INSTITUTE OF PRIMATE RESEARCH
NATIONAL MUSEUMS OF KENYA
WHO COLLABORATING CENTRE

Proteomics and endometriosis

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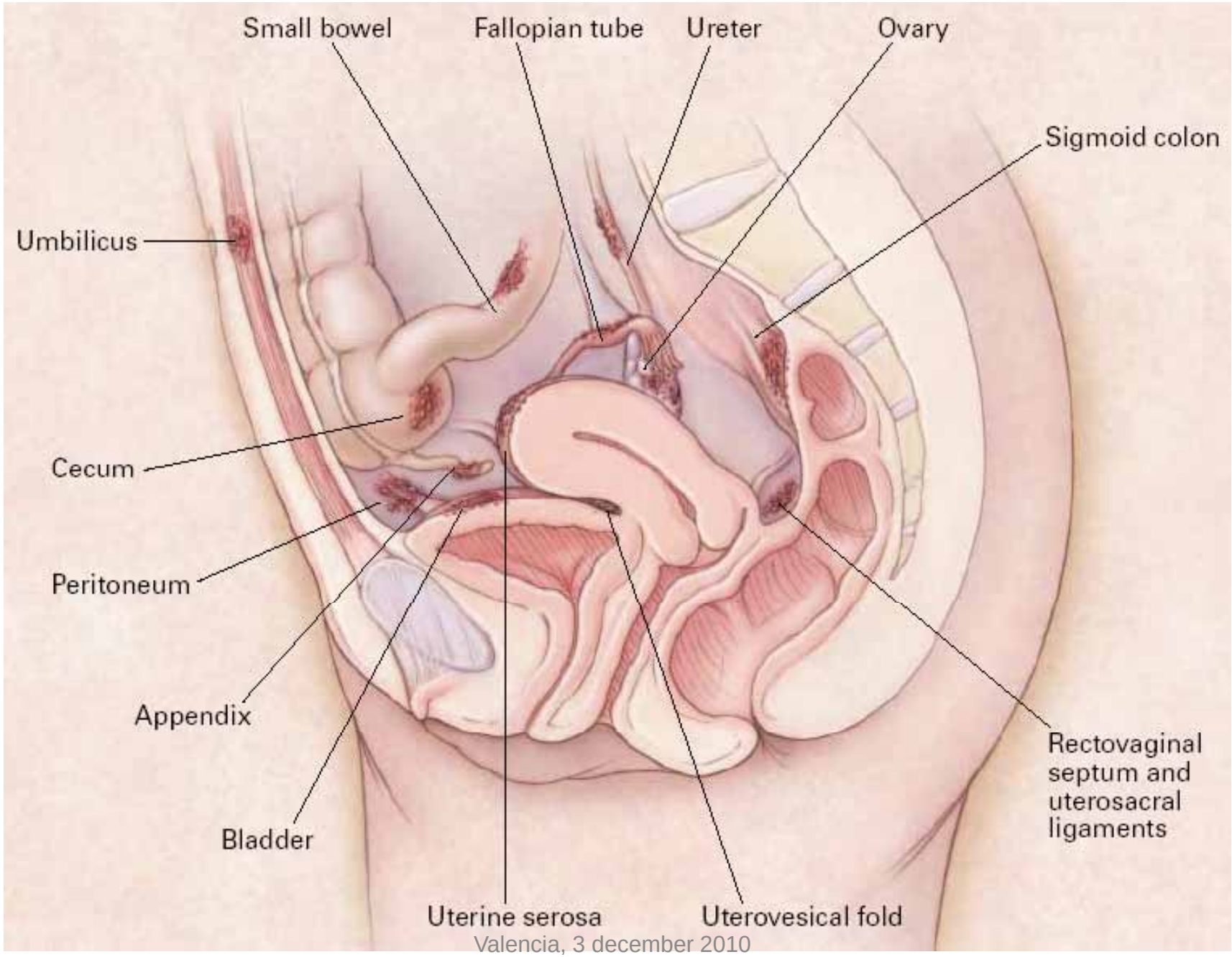
ESHRE Campus Course, Valencia, Spain
"The Maternal-Embryonic Interface"
2-3 December 2010

Presentation overview

- **Introduction endometriosis and proteomics**
- Proteomics by 2D –gel analysis
- Proteomics by SELDI-TOF analysis
- Conclusion and Future directions


Small bowel
Fallopian tube
Ureter
Ovary
Sigmoid colon
Umbilicus
Cecum
Peritoneum
Appendix
Bladder
Uterine serosa
Uterovesical fold
Rectovaginal septum and uterosacral ligaments

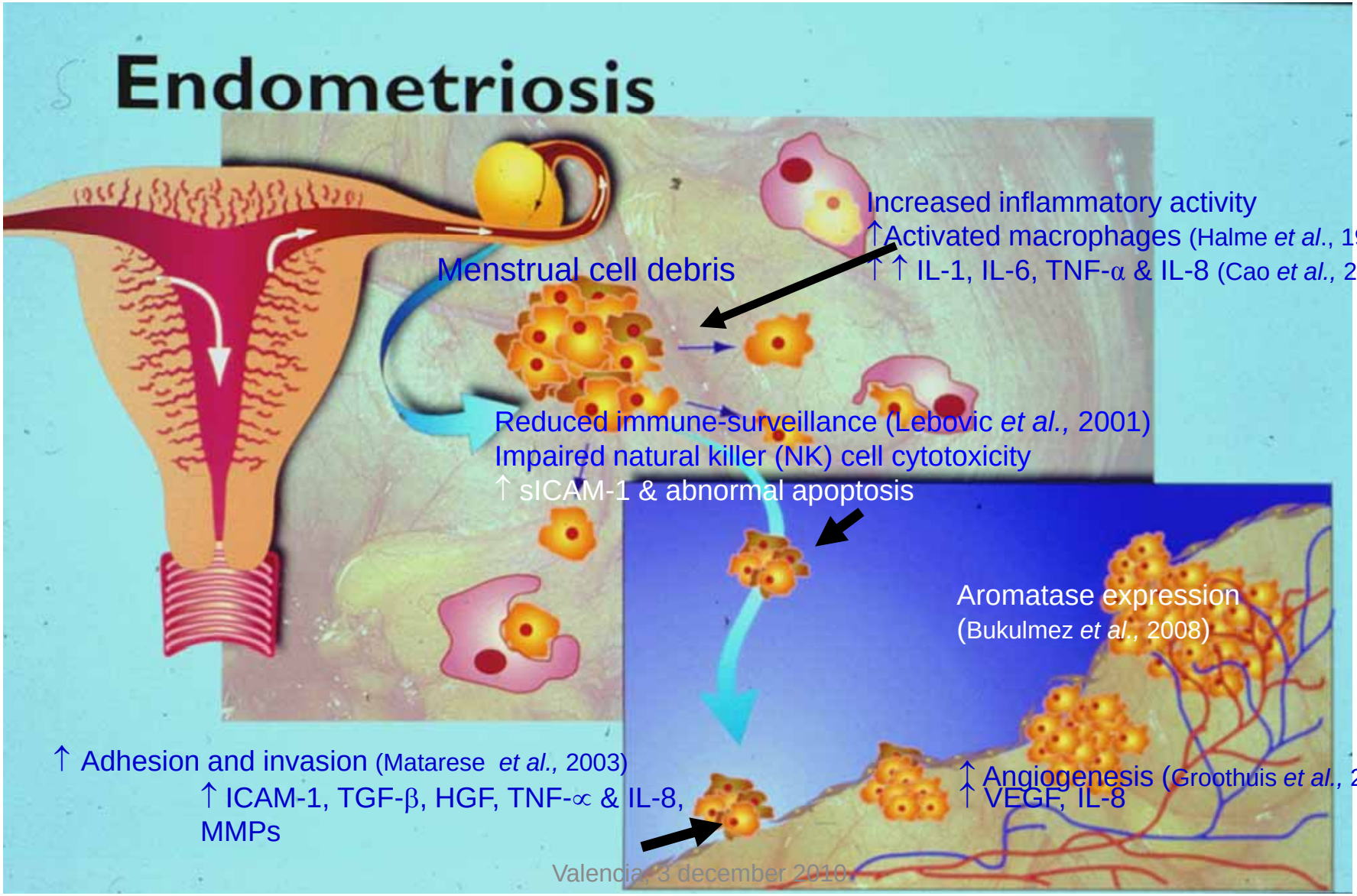
Endometriosis

- Defined as the presence of endometrial tissue (glands/stroma) outside the uterus
- Prevalence
  - 7-15% of reproductive age women
  - up to 50% patients with pelvic pain/infertility
- Estrogen dependent
  - rare before menarche or after menopause
- Progressive
  - >50% women/baboons after 1-2 years
- Most common theory is “retrograde menstruation” (Sampson Hypothesis -1927)

Prevalence of Endometriosis

- More than 70 million women worldwide
- 10% women of reproductive age
- 30% and 60% in women with infertility and pelvic pain respectively
- Endo cost considerably higher than cost related to Crohn's disease or to migraine in the USA for 2002 (Simoens et al., 2007)

Retrograde menstruation



Endometriosis = Pelvic inflammation

- Patients have chronic pelvic inflammation
  - ↑ PF volume and PF WBC concentration
  - ↑ activation of PF macrophages
  - ↑ PF inflammatory cytokines/growth factors

- ↑ pelvic inflammation in baboons after intrapelvic injection of endometrium (D'Hooghe et al, 2001)

Endometriosis = Pelvic inflammation with active endometrial and PERITONEAL contribution

- Endo versus controls:

1. RT PCR endometrium (Kyama et al, 2005, FS
   Menstrual EM: increased expression of
   TNF-alpha, IL-8 and MMP-3
   Luteal EM: increased expression of
   IL-1beta and RANTES
2. RT PCR peritoneum (Kyama et al, 2005)
   Menstrual peritoneum: increased expression of
   ICAM-1, TGFbeta, IL-6 and IL-1beta

Systemic inflammation biomarkers for endometriosis?

- Glycoprotein markers: CA-125, CA-19-9
- Cytokine markers: IL-6, TNF-alpha, MCP-1;MIF
- Adhesion molecules: sICAM-1
- Angiogenic factors: VEGF, leptin
- Anti-endometrial antibodies
- CCR1
- Novel candidates of biomarkers:

  HSP-90-beta; annexin A2, A5;

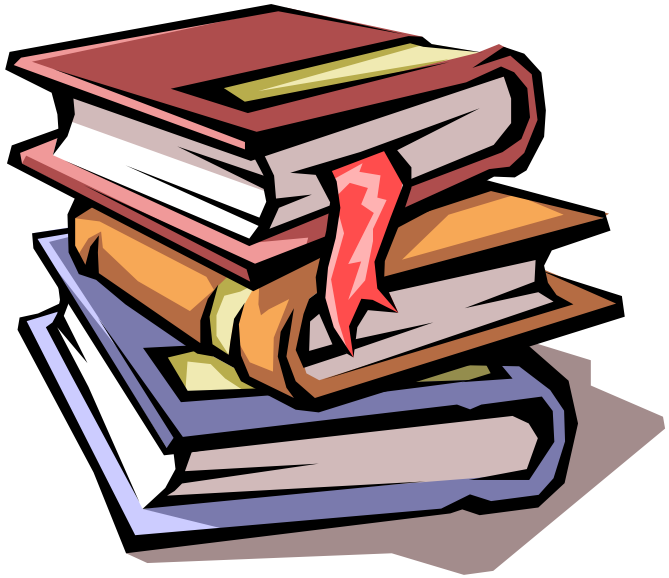
  glycodelin; Apo A1; transgelin

Ideal non-invasive test for endometriosis

- Symptomatic women (pain/infertility)
- Diagnostic delay 8-11 years
- 100% sensitivity, even if specificity only 50%
- Identify patients who might benefit from laparoscopic surgery (endometriosis/other fertility problems) (D'Hooghe et al, 2006)
- Do not miss patients with endometriosis, since surgery may double their MFR and improve their pain (ESHRE Guidelines; Kennedy et al, 2005)

Proteomics

The study of the protein library



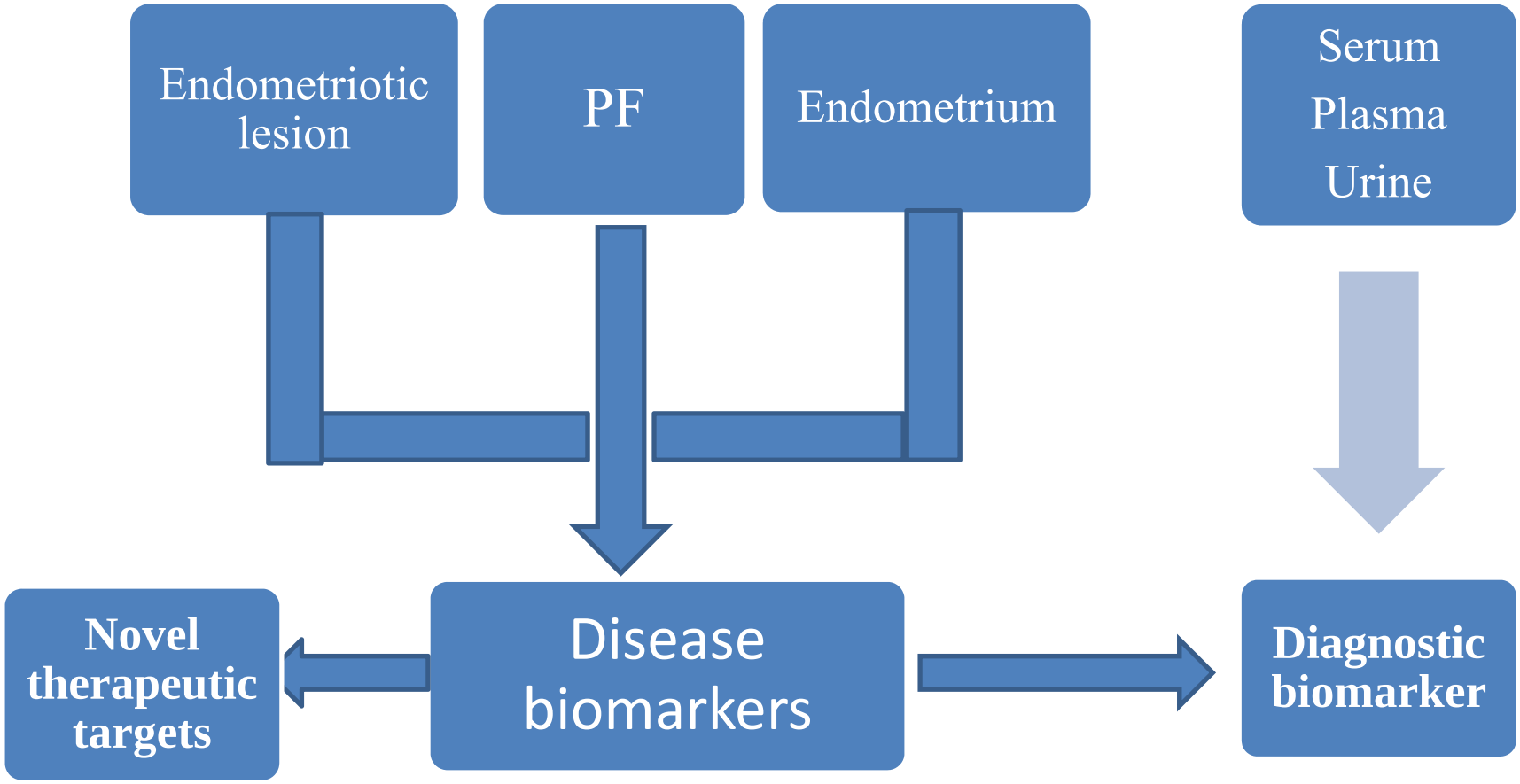
Why Proteomic Analysis in endo?

By screening the whole protein fraction:
discover new proteins/peptides relevant to

1. Pathogenesis of endometriosis:
2. Non-invasive or semi-invasive diagnosis (blood, urine, saliva; endometrium; peritoneal fluid).
3. Identify new molecular targets in order to develop new medical treatment.

! Better understanding of how mRNA microarray profiles translate into proteomic profiles

Use of proteomics in search of biomarkers for endometriosis



Proteomic tools used in endometriosis

2DE, MALDI -TOF – MS, SELDI-TO-MS

Protein profiling in women with endometriosis when compared with controls showed differentially expressed proteins/peptides [Chehna-Patel et al., 2010; Fowler *et al.*, 2007; Stephens et al., 2010; ten Have et al., 2007; Zhang *et al.*, 2006]

SELDI-TOF-MS profiling coupled to a learning algorithm has shown to offer diagnostic value in endometriosis [Ding et al., 2010, Jing *et al.*, 2008, Liu *et al.* 2007; Seeber et al., 200;, Wang *et al.*, **2007,** 2008 and 2010; Woelfer et al., 2008; Zhang et al., 2009]

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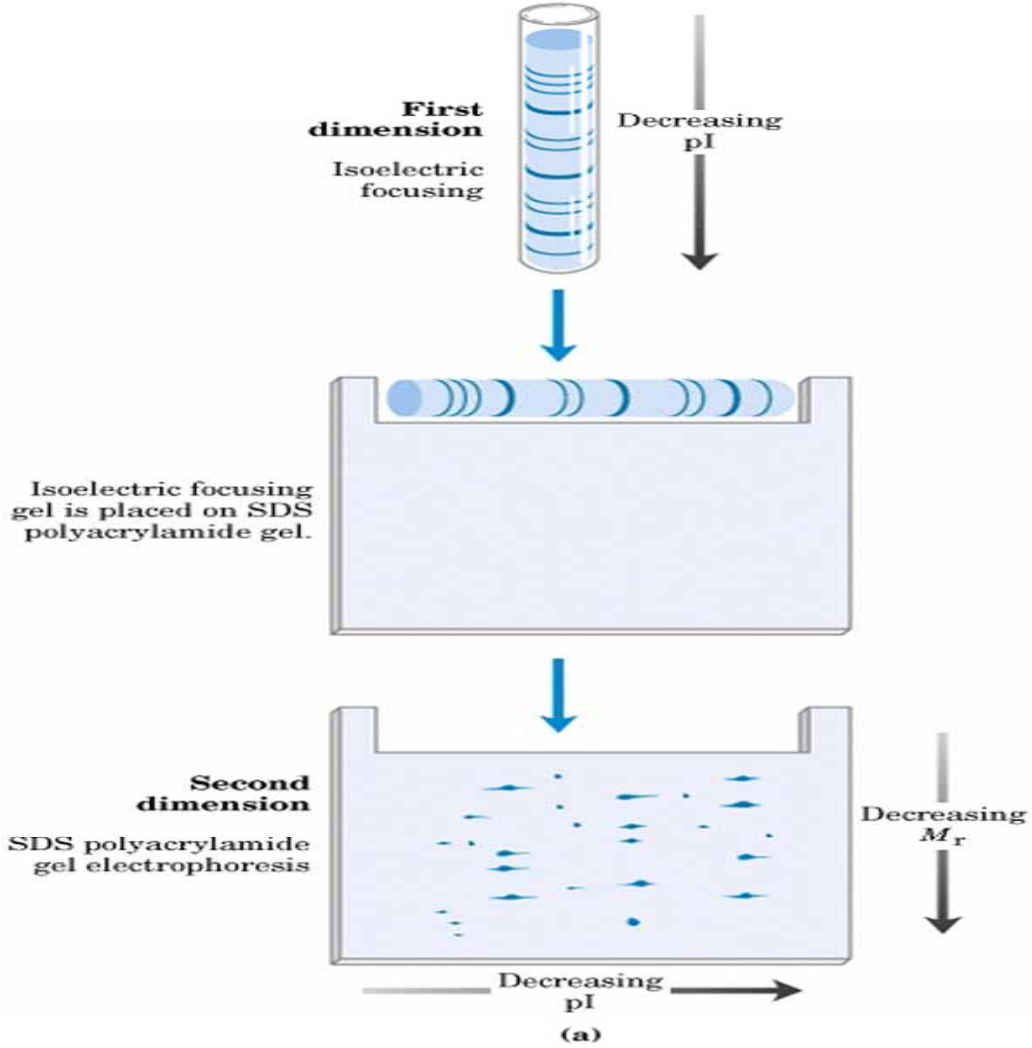
Two-D Gels


First dimension
Isoelectric focusing
Decreasing pI
Isoelectric focusing gel is placed on SDS polyacrylamide gel.
Second dimension
SDS polyacrylamide gel electrophoresis
Decreasing M_r
Decreasing pI
(a)


(b)

2DE Two-dimensional gel electrophoresis

Advantages

- hundreds to thousands of polypeptides can be analyzed in a single run
- High resolution between 30kDa-150KDa
- Proteins can be separated in pure form from the resultant spots
- Spots can be quantified and further analyzed by mass spectrometry

Disadvantages

- Large amount of sample handling (100-450µg of total protein concentration)
- Resolution <30kDa
- Limited reproducibility
- Not automated for high throughput analysis

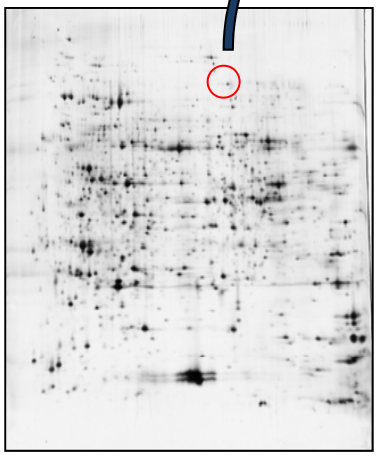
Mass spectrometry techniques to identify proteins/peptides

- Matrix- assisted laser desorption/ionization Time of flight mass spectrometry

  (MALDI-TOF MS)
- Electrospray ionization (ESI)
- Triple quadrupole (TQ) time of flight (TOF)
- Fourier Transform ion cyclotron resonance (FT-ICS)

MALDI TOF MS identification of proteins from 2DE

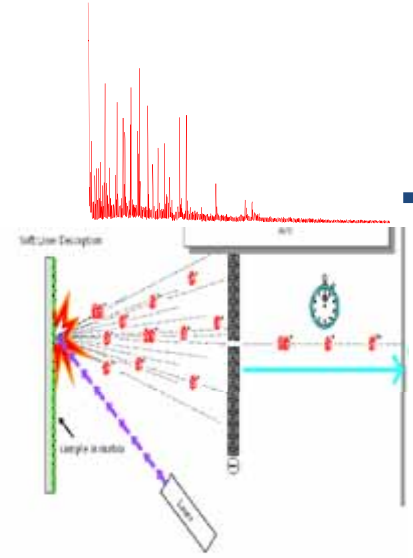
2D Gel



Identify differentially expressed proteins

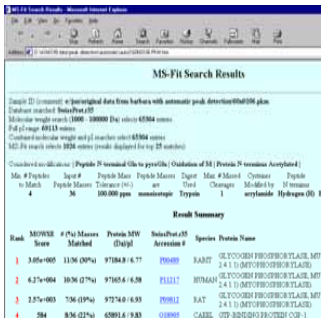
Excise spot of interest, destain, digest, extract peptides

MALDI-TOF-MS



Spot onto surface and mass analyze

Protein ID



Search spectra against protein databases

2DE/MALDI TOF MS results in endometriosis research

Reference	Sample Size	Technique	Results
Chehna-Petal et al., 2010	N=20 Paired endometriosis ectopic &eutopic endometrium (n=11) Controls (n=9)	2DE,western blotting, MALDI-TOF MS, immunohistochemistry	53 spots present in ectopic not in eutopic endometrium Validated proteins: 1. haptoglobin, 2. Rho-GDIα, 3. SM-22α, 4. Rab37
Fowler et al., 2007	N=35 pooled eutopic endometrium samples Endometriosis (n=18) Controls (n=17)	2D PAGE, MALDI-TOF MS	1. Apolipoprotein A1 2. peroxiredoxin 2 3. heat shock protein 90 4. annexin A2 5. Proteins associated with DNA metabolism and catabolism

Reference	Sample Size	Technique	Results kDa
Stephens et al., 2010	N=8 eutopic endometrium Endometriosis (n=4) Controls (n=4)	2DE, western blotting, Immunohistochemistry , MALDI-TOF MS	20 differentially expressed proteins <u>Validated proteins</u> 1. Vimentin, 2. RNH1 **3. PRDX6** (undetectable in normal endometrium) ↑2DE↓western blotting
Ten Have et al., 2007	N=18 eutopic endometrium Endometriosis (n=6) Controls(n=12)	2D PAGE, MALDI-TOF MS	21 proteins only present in disease samples Apoptosis, immune reaction, glycolytic pathway, cell structure , transcription factor
Zhang et al., 2006	N=12 serum& eutopic endometrium Endometriosis(n=6) Controls(n=6)	2DE,western blotting, MALDI-TOF MS	13 differentially expressed proteins IDENTIFIED proteins (serum): 1. vimentin 2. beta-actin 3. ATP synthase beta subunit

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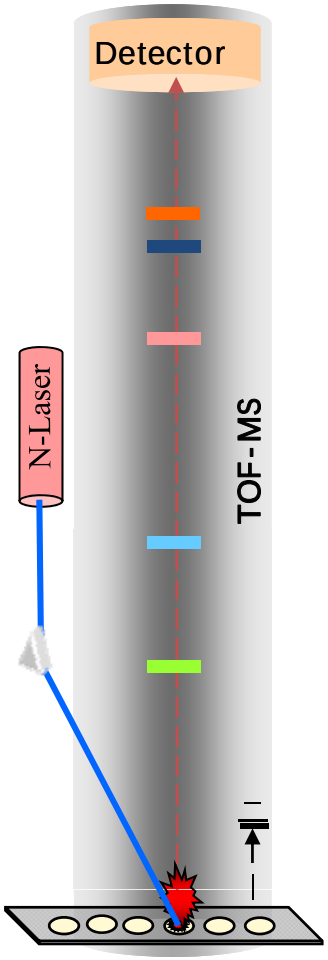
SELDI –TOF MS

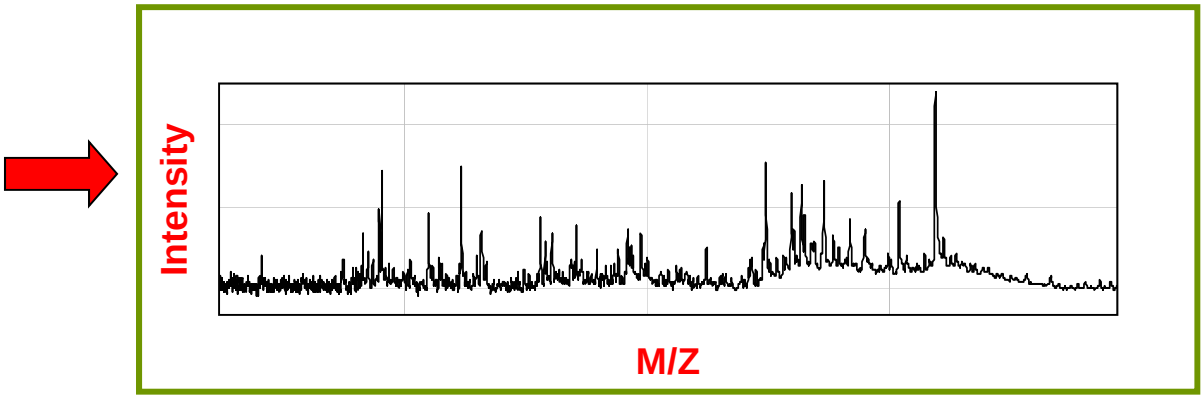
- Surface Enhanced Laser Desorption Ionisation-Time of Flight Mass Spectrometry

  - Analysis of protein mixtures
  - Body fluids (Blood) or tissues (EM)
  - Comparison of protein levels between patients with and without endo

Time-Of-Flight Mass Spectrometry



- Retained proteins are “eluted” from the ProteinChip Array by Laser Desorption/Ionization
- Ionized proteins are detected and their mass accurately determined by Time-of-Flight Mass Spectrometry

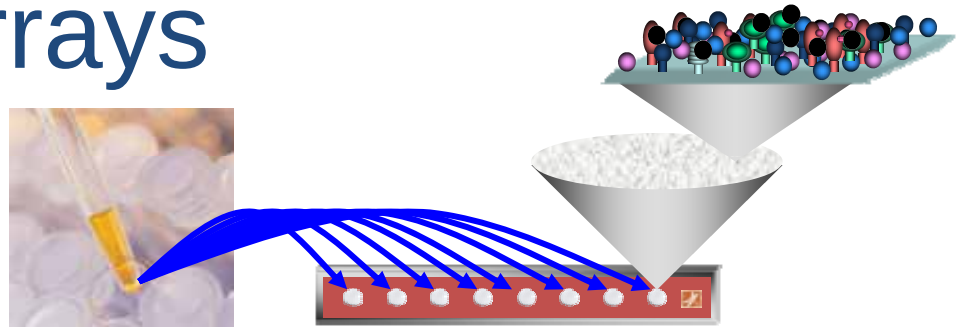


A mass spectrum

Preparation of Chromatographic arrays

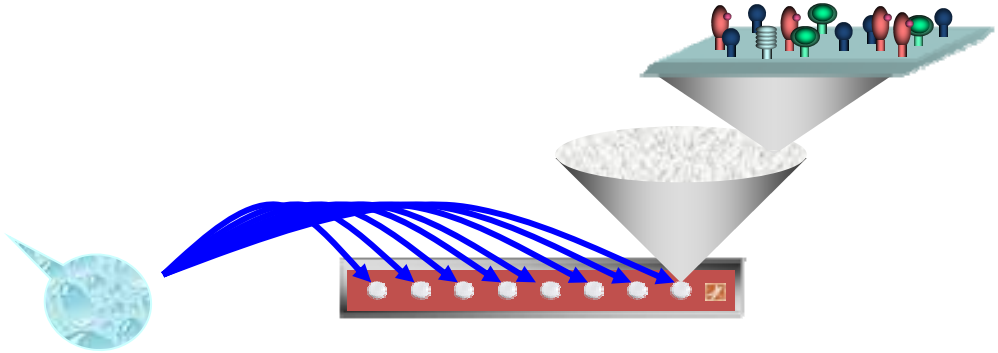
1. Apply Crude Sample

   Proteins bind to chemical or biological "docking sites" on the ProteinChip surface through an affinity interaction



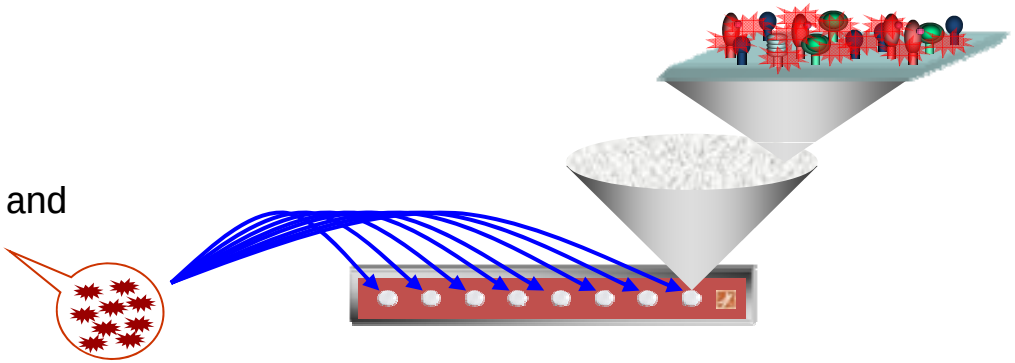
2. Wash ProteinChip

   Proteins that bind non-specifically and buffer contaminants are washed away, eliminating sample "noise"

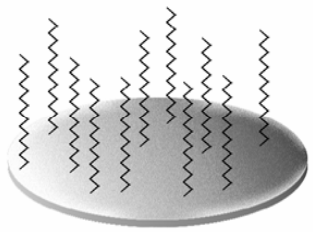


3. Add Energy Absorbing Molecules or "Matrix"

   After sample processing the array is dried and EAM is applied to each spot to facilitate desorption and ionization in the TOF-MS

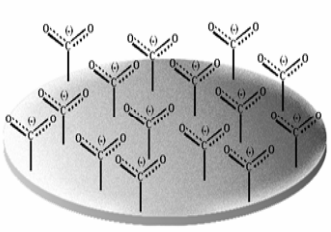


Different type of surfaces



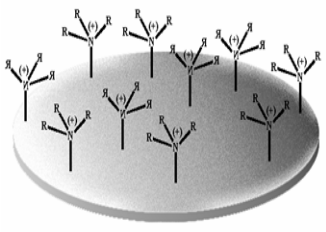
H50 /H4

hydrophobic



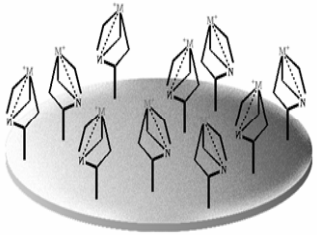
CM10- Anionic

surface



Q10- Cationic

surface



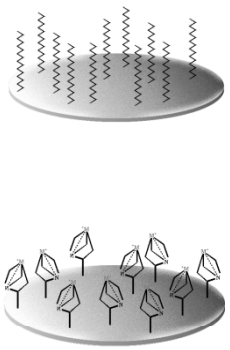
IMAC-30-Metal

affinity surface

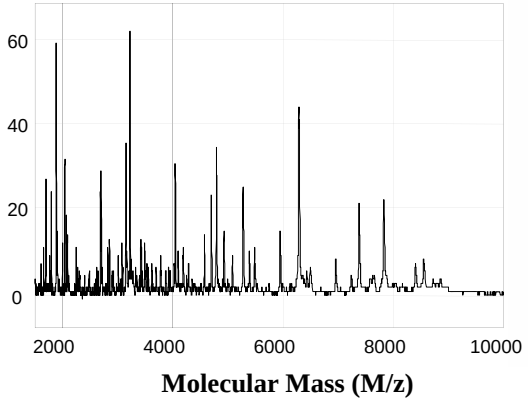
What is Protein Chip SELDI Technology

Retentate Chromatography

Mass Spectrometry



+



An extremely powerful tool for the HTP analysis of proteins and peptides

Advantages SELDI TOF MS

- Simple and fast
- High Throughput: up to 400 samples a day
- Sensitivity: Down to femtomole level
- Low amounts of samples required for analysis:

  2µg/ml total protein in min amount of 10µl.

Disadvantages SELDI TOF MS

- ? Reproducibility (intra- and inter assay) due to lack of standardized validated protocol
- Need to remove highly abundant proteins before analysis (Hb in EM; Albumin and IgG in plasma): experimental
- Less resolution if MW >20kDa
- Expensive
- Protein/Peptide Identification: extra step

Differences between SELDI-TOF MS and MALDI-TOF/TOF MS

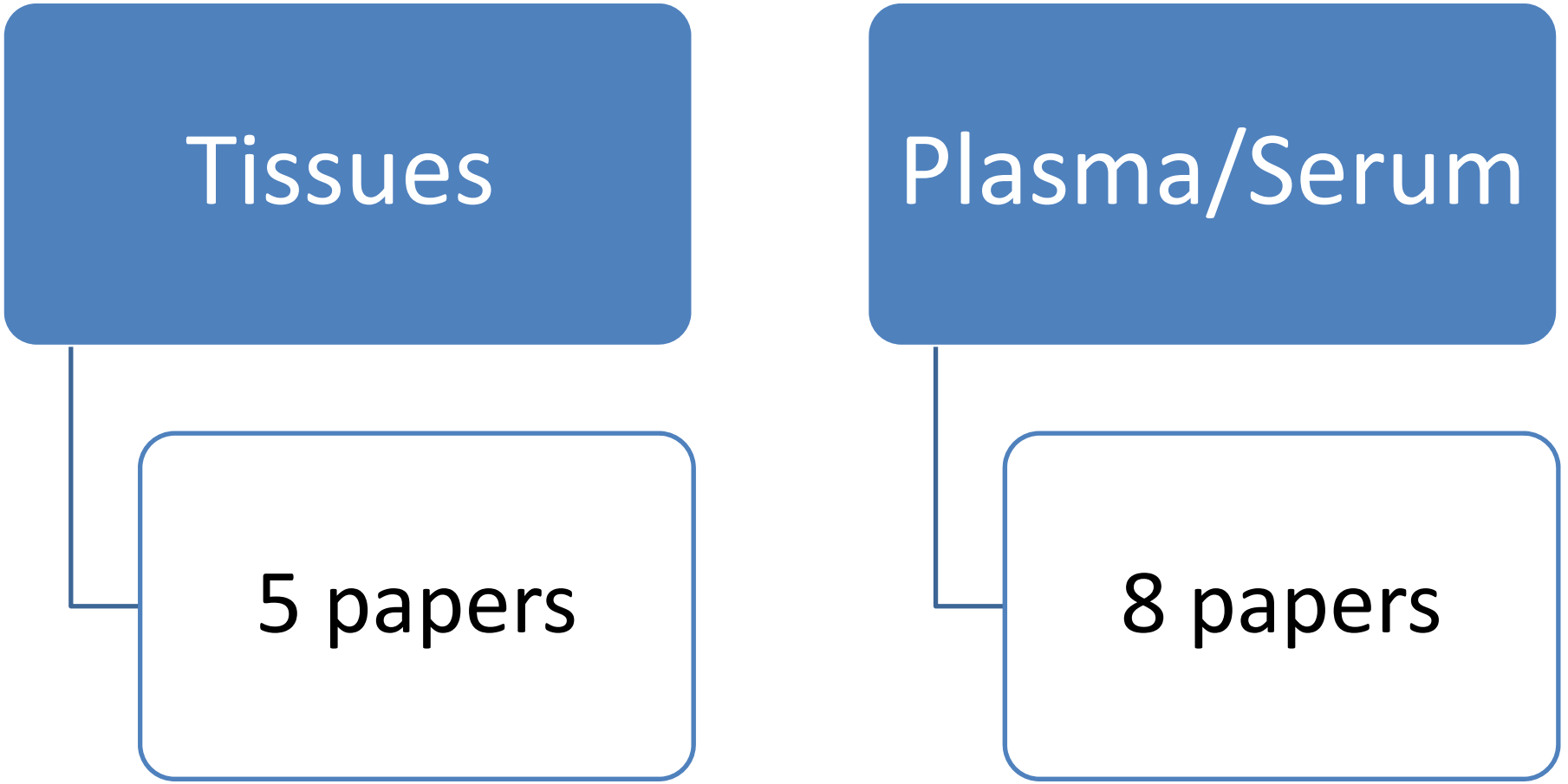
SELDI-TOF MS

- Binds proteins to array surfaces according to physico-chemical properties of array
- Array not reuseable
- more sensitive in the low mass ranges (< 20 kDa)
- Required amount of total protein concentr:2µg/ml

MALDI-TOF/TOF MS

- Proteins not selectively bound to array surface
- Arrays are reuseable
- more sensitive for the high mass ranges (>15 kDa)
- Required amount of total protein concentr: 1-2 µg/ml

PUBMED SEARCH (25nov2010)
SELDI TOF and ENDOMETRIOSIS



Literature (Serum)

Reference	Sample Size	Surface	Results kDa	Sensitivity	Specificity
Jing et al., 2009	N=120 Stage I-II (n=29) Stage III-IV (n=30) Controls (n=31) Healthy volunteers (n=30)	IMAC 30	1. 5.830 2. 8.865	89.66% 89.67% (after blind test)	96.67% 96.77% (after blind test)
Wang et al., 2007	N=32 Stage I-II (n=10) Stage III-IV(n=6) Controls (n=16)	H4	1. 3269 2. 6096 3. 5894 4. 8141	-	-

Literature (Serum)

Reference	Sample Size	Surface	Results kDa	Sensitivity	Specificity
Wang et al., 2008	N=66 Stage I-II(n=22) Stage III-IV (n=14) Controls (n=30)	H4	1. 8.142 2. 5.640 3. 5.847 4. 8.940 5. 3.269	91.7%	90%
Zhang et al., 2009	N=80 Endometriosis (n=48) Controls (n=32)	CM10	1. 4.974 2. 5.813 3. 4.290	91.7% 91.7% (Validated)	95.8% 75% (Validated)

Literature (Serum)

Reference	Sample Size	Surface	Results kDa	Sensitivity	Specificity
Seeber et al., 2010	N=141 Stage II-IV (n=63) Controls (78)	CM 10	1. 1.629 2. 3.047 3. 3.526 4. 3.774 5. 5.046 6. 5.086	66%	99%
Woelfer et al., 2009	N=91 Stage I-II(n=19) Stage III-IV(n=32) Controls (n=39)	Q10	1. 4.159 2. 5.264 3. 5.603 4. 9.861 5. 10.533	81.3%	60.3%

Literature (Plasma)

Reference	Sample Size	Surface	Results kDa	Sensitivity	Specificity
Liu et al., 2009 (Article only in Chinese)	N=71 Endometriosis (n=36) Controls (n=35)	Not mentioned	1. 3.956 2. 11.710 3. 6.986	92% 88%(after blind test)	83% 82%(after blind test)
Liu et al., 2007	N=87 Endometriosis (n=52) Controls (n=46)	CM 10	1. 3,956 2. 11,710 3. 6,986	87.5%	85.7%

Literature (Eutopic EM)

Reference	Sample Size	Surface	Results kDa	Sensitivity	Specificity
Ding et al., 2010 Mitochondrial protein expression	N=53 Stage I-II (n=19) Stage III-IV (n=5) Controls (n=29)	Cm10	1. 15.334 2. 15.128 3. 16.069	87.5%	86.2%
Wang et al., 2010	N=26 Stage I-II (n=8) Stage III-IV (n=5) Controls (n=13)	H4	1. 6898 2. 5891 3. 5385 4. 6448 5. 5425	91.7%	90%

Reference	Sample Size	Surface	Results	Sensitivity	Specificity
Fassbender et al., 2010 N=16	eutopic EM Stage I-II (n=5) Stage III-IV (n=5) Controls (n=6)	CM10 IMAC 30	32 peaks differentially expressed proteins in EM endo versus controls No relation with same sample mRNA array data	-	-
Kyama et al., 2006 N=6	Stage II (n=3) Paired eutopic EM & perit & perit endo lesion Controls (n=3) Eutopic EM	CM10 H50 IMAC 30 Q10	**Transgelin** 22-23kDa Upregulated in ectopic EM when compared to normal peritoneum	-	-
Kyama et al., 2010 N=29	Eutopic EM Stage I-II (n=9) + StIII-IV (n=10) Controls (n=10)	Q10 IMAC 30	•**T-Plastin** 90.675 •**Annexin 5** 39.956	100%	100%

Interesting Results

- Jing et al., 2009
  - Analyzed pre-postoperative serum samples
  - 5.830Da up-regulated in pre- versus post surgery and absent in the spectra of healthy volunteers
  - Specificity 100% and Sensitivity 89.65%

Interesting Results

- Woelfer et al., 2009
  - Prospective exploratory cohort study
  - low sensitivity & specificity which disqualifies the screening for serum proteins patterns by SELDI-TOF MS as a “quick fix” diagnostic test

SELDI-TOF MS in endometriosis Leuven group results :

Identified proteins:

- Transgelin (Ectopic EM > Nl Peritoneum)
- Annexin 5, T-plastin (EM endo > Endo Co)

(100% specificity and 100% sensitivity)

Kyama et al., 2006 & 2010

Potential role in endometriosis

Transgelin endo lesions (Kyama et al, 2006)

- 22-23kDa protein - a smooth muscle-actin binding protein
- Unknown in the development of endometriotic lesions
- Smooth muscle actin cells are present around the endometriotic lesions but absent in unaffected peritoneal site and eutopic endometrium (Anaf et al., 2000)

Potential role in endometriosis

Annexin 5

(Secretory phase endometrium)

(Kyama et al, 2010)

- In cancer: possible role in proliferation and/or cell mobility and have metastatic potential

- In endometriosis: possible role in early invasion of endometrial cells into the mesothelium after initial attachment to the peritoneal wall

Potential role in endometriosis

T-Plastin

(Secretory phase endometrium)

(Kyama et al, 2010)

- Plays a role in cellular motility, formation of actin bundles that are required for cell locomotion and maintenance of the cellular architecture
- Possible role in early development of endometriosis lesion (adhesion/attachment/invasion)

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Proteomics and endometriosis: known

- Tissues studied: PB (plasma/serum), EM, Endo lesions, Nl Peritoneum

- Techniques used: 2DE + MALDI-TOF; SELDI-TOF

- Fishing expedition which may result in discovery of new proteins/peptides relevant to pathogenesis of endometriosis with potential to lead to new biomarkers and/or new targets for medicial treatment. HIGH NOVELTY POTENTIAL.

Proteomics and endometriosis: known

• 2DE and SELDI TOF MS:
N of differentially expressed proteins in EM/serum (plasma)
from women with and without endometriosis:

If identified (2DE papers):
? Function ? New Biomarker potential by ELISA (serum) or IH (EM)

If not identified (most SELDI TOF MS papers):
? Biomarker potential: “peptide peak profile”

Proteomics and endometriosis: future research needs

- Better understanding of relationship between proteomic results and mRNA/miRNA microarray in same samples
- Explore effects of menstrual cycle on proteomic results
- Sufficient N samples corrected for cycle phase required
- Different types of chip surfaces need to be studied in SELDI TOF MS

Future studies SELDI TOF MS

Assay improvement

- More chip surfaces
- Intra- and Interassay variability
- Use and validation of depletion methods
- Need for standardization of technique

Sample Population

- Large sample size
- Control for cycle phase
- Need for training and test set (validation in mono- and multicenter context)
- Advanced bio-informatics

Protein/peptide Identification

- MALDI-TOF/TOF MS
- Confirmation tests using ELISA, IH,Western Blots,..
- Development of novel markers (? nonID profiles) as possible diagnostic test

Acknowledgments

Leuven University Fertility Centre
Alexandra Vodolazkaia M.D.
Srinu Pathivada, MSc
Cleophas Kyama, PhD
Attila Bokor M.D.
Peter Simsa Msc.
Attila Mihalyi Ph.D.
Christel Meuleman M.D.
Carla Tomassetti M.D.
Sofie Pelckmans M.D.
Karen Peerear M.D.

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Dept of Electrical Engineering, ESAT-SCD
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Institute of Primate Research Nairobi-Kenya
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Postdocs Leuven

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A Fassbender

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PhD Students Leuven – int'ntl

P Simsa (Budapest)

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H Falconer (Karolinska)

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N Kulia

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Leuven Research coördinator

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G. Fried (Karolinska, Stockholm, SE)

G. Dunselman (Maastricht, NL)

A. Sharkey (Cambridge, UK)

F. Vilmos (Budapest, HUN)

K. Coleman (Oregon Primate Center, USA)

EU Network for Endometriosis (ENE)

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WHO COLLABORATING CENTRE

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International Collaboration

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  WHO Collaborating Center
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- Oxford and Cambridge Universities, UK
- European Network Endometriosis
- Karolinska University, Stockholm, Sweden (H. Falconer)
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- Endometriosis Association, Milwaukee, USA
- World Endometriosis Research Foundation, London, UK

Funding since 1998

- **Leuven University Research Council**
- **Leuven IRO (International Council for Development Collaboration)**
- **Leuven University Hospital Clinical Research Foundation**

- **Belgian Fund for Scientific Research (FWO)**
- **Belgian Institute for Science/Technology (IWT)**
- **Flemish Government (endocrine disrupters)**

- **Endometriosis Association USA**
- **University Michigan Ann Arbor; University Milwaukee, WI, USA**
- **World Endometriosis Research Foundation**
- **EU Public Health Grant (European Network for Endometriosis)**

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