressed *m/z* species between healthy and tumor regions simultaneously that allows a straightforward correlation of the expression pattern within the tissue morphology (b) Comparative representative spectra of *m/z* 1111.553Da ± 0.250Da between healthy and tumor regions"

Figure 3: MALDI-MSI of a EC FFPE sample. The expression pattern of *m/z* 1111.553Da ± 0.250Da is visualized between healthy and tumour regions (B), when comparison is made with corresponding haematoxylin and eosin (H & E) stained annotated image (A). Visualization of difference in the intensities was performed in SCiLS lab software (V2014b, Bruker Daltonics, Bremen, Germany) with edge preserving image denoising and automatic hotspot removal applied. This *m/z* species is seen to be specifically downregulated in tumour region as comapre to healthy region.

Figure 4: The ROC curve of *m/z* 1111.553Da ± 0.250Da (AUC = 0.985) for healthy versus tumour region

706 **Table 1:** Classification of carcinoma of the endometrium, International Federation of Gynaecology

707 and Obstetrics (FIGO 2010), Table modified from Creasman *et al*. (102)

FIGO stage	**Tumour localisation**
Stage I	Tumour confined to the corpus uteri
IA	No or less than half myometrial invasion
IB	Invasion equal or more than half of the myometrium
Stage II	Tumour invades cervical stroma, but does not extend beyond the uterus
Stage III	Local and/or regional spread of the tumour
IIIA	Tumour invades the serosa of the corpus uteri and/or adnexa
IIIB	Vaginal and/or parametrial involvement
IIIC	Metastasis to pelvic and/or para-aortic lymph nodes
IIIC1	Positive pelvic nodes
IIIC2	Positive para-aortic lymph nodes with or without positive pelvic lymph nodes
Stage IV	Tumour invades bladder and/or bowel mucosa, and/or distant metastasis
IVA	Tumour invades bladder and/or bowel mucosa
IVB	Distant metastasis, including intra-abdominal metastasis and/or inguinal lymph nodes

708

Figure 1

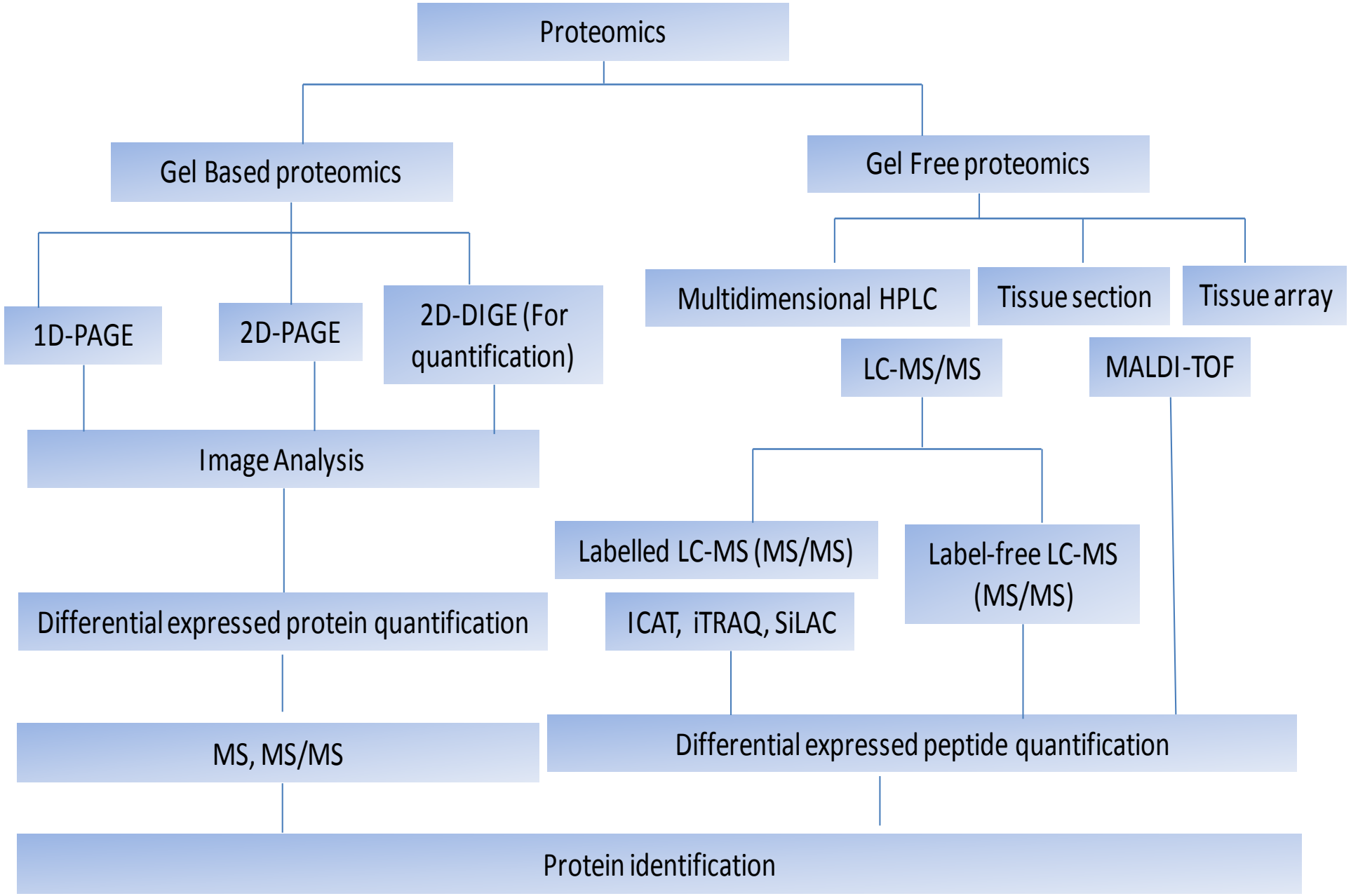

Proteomics
Gel Based proteomics
Gel Free proteomics
1D-PAGE
2D-PAGE
2D-DIGE (For quantification)
Multidimensional HPLC
Tissue section
Tissue array
LC-MS/MS
MALDI-TOF
Image Analysis
Labelled LC-MS (MS/MS)
Label-free LC-MS (MS/MS)
Differential expressed protein quantification
ICAT, iTRAQ, SiLAC
MS, MS/MS
Differential expressed peptide quantification
Protein identification

Figure 2a

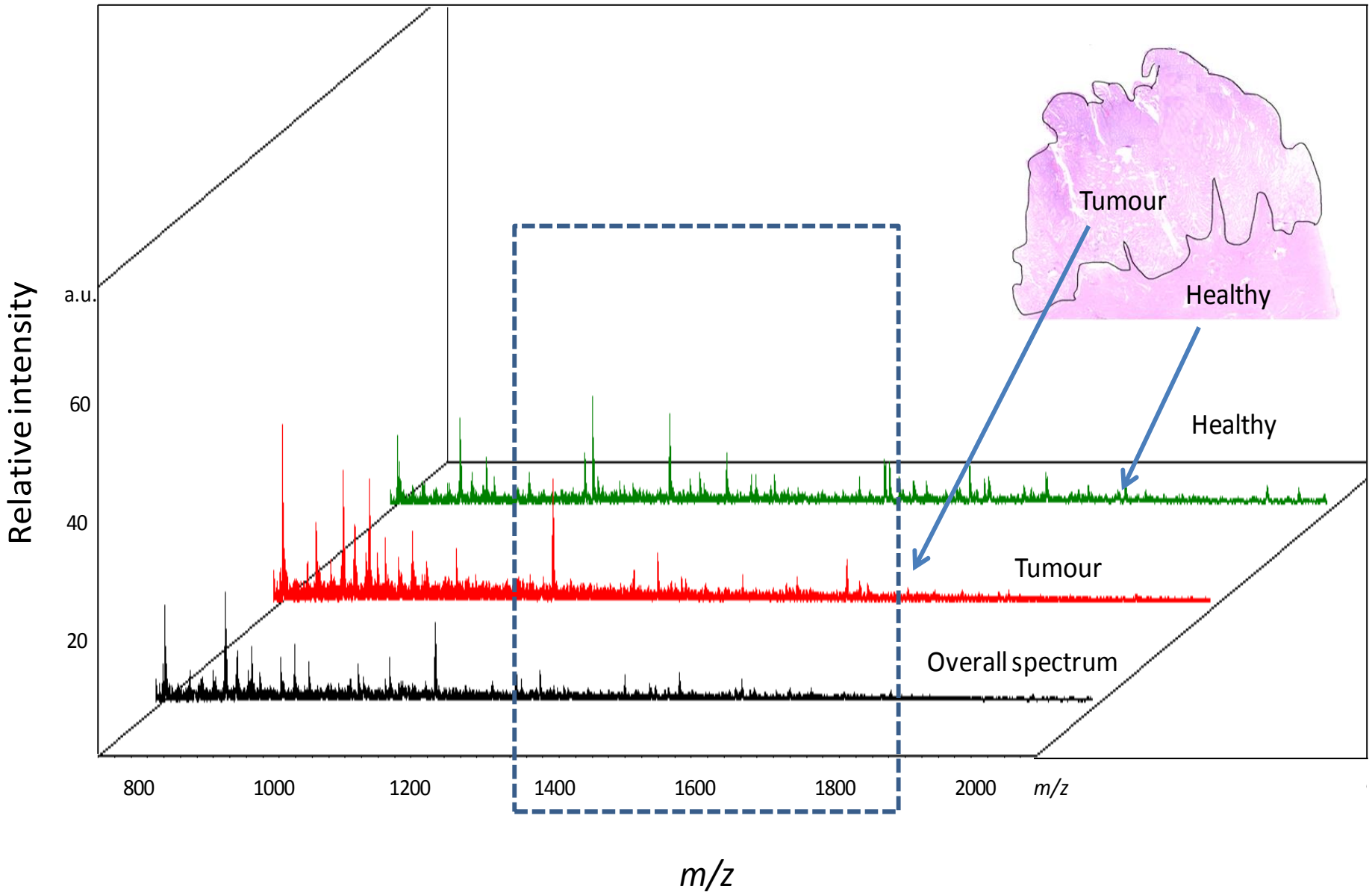

Relative intensity
a.u.
60
40
20
800
1000
1200
1400
1600
1800
2000
m/z
Tumour
Healthy
Healthy
Tumour
Overall spectrum
m/z

Figure 2b

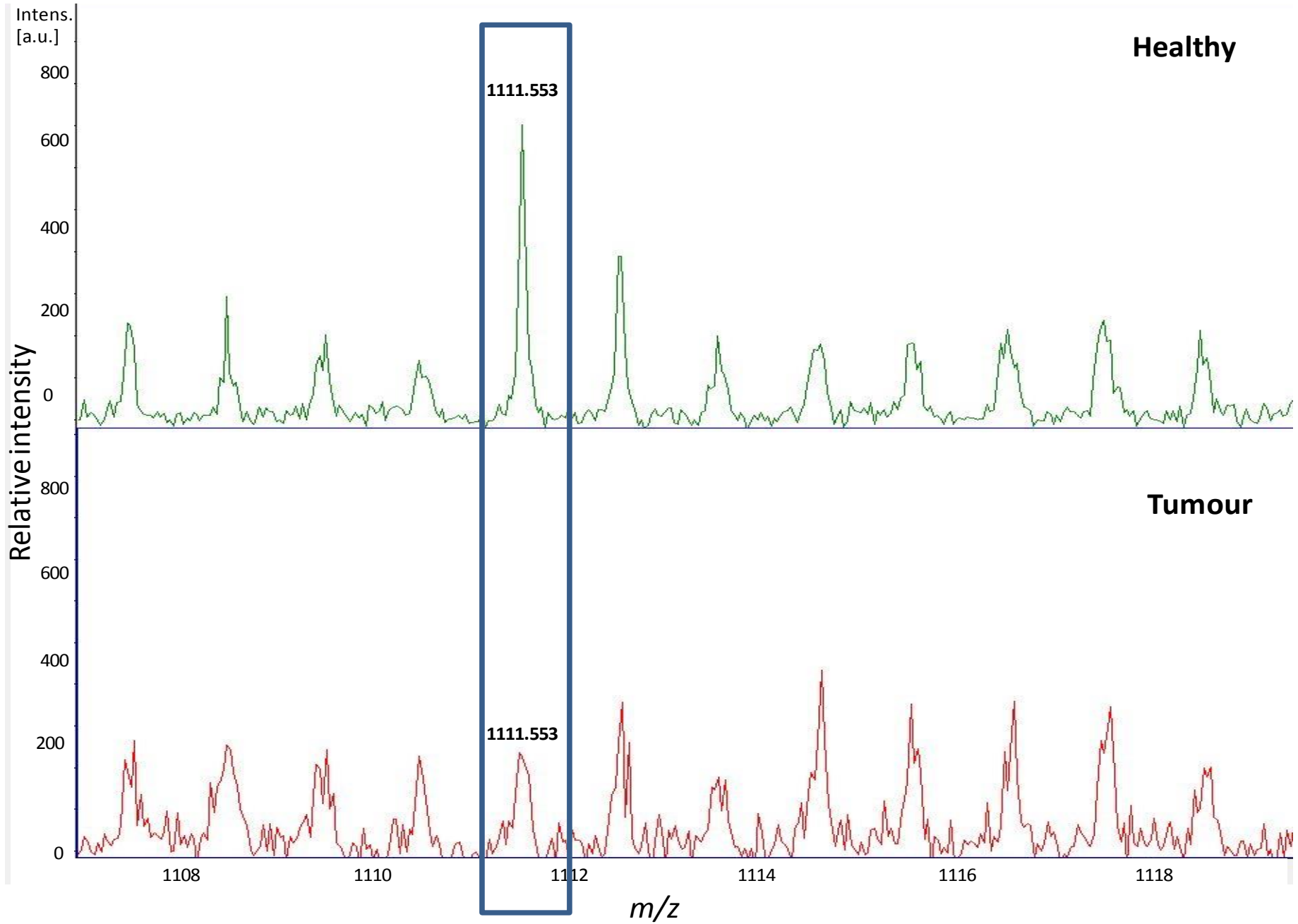

Intens.
[a.u.]
Healthy
1111.553
800
600
400
200
0
Relative intensity
Tumour
1111.553
800
600
400
200
0
1108
1110
1112
1114
1116
1118
m/z

Figure 3

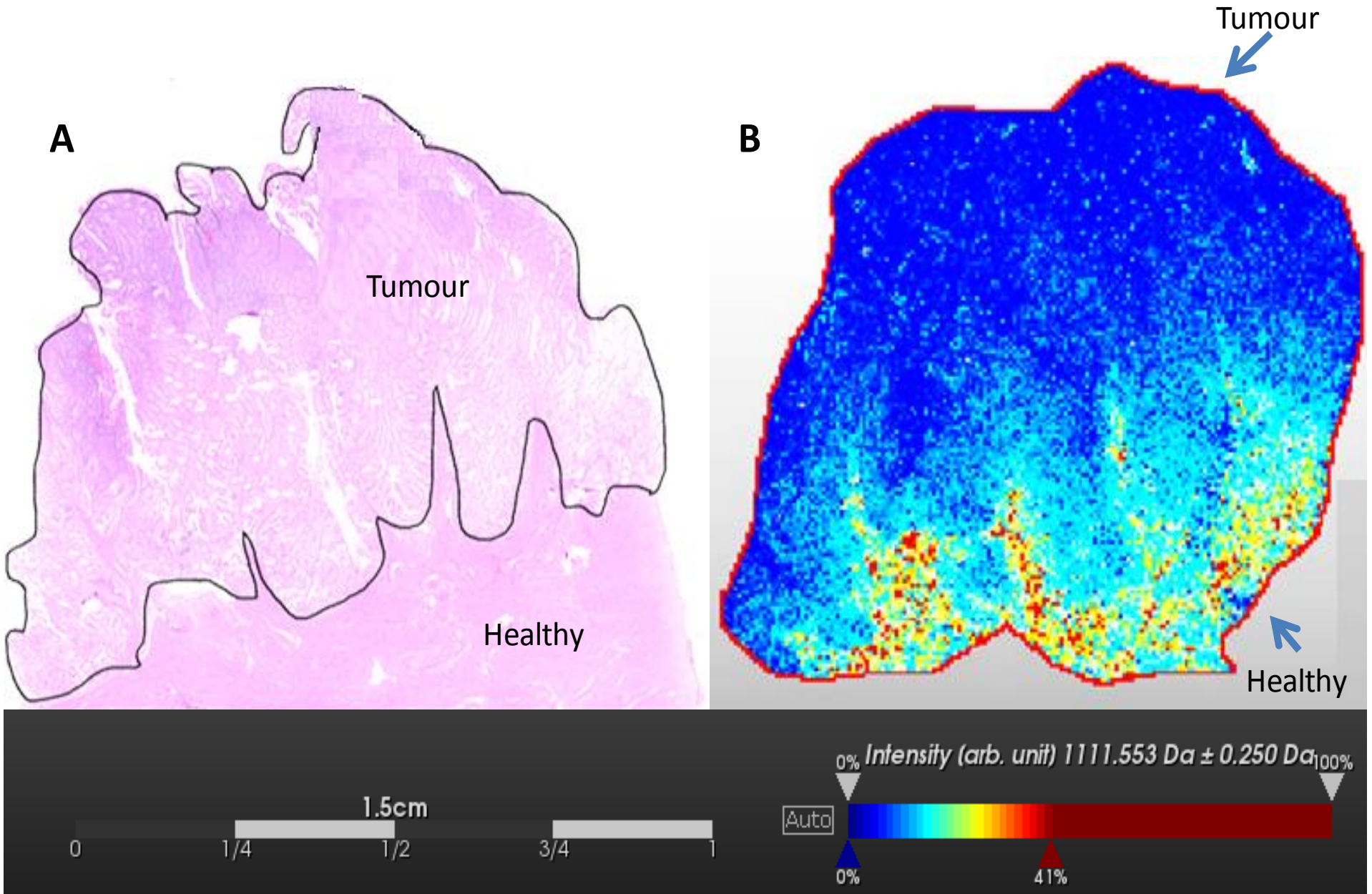
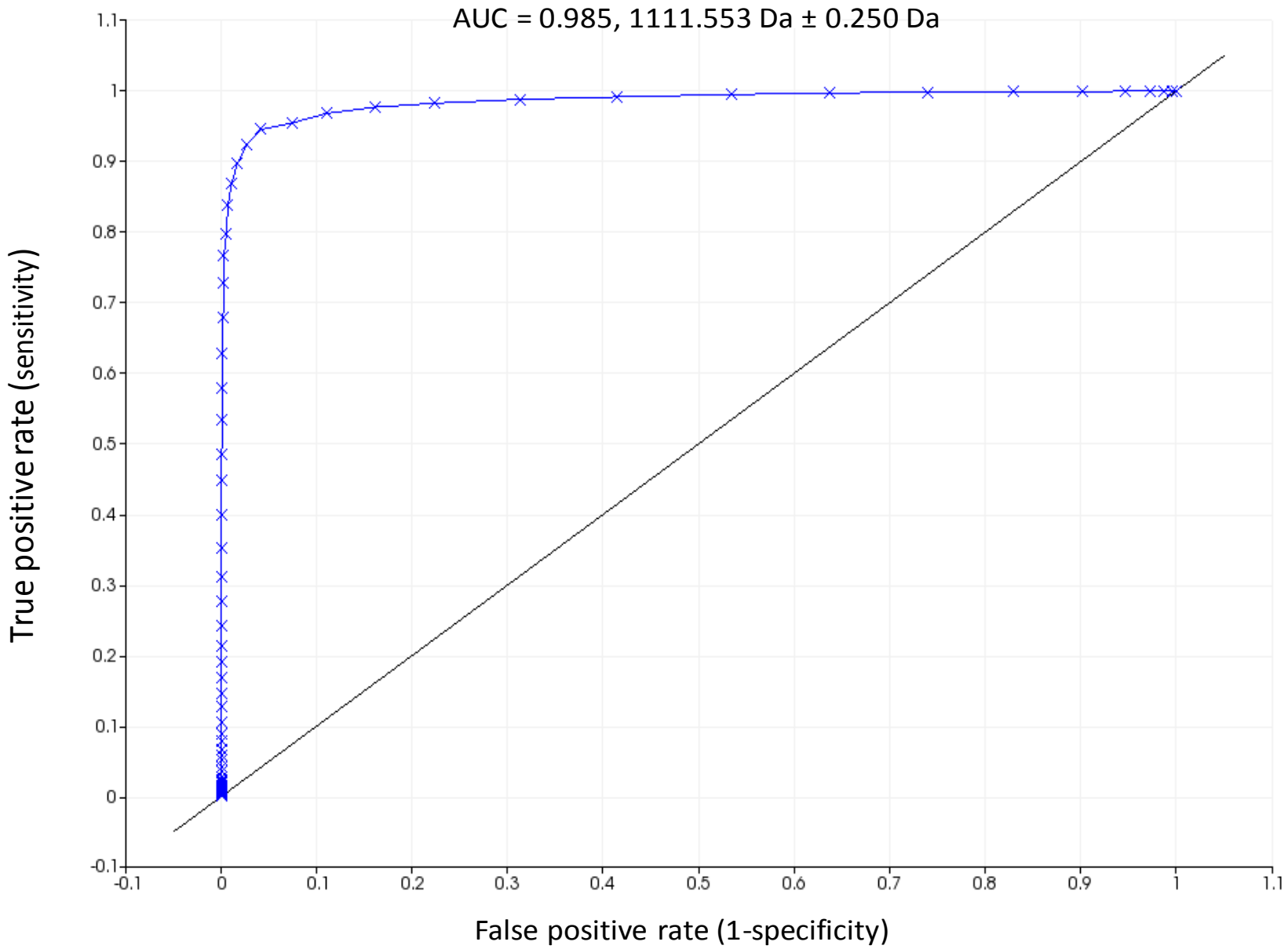

A
Tumour
Healthy
B
Tumour
Healthy
1.5cm
0
1/4
1/2
3/4
1
0%
Intensity (arb. unit) 1111.553 Da ± 0.250 Da
100%
Auto
0%
41%

Figure 4


AUC = 0.985, 1111.553 Da ± 0.250 Da
True positive rate (sensitivity)
False positive rate (1-specificity)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1