Proteomics in endometriosis

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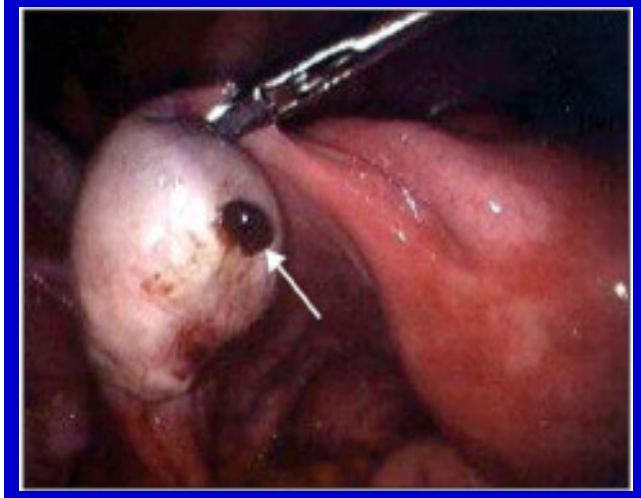
Learning Objectives

- Appraise the need for new approaches in endometriosis research
- Provide an overview of the proteomic platform used in endometriosis studies
- To appreciate the potential of proteomics in biomarker discovery

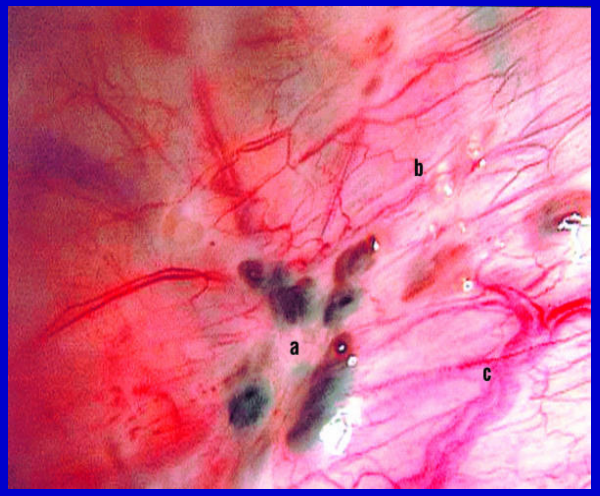
Endometriosis

- **Defined as the presence of endometrial-like cells outside the uterus**
- **Estrogen dependent**
  - **rare before menarche or after menopause**
- **Progressive**
  - **>50% women/baboons after 1-2 years**
- Prevalence:
  - **4% in asymptomatic women having sterilization**
  - **5-20% in women with pelvic pain**
  - **20-40% among infertile women**

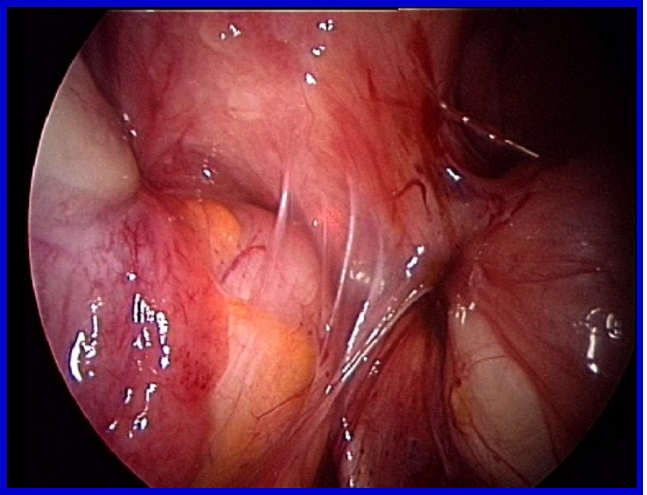
Ovarian endometrioma

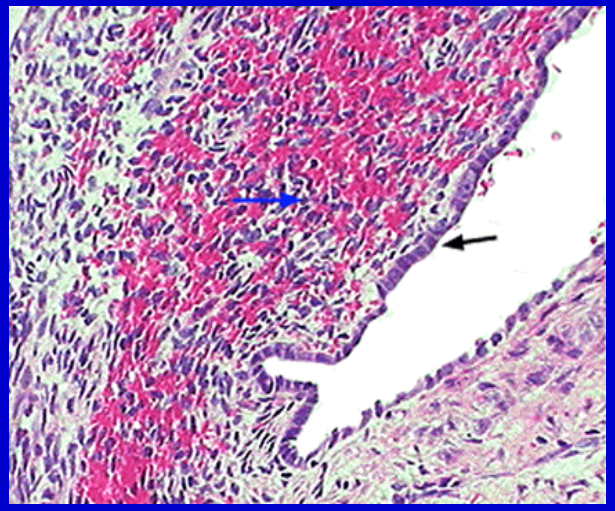


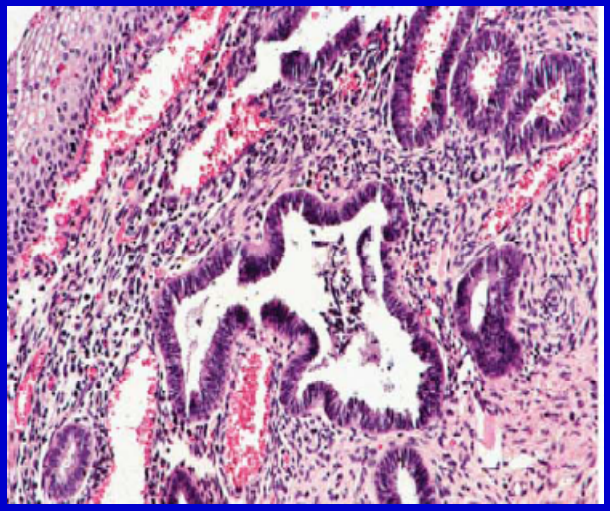
Peritoneal endometriosis

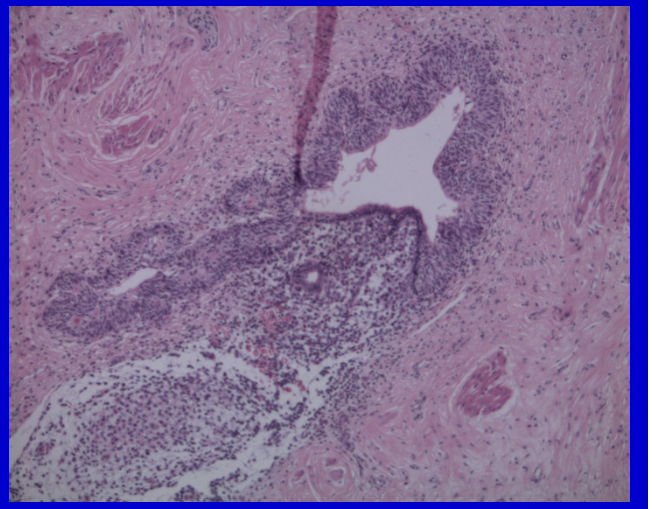


Endometriosis adhesion



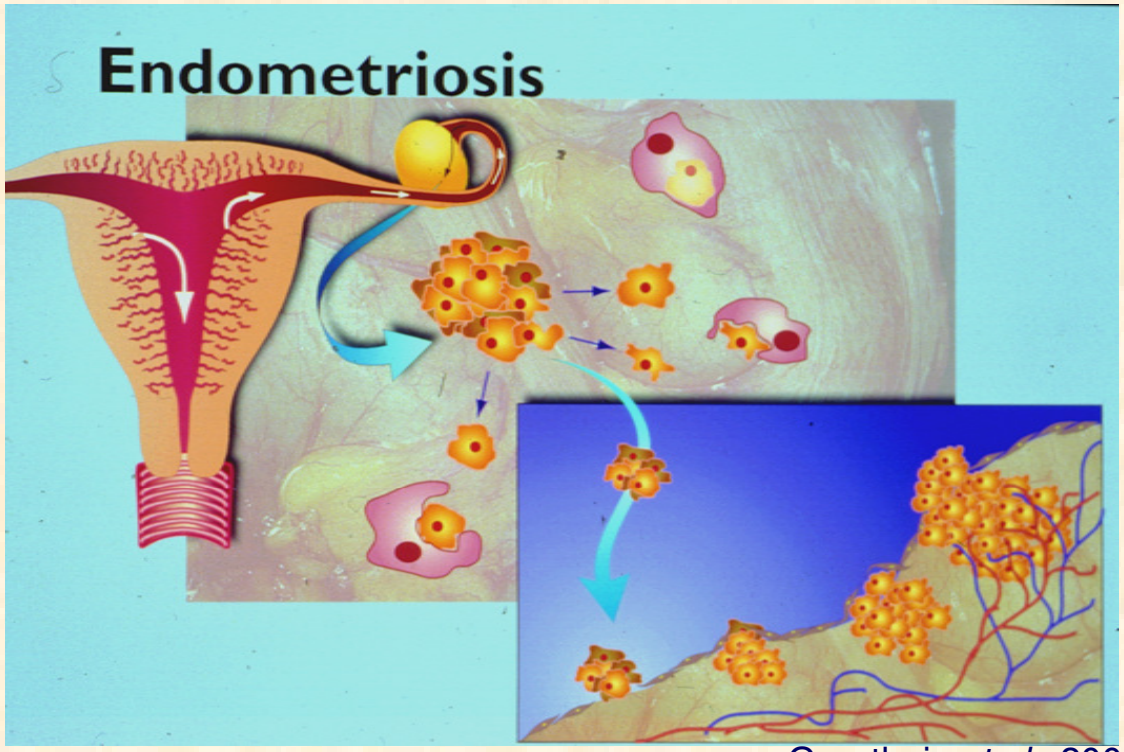






Clement., 2007; Hart., 2003; Prentice., 2001

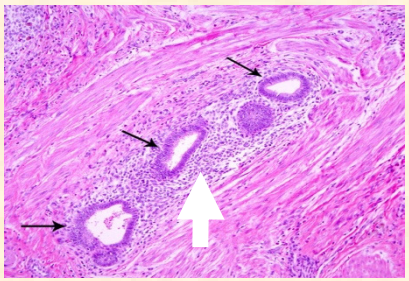
Pathogenesis of Endometriosis



Groothuis *et al.*, 2005

Peritoneal mesothelial cells

Metaplastic change



Endometriosis

Principal theories of histogenesis

Retrograde menstruation (Sampson, 1927)

Metaplasia theory (Iwanoff, 1898)

Induction theory (Levander and Normann, 1955)

Diagnosis of Endometriosis

- Laparoscopic surgery + histology
- Ovarian endometriomas: ultrasound or MRI can be sufficient (Kennedy et al., 2005)

• The delay in diagnosis in patients with pelvic pain or infertility is on average 11.7 and 3.5 years respectively (Arruda *et al*., 2003)

Molecules	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
CA125	27% 61%	94% 95%	86 91%	50 (Somigliana et al., 2004) 75% (Gagne *et al*., 2003)
CA19-9	16%	91%	70%	46% (Somigliana et al., 2004)
IL-6	11%	91 %	62%	44% (Somigliana et al., 2004)
CCR1	90%	74%	82%	85% (Agic et al., 2007)

•No accurate non-invasive diagnostic test (Othman *et al.,* 2008)

Need for new approaches in endometriosis research

- Aetiology is not precisely known
- Natural process of disease development is still poorly explored
- Occurs in women and non-human primates
- Controlled invasive studies cannot be done in humans
- No biomarkers to predict endometriosis non-surgically
- Search for novel candidates biomarkers:

  - Use of Proteomic tools

Proteomics in pathogenesis of endometriosis

- **Proteomics: global analysis of proteins**

- Proteomics is based on proteome as a complete set of proteins produced by given cell or organism under defined set of condition

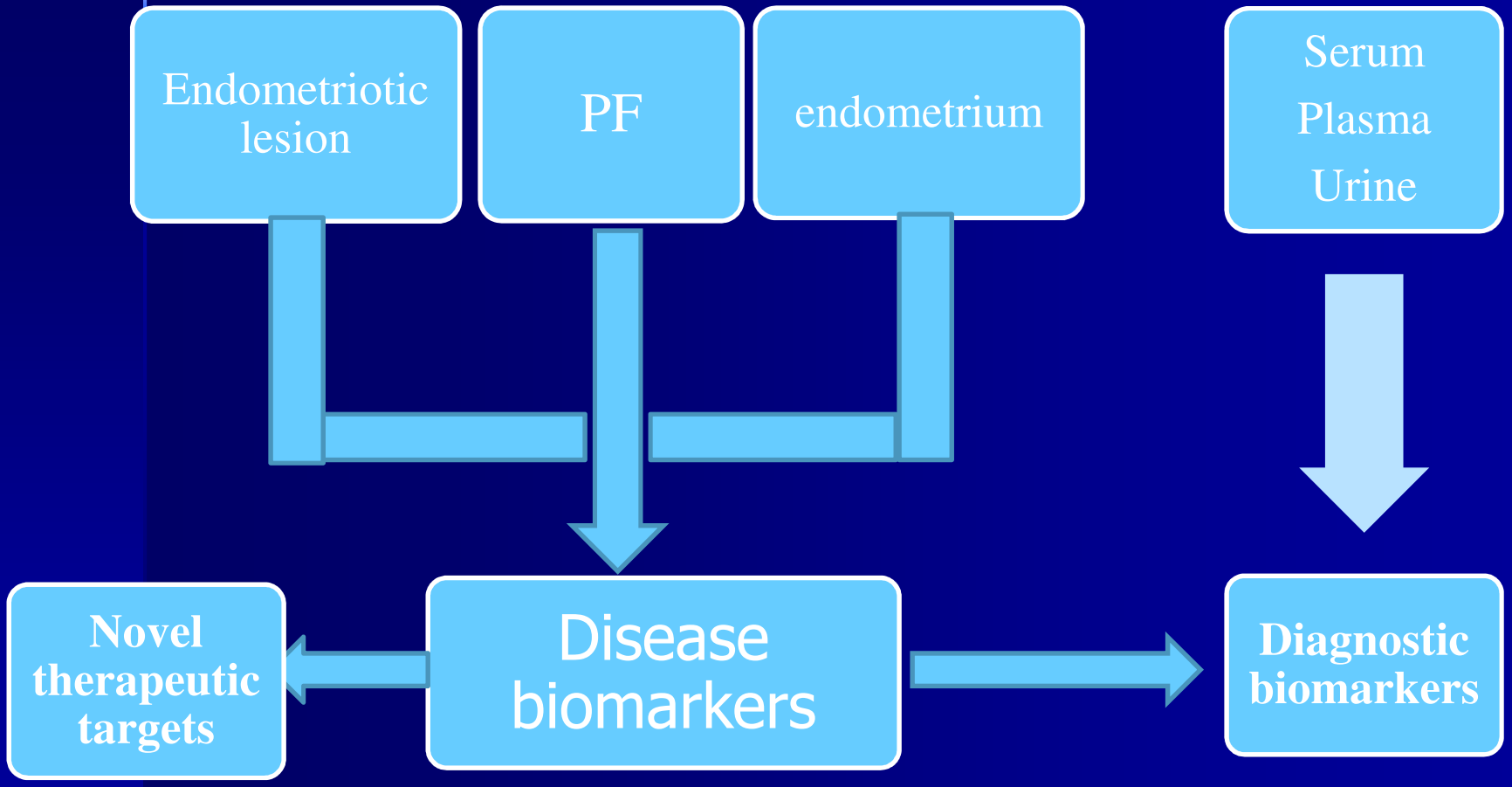
- Proteomics has the potential for biomarker discovery as well as addressing the pathogenesis of endometriosis

- Since it provides a robust platform for the study of clinically relevant samples

Why Use Proteomics?

- Have a better understanding of the function of gene products in the disease process
- Allow for the novel design of new therapies
- Provide new and specific biomarkers of endometriosis disease

Use of proteomics in search of biomarkers for endometriosis



Proteomic tools used in endometriosis

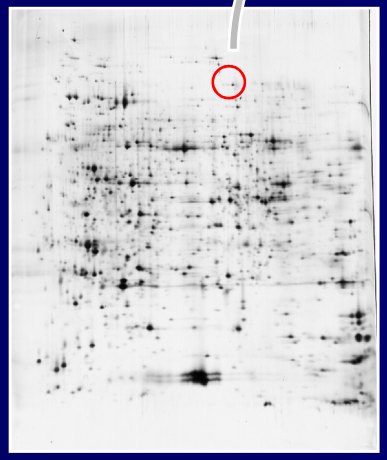
- 2D-GEL; LC-MS/MS
- MALDI-TOF-MS; SELDI-TOF-MS

Protein profiling in women with endometriosis when compared with controls showed differentially expressed proteins/peptides [Zhang *et al.,* 2006; Ametzarurra *et al*. 2009; Ferrero *et al*., 2008; Fowler *et al.,* 2007]

SELDI-TOF-MS profiling coupled to a learning algorithm has shown to offer diagnostic value in endometriosis [Liu *et al*., 2007; Wang *et al*., 2008; Jing *et al*., 2008; Wolfler *et al.,* 2008]

Proteomic platform used in endometriosis

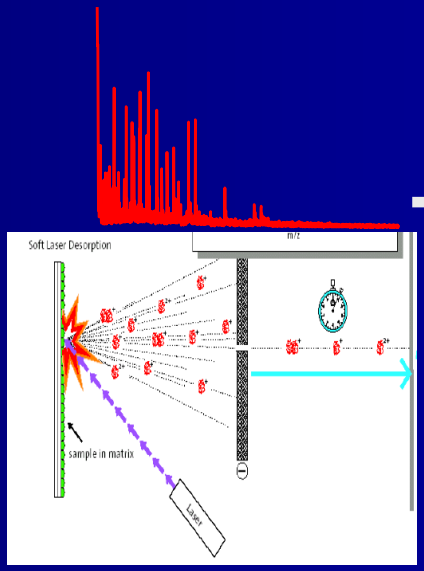
2D Gel



Identify differentially expressed proteins

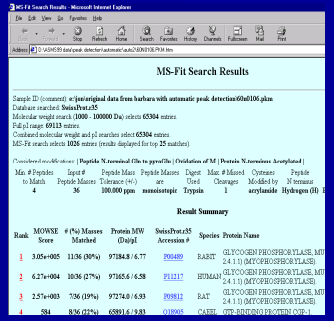
Excise spot of interest, destain, digest, extract peptides

MALDI-TOF-MS



Spot onto surface and mass analyze

Protein ID



Search spectra against protein databases

Proteomics platform used in endometriosis

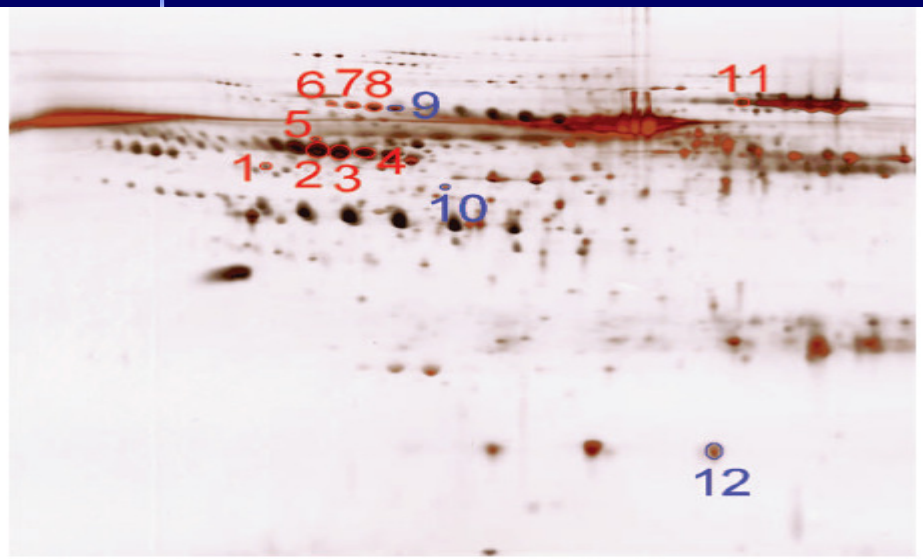


Figure 1. Silver-stained two-dimensional gel of peritoneal fluid. The protein spots circled in blue had significantly higher expression in the peritoneal fluid of women with stage I–II endometriosis (as defined by the American Society for Reproductive Medicine, ASRM) than in those with ASRM stage III–IV disease. The protein spots circled in red had significantly higher expression in the peritoneal fluid of women with ASRM stage III–IV endometriosis than in those with ASRM stage I–II disease. 1, α1-Antitrypsin (pI = 4.87, M_r = 50.32 kDa); 2, α1-antitrypsin (pI = 4.95, M_r = 54.33 kDa); 3, α1-antitrypsin (pI = 5.00, M_r = 54.76 kDa); 4, α1-antitrypsin (pI = 5.05, M_r = 54.33 kDa); 5, S100-A8 (pI = 4.97, M_r = 58.69 kDa); 6, α-1b-glycoprotein (pI = 5.05, M_r = 75.11 kDa); 7, α-1b-glycoprotein (pI = 5.09, M_r = 74.13 kDa); 8, α-1b-glycoprotein (pI = 5.13, M_r = 73.86 kDa); 9, α-1b-glycoprotein (pI = 5.19, M_r = 73.17 kDa); 10, unknown (pI = 4.66, M_r = 45.93 kDa); 11, serotransferrin (pI = 6.40, M_r = 80.75 kDa); 12, haptoglobin α chain (pI = 6.06, M_r = 16.90 kDa).

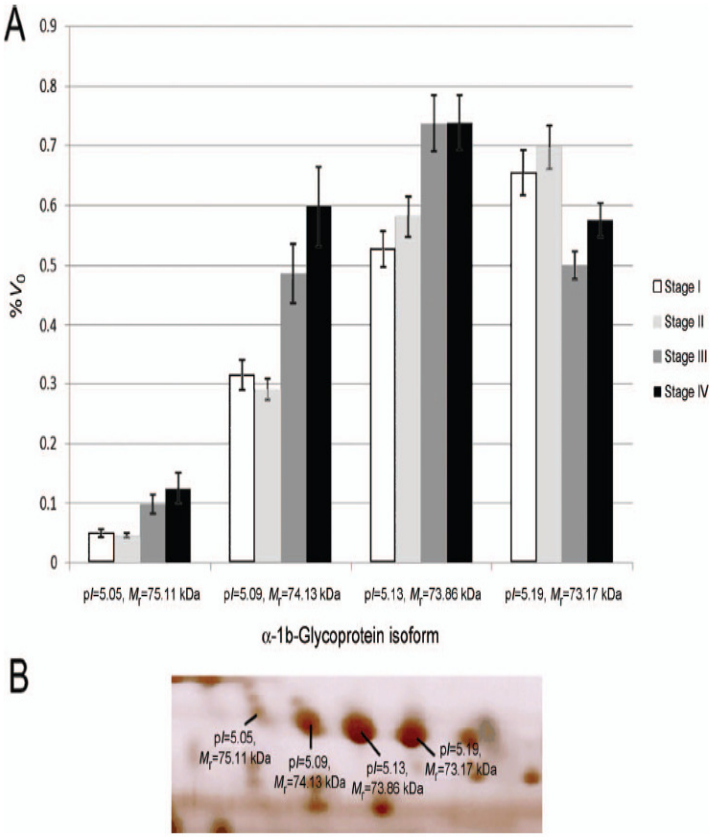


Figure 2. (A) Expression (%V_0) of four α-1b-glycoprotein isoforms according to the stage of endometriosis as defined by the American Society for Reproductive Medicine. (B) Detail of two-dimensional gel of peritoneal fluid showing the four isoforms of α-1b-glycoprotein.

Ferrero *et al.*, 2008

SELDI-TOF-MS platform in endometriosis

Selection and distribution of controls and endometriosis patients.

Group	n	Phase 1	Phase 2
Control group	61	30	31
Normal women	30	15	15
Benign ovarian tumor	13	6	7
Myoma	18	9	9
Endometriosis group	59	29	30
Stage I	9	4	5
Stage II	20	10	10
Stage III	14	7	7
Stage IV	16	8	8

Jing. Biomarkers for EM using SELDI-TOF-MS. Fertil Steril 2008.

↑8.865kDa
↑5.830kDa

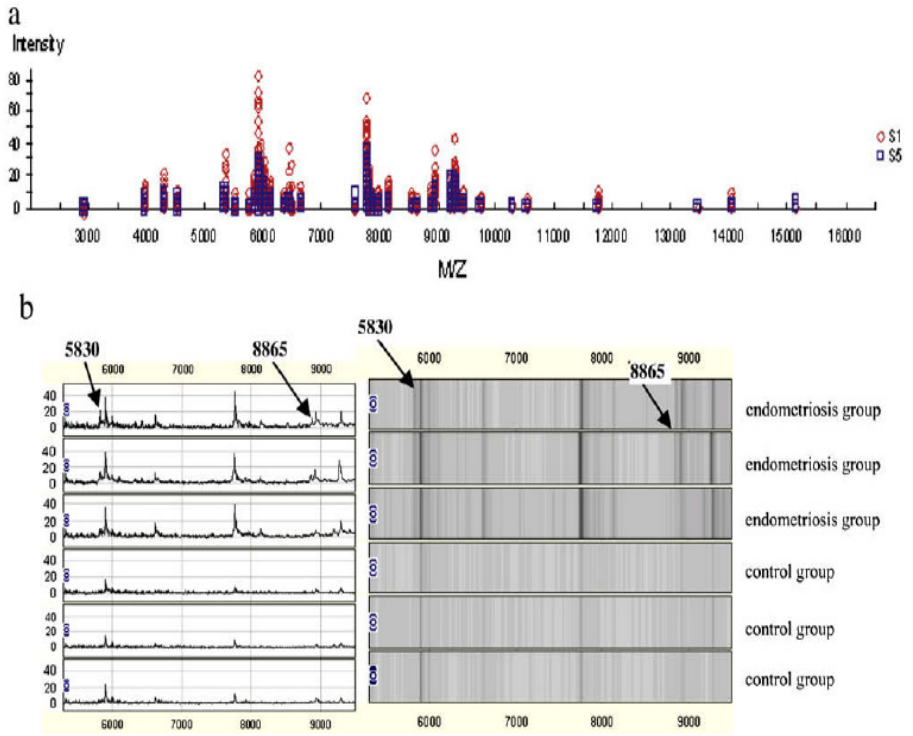
Control vs Endometriosis

86.7% Sensitivity

96.8% Specificity

Jing *et al.*, 2008

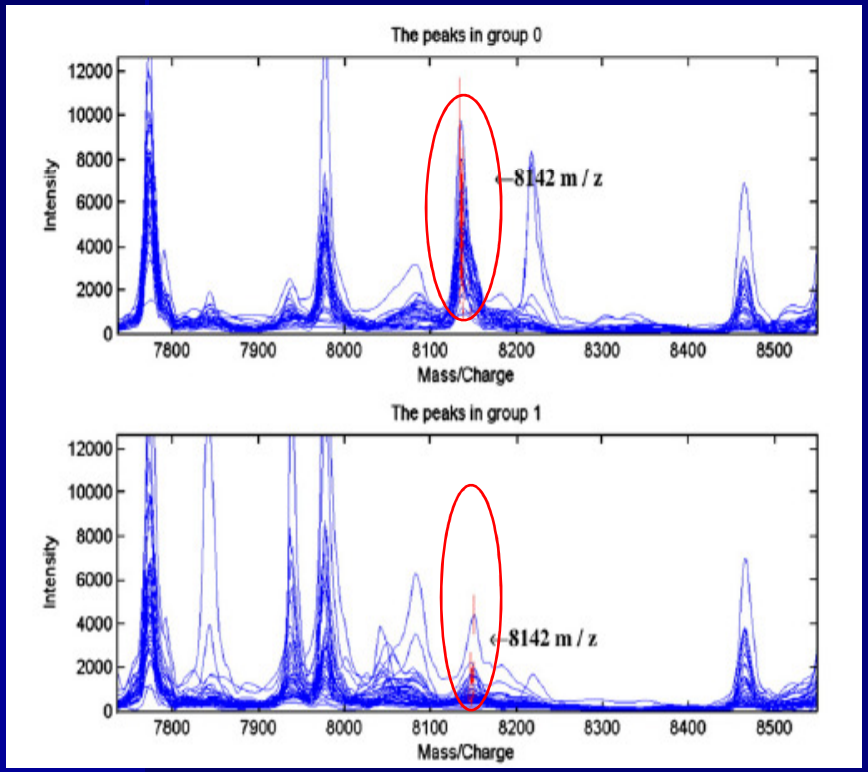
(**a**) Peak intensity of proteins analyzed with Biomarker Wizard software. Diamond (S1) represents group of endometriosis patients, square (S5) represents control group. Peaks of 34 of 98 proteins were significantly different between the two groups. (**b**) Serum proteomic pattern in endometriosis and control samples with mass spectra (*left*) and gel view (*right*) of SELDI analysis. Two serum proteins were screened and selected as potential biomarkers of endometriosis by SELDI-TOF-MS.

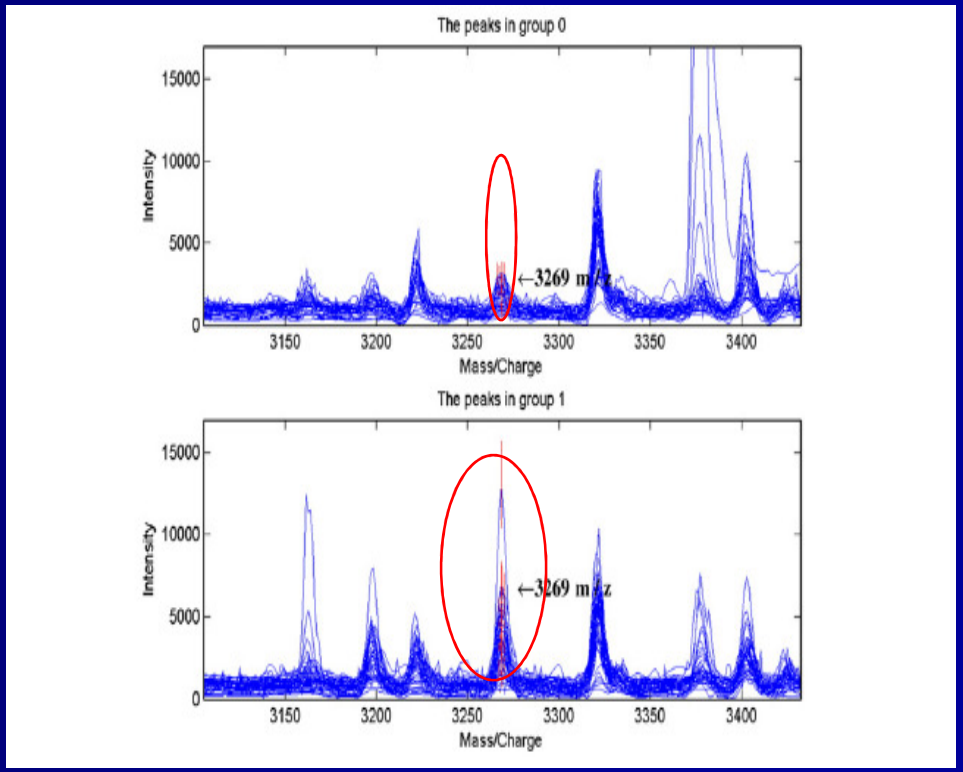


Jing. Biomarkers for EM using SELDI-TOF-MS. Fertil Steril 2008.

SELDI-TOF-MS platform in endometriosis

Controls	Endometriosis
20	24





Wang *et al.*, 2008

SELDI-TOF-MS platform in endometriosis

Table 1 Statistics for the candidate biomarkers

m/z peak	Serum sample group		*P* value
	Endometriosis	Validation	
↓8141	889.19 ± 496.94	3967.17 ± 1772.43	0.0000000000
5640↑	15237.24 ± 5224.35	7158.66 ± 1993.55	0.0000000000
5847↑	1175.55 ± 354.05	589.67 ± 143.16	0.0000000000
3269↑	3992.22 ± 1969.59	1810.39 ± 755.51	0.0000000375
↓8940	1504.19 ± 585.06	4403.19 ± 2416.99	0.0000000004

Values are given as mean ± SD.

Controls	Endometriosis
20	24

Table 2 Cross-validated results for endometriosis and validation samples

Sample group	Total, no.	Detected as endometriosis samples, no.	Detected as validation samples, no.	Predictive value, %
Endometriosis	12	11	1	91.7 (sensitivity)
Validation	10	1	9	90.0 (specificity)
Total	22	12	10	90.9 (positive value)

Wang *et al.*, 2008

Evaluation of protein expression in endometriotic lesion and endometrium

HYPOTHESIS

➢Test the feasibility of SELDI-TOF in women with and without endometriosis

Specific objectives

❖ Investigate differential protein expression in women with endometriosis compared to controls

❖ Investigate differential protein expression in paired peritoneum compared to endometriotic lesion in women with endometriosis

❖To identify selected mass peak

EXPERIMENTAL DESIGN

Patients (n=9) luteal phase (day 20 –22)

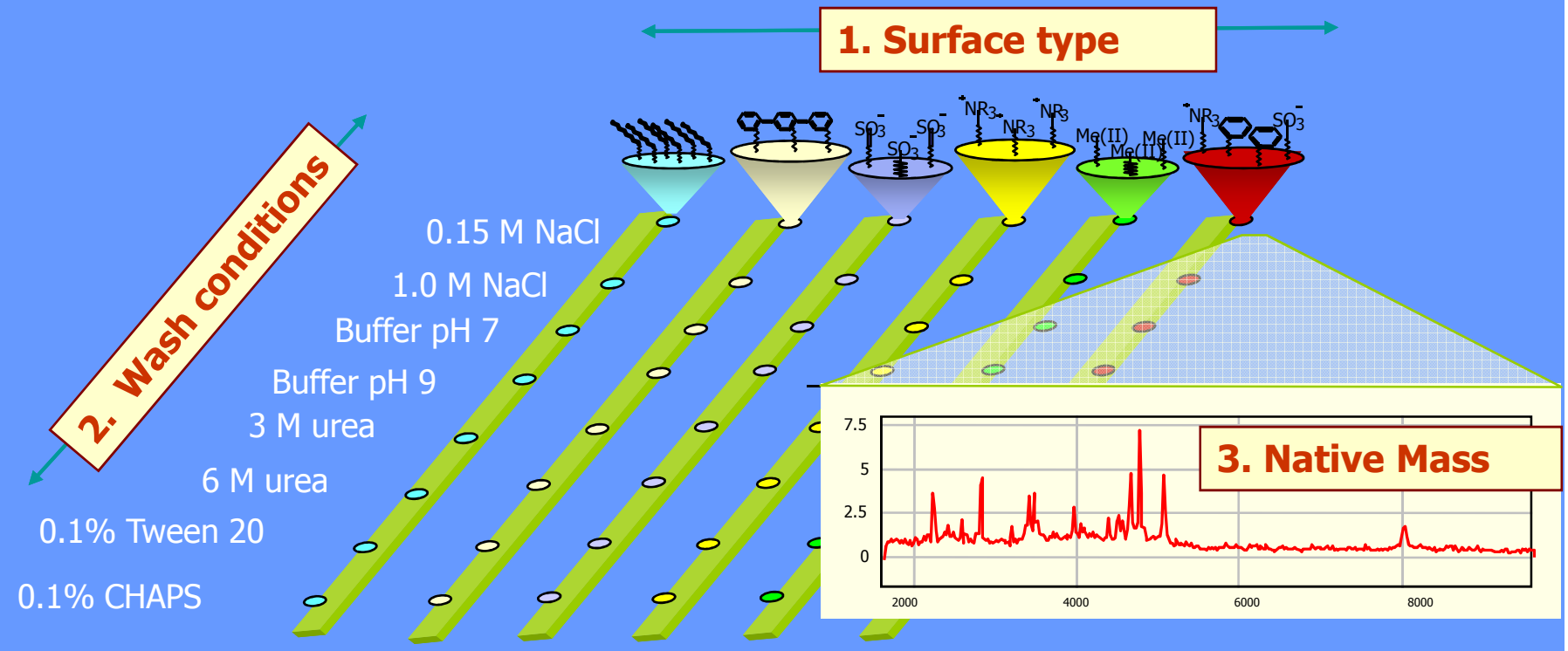
Comparison between tissue samples

a) endometrial biopsy samples (n= 3) endometriosis vs endometrial biopsy (n= 3) controls

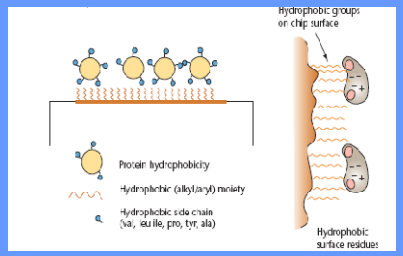
b) endometriotic lesion samples (n= 3) vs normal peritoneal biopsy (n= 3) from women with endometriosis

- All samples were collected during surgery intervention, and were stored as such, without preceeding washes with isotonic solution to remove blood
- Snap frozen in liquid nitrogen
- Stored at -80°C

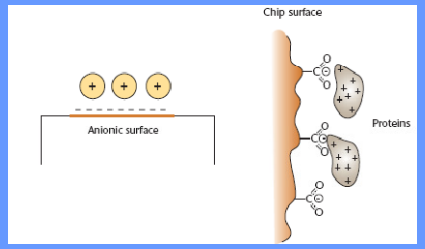
ProteinChip Arrays for Biomarker Discovery



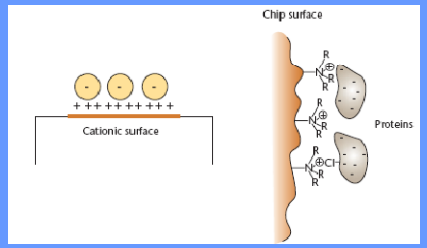
ProteinChip Surfaces



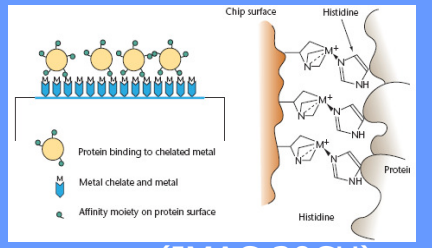
(H50-Hydrophobic)



(CM10- Anionic surface{-})



(Q10- Cationic surface{+})



(IMAC-30CU)

Expression **Difference** Mapping Using Chromatography MS

Step 1: Complex protein sample is placed on a ProteinChip Array

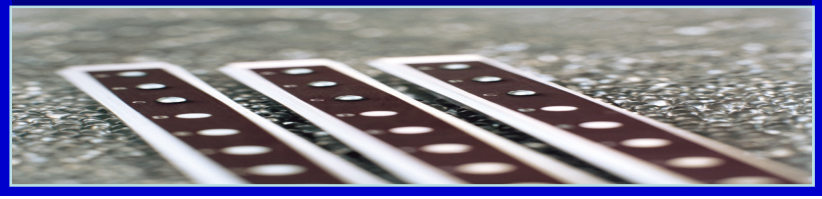
- Affinity **Capture** – Proteins bind to chemical or biological sites on the ProteinChip surface

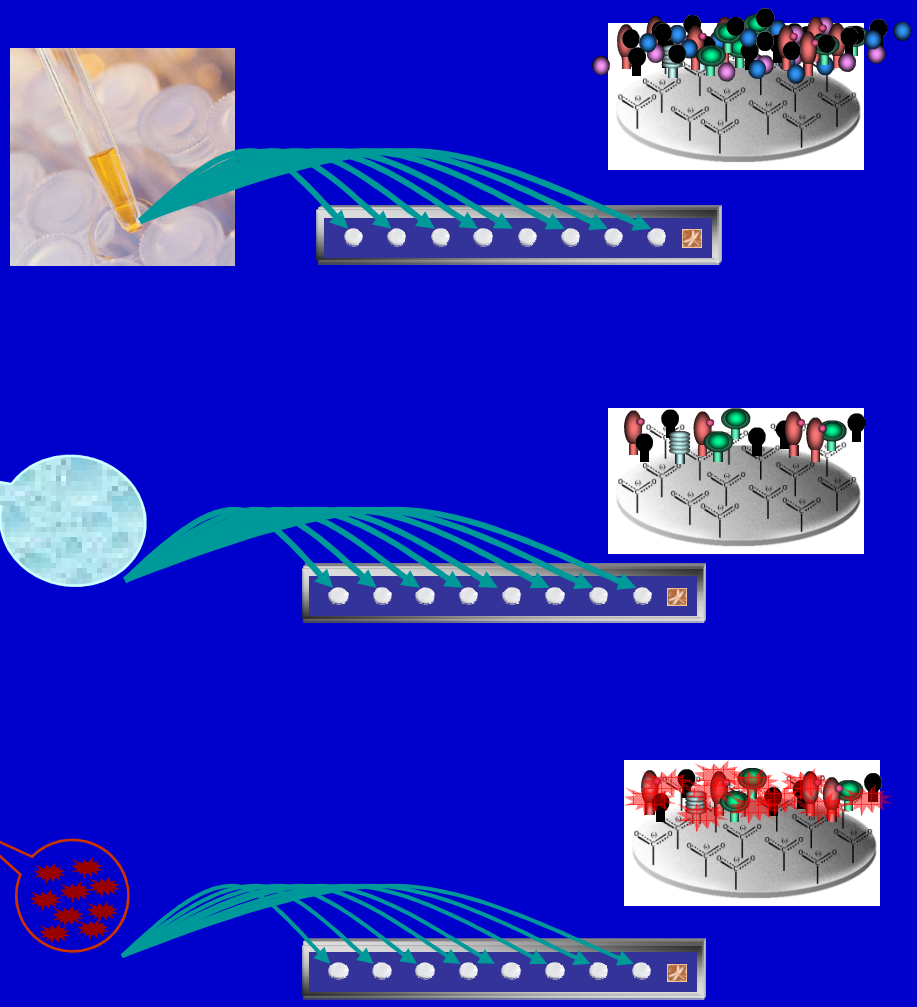
Step 2: Remove unbound proteins

- **Wash** the ProteinChip with appropriate stringency buffer
- Bound proteins are retained

Step 3: Add Energy Absorbing Molecules or "Matrix"

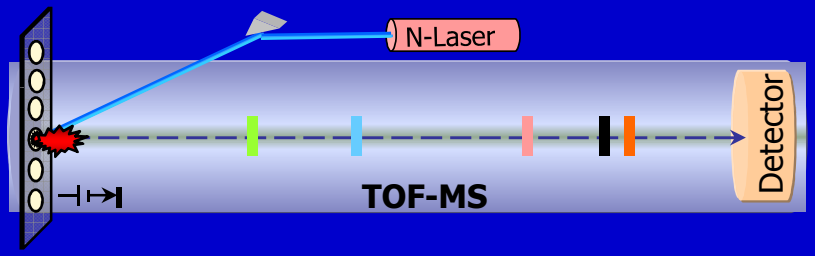
- **EAM** is applied to each spot to facilitate desorption and ionization in the TOF-MS Chip Reader.





ProteinChip Technology: PCS4000 TOF MS Detector

- Retained proteins are "eluted" from chip by Surface Enhanced Laser Desorption and Ionization (SELDI)



- Ionized proteins detected and mass accurately determined by Time-of-Flight Mass Spectrometry (TOF MS)

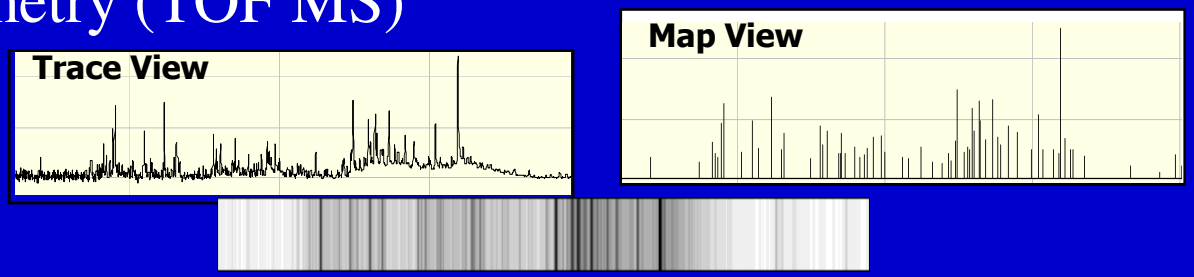


TABLE 1

Mean signal intensities of various peptides comparing endometrium of women with a normal pelvis vs. endometriosis and peritoneal biopsy vs. endometriotic lesion of women with endometriosis.

Analysis condition		MW	Mean signal intensity	Mean signal intensity	Ratio mean signal intensity
CM10, pH 4	CHCA	6,755	0.4 (PB)	3.9 (EL)	13
CM10, pH 4	CHCA	6,513[a]	10.1 (PB)	4.4 (EL)	2.4
CM10, pH 9	SPA high	20,436	0.2 (PB)	0.8 (EL)	7.8
CM10, pH 9	SPA high	20,626	0.1 (PB)	0.9 (EL)	12.7
CM10, pH 9	SPA high	20,821	0.1 (PB)	0.7 (EL)	7.7
CM10, pH 9	SPA high	22,521	0.2 (PB)	1.3 (EL)	11.1
CM10, pH 9	SPA high	22,827	0.1 (PB)	2.2 (EL)	31
CM10, pH 9	SPA high	23,014	0.1 (PB)	1.9 (EL)	26.5
CM10, pH 9	SPA high	23,211	0.1 (PB)	1 (EL)	19.2
CM10, pH 9	SPA high	18,425	0.3 (PB)	1.2 (EL)	5.5
CM10, pH 9	SPA high	18,615	0.3 (PB)	0.8 (EL)	3.6
CM10, pH 9	SPA high	96,212	0.2 (PB)	0.3 (EL)	2.4
H50	CHCA	6,318	0.6 (PB)	1.5 (EL)	2.9
H50	CHCA	6,440	0.7 (PB)	2.8 (EL)	4.6
H50	CHCA	6,630	0.5 (PB)	2 (EL)	4.1
IMAC30-CU	CHCA	6,760	2.7 (PB)	3.6 (EL)	1.4
IMAC30-CU	CHCA	6,513	3.7 (PB)	5.7 (EL)	1.6
IMAC30-CU	SPA high	20,436	0.1 (PB)	0.8 (EL)	19.7
IMAC30-CU	SPA high	20,619	0.1 (PB)	0.9 (EL)	26.5
Q10, pH 9.0	CHCA	3,175	7.5 (PB)	29.6 (EL)	4
Q10, pH 9.0	CHCA	4,867	2.5 (PB)	9.1 (EL)	3.8
Q10, pH 9.0	CHCA	4,926	1.7 (PB)	10.5 (EL)	6.3
Q10, pH 9.0	CHCA	4,970	2.2 (PB)	14.8 (EL)	7.1
Q10, pH 9.0	SPA high	16,800	8.4 (PB)	14.8 (EL)	1.8
IMAC30-CU	CHCA	2,948[a]	15 (C)	1.4 (Endo)	11.4
IMAC30-CU	SPA low	3,555[a]	15.3 (C)	1.2 (Endo)	13.7
IMAC30-CU	CHCA	3,011[a]	5.1 (C)	0.3 (Endo)	24.5
IMAC30-CU	SPA low	3,024[a]	5.9 (C)	2 (Endo)	3
H50	CHCA	3,638[a]	2 (C)	0.6 (Endo)	3.7
H50	CHCA	3,622[a]	3.4 (C)	0.8 (Endo)	4.8
IMAC30-CU	SPA low	3,002[a]	6.5 (C)	1.3 (Endo)	5.2
IMAC30-CU	SPA low	8,355[a]	4.2 (C)	0.8 (Endo)	5.5
IMAC30-CU	SPA low	3,809[a]	9.2 (C)	1.7 (Endo)	5.6
IMAC30-CU	CHCA	2,886[a]	29.1 (C)	4.8 (Endo)	6.2
IMAC30-CU	CHCA	3,623[a]	4.8 (C)	0.8 (Endo)	6.2
IMAC30-CU	SPA low	5,568[a]	15.5 (C)	2.5 (Endo)	6.4
IMAC30-CU	SPA low	2,816[a]	51.5 (C)	7.8 (Endo)	6.7
IMAC30-CU	SPA low	2,799[a]	18.3 (C)	2.7 (Endo)	6.9
IMAC30-CU	SPA low	4,718[a]	6.9 (C)	1 (Endo)	6.9
IMAC30-CU	SPA low	5,132[a]	7.8 (C)	1.2 (Endo)	7
IMAC30-CU	CHCA	2,872[a]	8.8 (C)	1.1 (Endo)	8.8

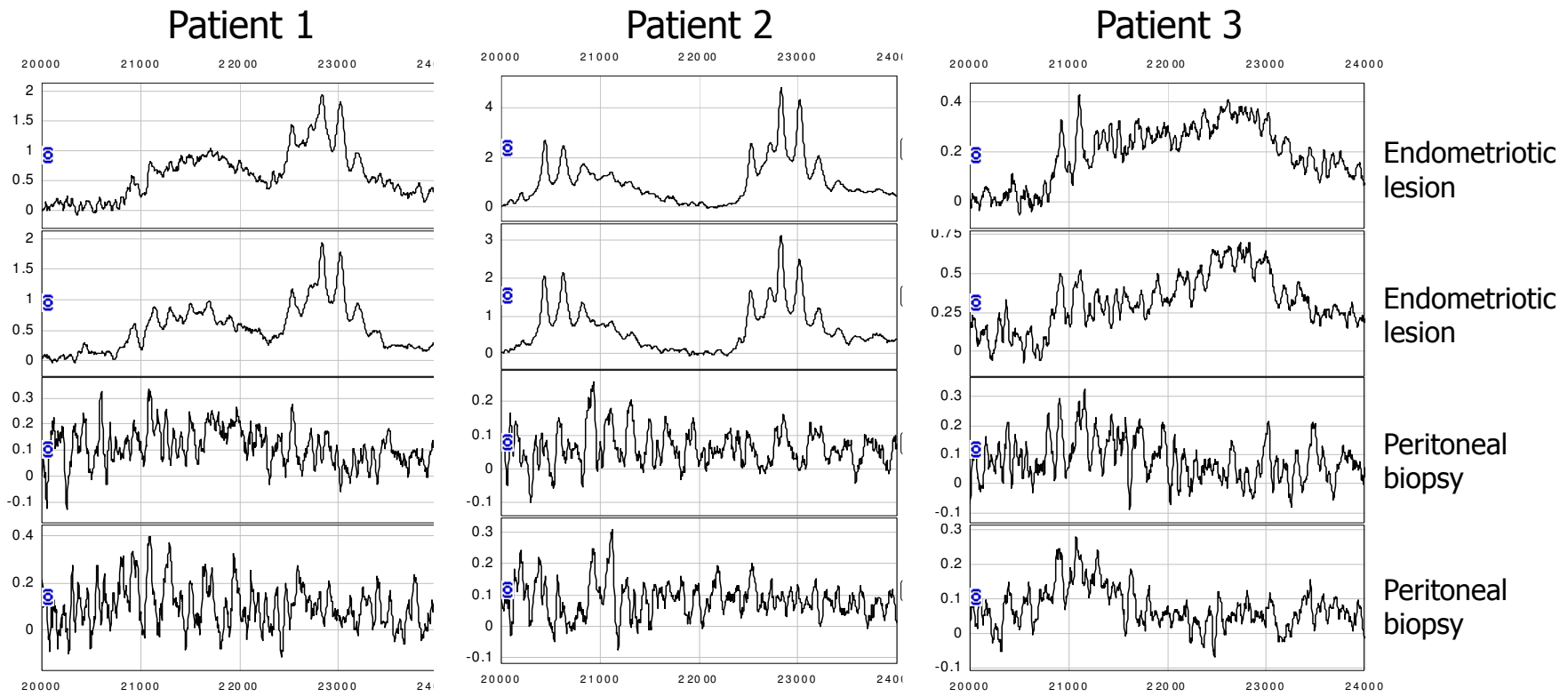
Note: PB = peritoneal biopsy; EL = endometriotic lesion; C = a normal pelvis (Controls); Endo = endometriosis. The two columns under "Analysis condition" serve the following roles: the first column represents the type of ProteinChip surface used and the second column represents the type of energy absorbing molecule (EAM) used. The horizontal line within the table separates the results of the two comparisons, i.e. C vs. Endo, and PB vs. EL.

[a] Indicates down-regulated proteins and peptides, whereas the rest were up-regulated.

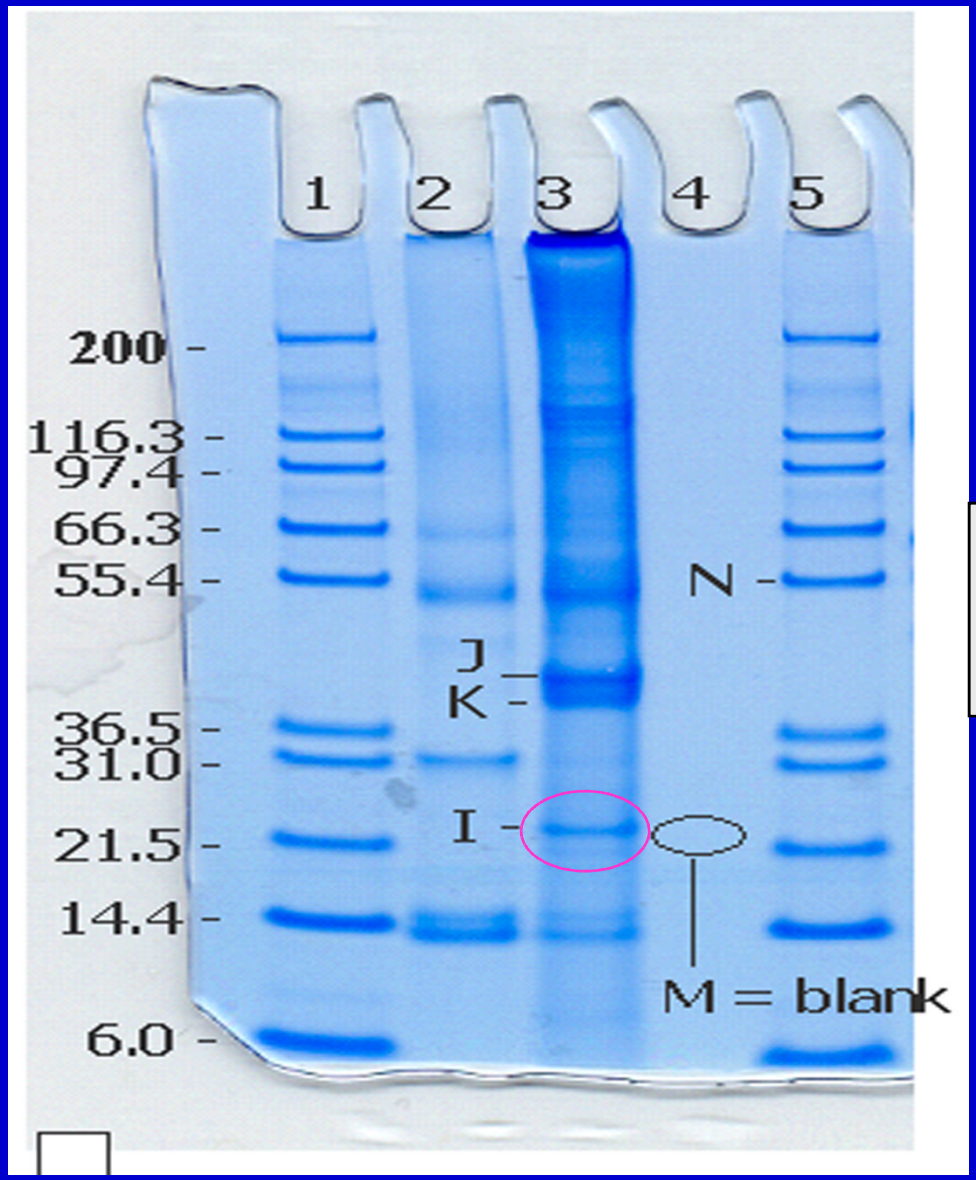
Kyama. SELDI-TOF MS in endometriosis reaction. Fertil Steril 2006.

Upregulation of 22 - 23 kDa molecules cluster in endometriotic lesions

Cation exchange CM10 surface, binding buffer pH 9.0, EAM = sinapinic acid



Identification of 22-23kDa in endometriotic lesion lysates



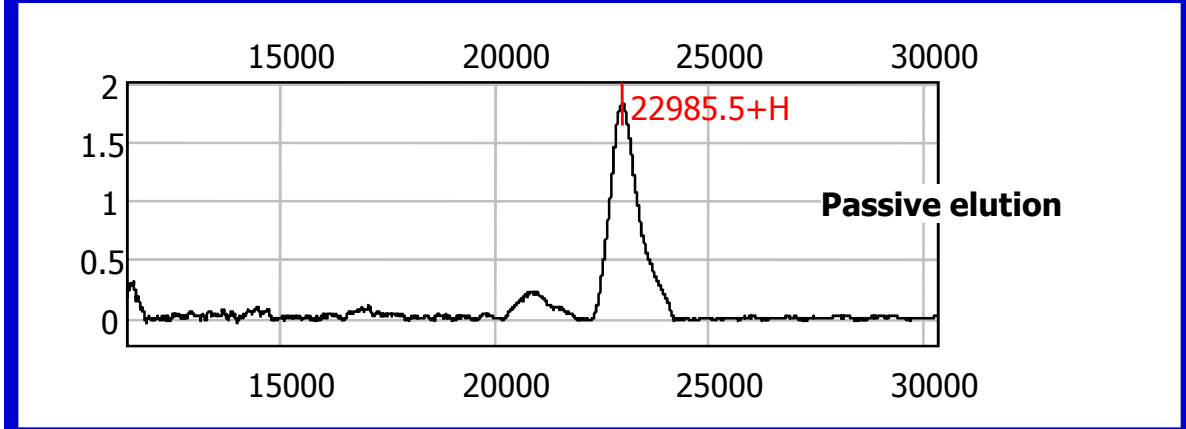
NuPage 4 -12% bis-tris, run with MES buffer
Lane 1and 5 contains Mark12 MW marker. Lane 2 contains the patient 2 Peritoneal Biopsy lysate, Lane 3 is loaded with Endometriotic Lesion biopsy of the same affected woman.

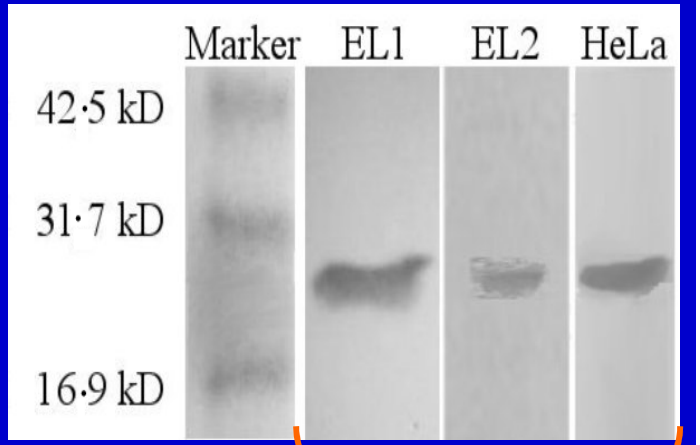
Protein in Band I identified by peptide mapping on PBSIIc and confirmed by passive elution: Transgelin, smooth muscle actin-binding protein

After 2 hours of trypsin digest matched upto;

Sequence coverage = 82 %

Est'd Z value = 2.27





Kyama *et al.*, Fertil Steril. 2006 ; 86: 203 - 209

SUMMARY OF RESULTS

Women with endometriosis vs controls

- ↓ 2.8kDa – 12.3kDa peptide/proteins in endometrium

Endometriotic lesion vs normal peritoneum

- ↑ 3.175kDa - 96kDa & ↓ 6.513kDa peptide/proteins

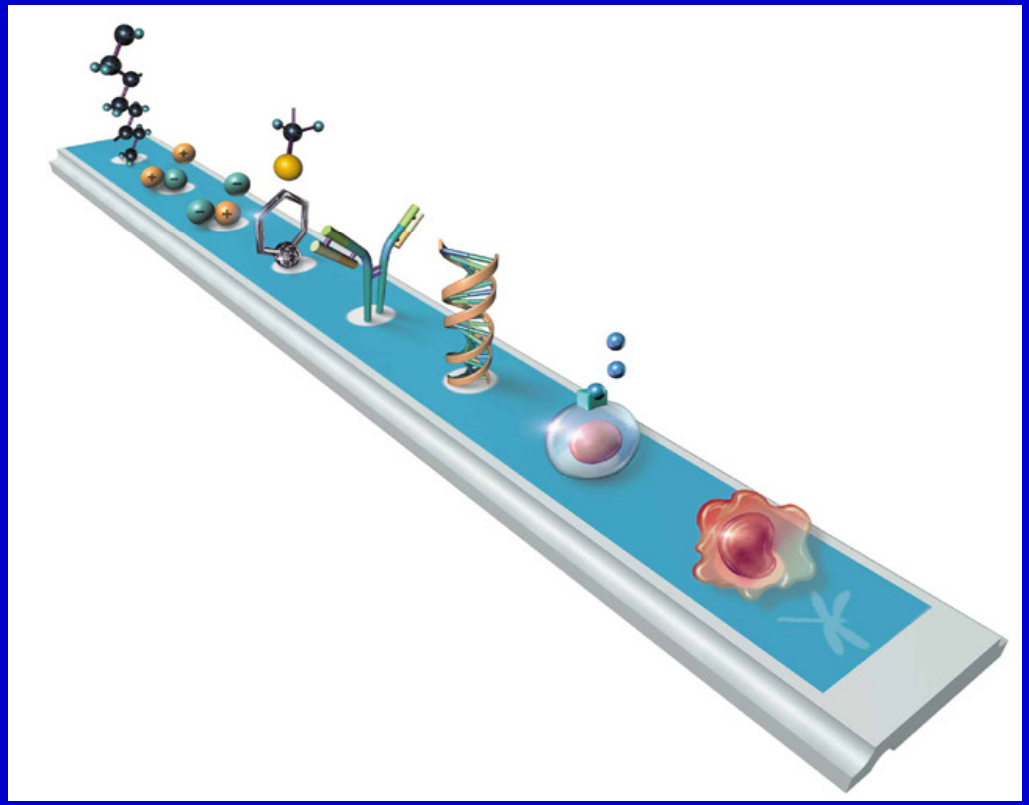
- Transgelin remarkably upregulated in endometriotic lesions

CONCLUSION

ProteinChip technology is a promising method to distinguish protein expression in disease and control tissues

Kyama *et al.*, Fertil Steril. 2006 ; 86: 203 - 209

Evaluation of endometrial biomarkers for semi-invasive diagnosis of endometriosis



HYPOTHESIS

- Women with endometriosis express specific proteins or peptides in secretory eutopic endometrium compared to controls
- Women with endometriosis express specific proteins or peptides in secretory eutopic endometrium at specific stages of the disease (minimal-mild and moderate-severe)

Specific objectives

❖ Investigate differential protein expression in women with endometriosis compared to controls

❖ To develop diagnostic models using leave-one-out - support vector machine algorithm and logistic regression classification models

❖ To identify selected mass peak as potential biomarkers

EXPERIMENTAL DESIGN

Study population

	Stage I-II	Stage III-IV	Total
Cases	9	10	19
Controls	---	---	10
			29

Cycle phase

Secretory	**Day 16 – 26**

Sample processing

- Endometrial biopsy collected during surgery, snap frozen in liquid nitrogen and stored at -80 ℃

Search for biomarkers

- Global protein profiling

SUMMARY OF RESULTS

Data analysis:

Ciphergen's ProteinChip Software v3.1.1. **P<0.05**

	Qualified Mass Peaks ↓↑ regulation
Controls vs Stage I-II	30
Controls vs Stage III -IV	131
Controls vs endometriosis	73

1.923kDa – 133.8kDa

Diagnostic models

-Support Vector Machine (SVM) algorithm

- logistic regression classification models with Leave-One-Out – Cross Validation (LOO – CV)

- Ranking the significant mass peaks according to their classification power

Leave-One-Out –Cross Validation (LOO – CV)

Support Vector Machine (SVM)

Logistic model: Logistic ridge regression model: LS-SVM ranking Control versus Endometriosis

all selected features	features with odds ratio>2	average merit	std	average rank	std	M/Z	↑↓ regulation
10124,69Da	13777,09Da	70,793	2,325	3,2	2,32	11115,70Da	↑ Endo
8649,53Da	8649,53Da	69,034	6,365	5	6,37	8649,53Da	↓ Endo
11136,52Da	8659,24Da	67,379	4,366	6,6	4,37	1949,42Da	↓ Endo
11115,70Da	11072,13Da	66,517	7,541	7,5	7,54	13777,09Da	↓ Endo
1922,08Da	9645,82Da	66,207	2,34	7,8	2,34	8396,61Da	↑ Endo
8659,24Da	8171,33Dda	65,862	4,812	8,1	4,81	10124,69Da	↑ Endo
9645,82Daa	13907,42Da	65,724	4,912	8,3	4,91	5827,94Da	↓ Endo
5185,89Da	13784,25Da	65,276	3,433	8,7	3,43	8659,24Da	↓ Endo
13907,42Da	10743,37Da	64,414	5,568	9,6	5,57	10460,21Da	↑ Endo
8396,61Da	14262,32Da	64,138	5,063	9,9	5,06	13907,42Da	↓ Endo
14262,32Da	13814,75Da	62,103	6,593	11,9	6,59	13784,25Da	↓ Endo
12179,19Da	6323,97Da	61,897	7,265	12,1	7,27	16981,17Da	↑ Endo
5182,56Da	12179,19Da	61,241	4,561	12,8	4,56	5182,56Da	↓ Endo
1949,42Da	5182,56Da	60	7,259	14	7,26	9449,72Da	↑ Endo
	1949,42Da	59,069	7,781	14,9	7,78	14262,32Da	↓ Endo
	33373,35Da	57,621	2,219	16,4	2,22	14272,24Da	↓ Endo
		57,069	6,416	16,9	6,42	11136,52Da	↑ Endo
		54,862	8,792	19,1	8,79	8359,33Da	↑ Endo
		54,414	5,075	19,6	5,08	12547,33Da	↓ Endo
		53,379	3,943	20,6	3,94	1922,08Da	↑ Endo

Selected biomarker combination in women with endometriosis compared with controls during luteal phase

	Potential endometrial biomarkers	Sensitivity	95% Confidence Interval		Specificity	95% Confidence Interval	
			Lower Limit	Upper Limit		Lower Limit	Upper Limit
Control vs endometriosis	↓8.650 kDa, 8.659 kDa, 13.910 kDa, ↓5.183 kDa & 1.949 kDa	89.5%	0.654618	0.981555	90%	0.541155	0.994758
Control vs Stage I-II	↑90.675 kDa & 35.950 kDa, ↓1.924 kDa & 2.504 kDa	100%	0.628811	1	100%	0.655464	1
Control vs Stage III-IV	↑10.110 kDa ↓5.828 kDa, 12.172 kDa & 4.279 kDa	80%	0.442182	0.964573	70%	0.353671	0.919052

CONCLUSIONS

– SELDI-TOF –MS ProteinChip technology combined with bioinformatics analysis tools:

-help develop a diagnostic model test with a high sensitivity especially for minimal to mild endometriosis

– Confirmation of these data in a larger and independent patient population is needed

GENERAL CONCLUSIONS

– **Proteomic technology combined with bioinformatics tools:**

- help develop a diagnostic model test with a high sensitivity and specificity especially for minimal to mild endometriosis

– Proteomic technology in endometriosis may offer novel therapeutic targets

New breakthrough will need:

–Innovative technology

–Multidisciplinary approach

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