Endometrial biomarkers for endometriosis

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Nairobi, Kenya
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2nd Biomarker Meeting in Personalized Reproductive Medicine,
Valencia, Spain, April 12th, 2014
Disclosure:

- Co-Chair WHO Infertility Guidelines Development Group Steering Committee
- Board Member European Endometriosis Liga (EEL)
- Council Member Society Gynecol Investigation (SGI)
- Research Associate and Chair International Advisory Board, Institute of Primate Research, Kenya (WHO-CC)
- Grants from Merck Serono, Ferring, MSD, Besins, WERF
- Consultancy/KOL for Merck Serono, Ferring, MSD, Bayer, Abbott, Abbvie, Preglem, Gedeon Richter, Pharmaplex, Uteron Pharma, Roche, Proteomika
Leuven University, Belgium: founded 1425; 2000 beds
Center of excellence in endometriosis framework for long term multi-disciplinary patient management

Gynecological General Bowel Bladder Lung

Surgeons

Reproductive endocrinologists IVF ICSI IUI

Immunologists

Psychologists/counsellors

Nurses

WOMAN and GYNECOLOGIST
the decision making team

Patient support groups

Complementary therapies

Physiotherapy Massage Acupuncture Stress mgmt Exercise

Pain management

Telephone Online Meetings Literature Onsite support

D'Hooghe and Hummelshoj, Hum Reprod 2006;21(11):2743-8
Leuven University
Endometriosis Center of Expertise

Clinical Leuven

GYN
T D’Hooghe
C Meuleman
D De Neubourg
C. Tomassetti
K Peeraer
D. Timmerman
(US imaging)

S Pelckmans
P De Loecker
L. Meeuwis

URO
B. VCleynenbreugel

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A D’Hoore
A Wolthuis

Postdocs Leuven
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A. Fasbender (12)

PhD Students Leuven-Nairobi
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A Nyachieo (10)

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A Vodolazkaia (12)
C Meuleman (11)
E. Dancet (12)
O. D. (16)
D. Peterse (16)
C. Tomassetti (15)

PhD Students
Leuven – int’ntl
A Bokor (Budapest, 11)
H Falconer (Karolinska, 08)

Research Nairobi
J Mwenda
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A. Nyachieo
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E Omolo
H Saibulu

Veterinary staff
Animal attendants

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International collaborators
H. Taylor (Yale, USA)
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A. Sharkey
(Cambridge, UK)
J. Kremer (Nijmegen, NL)
F. Vilmos
(Budapest, HUN)
G. Montgomery
(Brisbane, AUS)
EU Network for Endometriosis (ENE)
World Endometriosis Research Foundation (WERF)
• Clinical presentations

Peritoneal endometriosis
Clinical presentations
Ovarian endometriotic cyst (endometrioma)
• Clinical presentations
Deep infiltrating endometriosis (DIE)
Endometriosis

- EM (glands/stroma) outside uterus

- Prevalence
  - 7% of reproductive age women (Treloar et al, 1999)
  - up to 50% patients with pelvic pain/infertility

- **Endo cost => cost for Crohn in USA and EU**
  (2002: 22 billion USD, Simoens et al., 2007)
  (2008: 10,000 Euro/pt: 1/3 direct and 2/3 indirect, Simoens et al, 2012)

- Diagnosis: laparoscopy (+ histology) → diagnostic delay

- Estrogen dependent
  - rare before menarche or after menopause

- Progressive
  - >50% women/baboons after 1-2 years

- Retrograde menstruation/Sampson Hypothesis - 1927
Learning objectives

At the conclusion of this presentation, participants should understand:

1. Pathogenesis of endometriosis: increased glycoproteins, adhesion and inflammation as target for endometriosis biomarkers
2. Which women would benefit from a noninvasive test for endometriosis.
3. Peripheral blood biomarkers
4. Endometrial biomarkers: microarray/proteomics
5. Endometrial biomarkers: nerve fibers
Biomarkers of endometriosis

Amelie Fassbender, Ph.D., a Alexandra Vodolazkaia, Ph.D., M.D., a Philippa Saunders, Ph.D., b Dan Lebovic, M.D., c Etienne Waelkens, Ph.D., M.D., d Bart De Moor, Ph.D., e and Thomas D’Hooghe, Ph.D., M.D. a,f,g

a Department of Development and Regeneration, Sexual, Pelvic, Reproductive, and Family Studies, University Hospital Gasthuisberg, Leuven, Belgium; b MRC Centre for Reproductive Health, Queen’s Medical Research Institute, Edinburgh, Scotland; c Department of Obstetrics and Gynecology, University of Wisconsin-Madison, Middleton, Wisconsin; d Department of Cellular and Molecular Medicine, Campus Gasthuisberg, Leuven, Belgium; e Department of Electrical Engineering (ESAT-SCD), Katholieke Universiteit Leuven, Leuven, Belgium; f Leuven University Fertility Centre, Department of Obstetrics and Gynaecology, University Hospital Gasthuisberg, Leuven, Belgium; and g Division of Reproductive Biology, Institute of Primate Research, Karen, Nairobi, Kenya
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Principal theories

• Retrograde menstruation (Sampson, 1927)
• Endometrial stem cell implantation (Gargett et al, MHR, 2014)
• Metaplasia theory (Iwanoff., 1898, Meyer., 1903)
• Induction theory (Levander and Normann., 1955)
• Immunological dysfunction (Matarese et al., 2003)
• Environmental influences (Rier and Foster., 2002)
• Genetic predisposition (Montgomery et al., 2008)
• Lymphatic or vascular distribution (Halban., 1924)
• Embryonic rests theory (Von Recklinghausen., 1896, Russell., 1899)
Genetics of endometriosis

• Familial aggregation in humans and NHPs (Moen et al, 1993; Stefansson et al, 2002; Nyholt et al, 2012)
• Complex genetic etiology
• Genetic factors contribute about half of the variance in endometriosis risk, with estimate of heridability of about 50%
• Robust assoc. with 7 risk loci including WNT4 (development femal repro tract); CDKN2B-AS1 (encodes 3 tumor suppressor proteins) and GREB1 (ER pathway) (Nyholt et al, 2012)
Mouse model
Pathogenesis of Endometriosis

Retrograde menstruation

Menstrual effluent

Induction of metaplastic change (Merrill, 1966) (Kyama et al, 2014)

Endometriosis

Cellular (pellet part)

Acellular supernatant
Early Development of endometriosis in baboons

Development of endo lesions after IP injection of menstrual endometrium is characterised by a specific time-dependent histological process

- Histologically, massive necrosis (D1-3) was followed by establishment of endo lesions by D 6-9
Menstrual cell debris

Increased inflammatory activity
- Activated macrophages (Halme et al., 1987)
  - ↑ IL-1, IL-6, TNF-α & IL-8 (Cao et al., 2004)

Reduced immune surveillance (Lebovic et al., 2001)
- Impaired natural killer (NK) cell cytotoxicity
  - ↑ sICAM-1 & abnormal apoptosis

↑ Adhesion and invasion (Matarese et al., 2003)
  - ↑ ICAM-1, TGF-β, HGF, TNF-α & IL-8, MMPs

Aromatase expression
(Bukulmez et al., 2008)

↑ Angiogenesis (Groothuis et al., 2005)
  - VEGF, IL-8
Intraperitoneal heme

- PF:
  Increased RBC conc during menses (Bokor et al, 2009)
  Increased Hgb in endo > co (Langendonckt, 2002)
- IP release of Hgb, heme, iron may activate:
  - cell adhesion molecules
  - cytokine production
  - cell proliferation
  - neovascularization
  - Oxidative stress
PF endometrial cells and endometriosis: does retrograde menstruation of EM cells exist?

- **PREVALENCE OF PF EM CELLS**
  - During menses (Reti et al, 1983): 24% (50% DII-III) NOT UNIVERSAL
  - During other phases of the cycle, most studies: 0-19% (23-67% after hysteroscopy or uterotubal flushing)

- **PROBLEMS WITH STUDY DESIGN:**
  - ? Cycle phase
  - ? Adequate PF cell preparation (cytospin vs cytoblock)
  - ? Adequate definition of morphology
  - ? Adequate immunohistochemical markers identifying EM epithelial, EM stromal, mesothelial cells and WBCs
  - Endometrial-peritoneal adhesions occurs within 24 hours (Witz et al, 2000)
Experimental in vivo data: positive correlation between weight of EM tissue used for intrapelvic seeding and extent of endometriosis in baboons (D’Hooghe et al, 1995)

Experimental in vitro data: EM fragments with intact microstructure express several adhesion molecules and adhere better to amniotic epithelium (van der Linden et al, 1995) and invade ECM earlier (Wild et al, 1994) than isolated or single EM cells.

Endometrial stem cell hypothesis (Gargett et al, MHR, 2014): EM epithelial progenitor cells and EM mesenchymal stem cells:
- clonogenic, highly proliferative, selfrenewal in vitro, differentiation in vivo
- Hx: increased in EM and PF of women with endometriosis
Epidemiology: increased risk for endometriosis if
- short cycle length (Cramer et al, 1986; Arumugam and Lin, 1997) or
  longer menstrual flow (Cramer et al, 1986; Vercellini et al, 1997)
- if obstructed menstrual outflow: endometriosis in
  . 66% (Olive and Henderson, 1987) or 77%
  (Pinsonneault and Goldstein, 1985) of women
  . 3/3 baboons (D’Hooghe et al, 1994)
QUANTITY OF PF EM CELLS (3):
CUMULATIVE RETROGRADE MENSTRUATION

• BABOON MODEL FOR ENDOOMETRIOSIS
• Increased duration of captivity --> increased prevalence of endometriosis (D’Hooghe et al, 1996a)
• Spontaneous endometriosis is a progressive disease when followed by laparoscopies every 6 months during 2 years (D’Hooghe et al, 1996b)
• Baboons with an initially normal pelvis develop in 64% histologically proven minimal endometriosis after 32 months as assessed by laparoscopies every 6 months (D’Hooghe et al, 1996c)
Endometrial alterations in endometriosis: a systematic review of putative biomarkers

K.E. May, J. Villar, S. Kirtley, S.H. Kennedy, and C.M. Becker

Cytokines
Immunology
Steroids and Hormones
Growth Factors
Cell Adhesion and Extracellular Matrix
Tissue Remodelling
Angiogenesis
Apoptosis and Cell Cycle Control
Reactive Oxygen and Nitrogen Species
Genetic Studies
Proteomics
Histology
Other
Menstrual Effluent and Uterine Fluid
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)
Inflammation →
local intralesional E2 production
(Noble et al, 1996)

IL1 beta → COX-2 → PG-E2 → aromatase → E2 → VEGF → VEGF

+ ER-beta overexpression/ER-alpha underexpression →
P resistance
+ increased oxidative stress due to increase in ROS (Reactive Oxygen Species) production by endometriotic cells and PF WBCs + decreased activity of antioxidant enzymes

Inflammation → activation of 2 pathways:
- MAPK/ERK (mitogen-activated protein kinase) and
- PI3K/AKT (phosphoinositolide -3 kinase)
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 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
- Other biomarker candidates: HSP-90-beta; annexin A2, Annexin 5; glycodelin; Apo A1; transgelin
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TVU in the diagnosis of endometriosis

• First-line imaging technique
• Adequate diagnostic method to detect ovarian endometriotic cysts (ESHRE guidelines, 2005 and 2013)
• Does not rule out peritoneal endometriosis or endometriosis-associated adhesions
• A limited role in diagnosis of uterosacral, vaginal and rectovaginal deep pelvic endometriosis (Bazot et al., 2004; 2009)
TVU / MRI in diagnosis of Deep Infiltrating Endometriosis (Bazot et al., 2009)

<table>
<thead>
<tr>
<th>Location of DIE</th>
<th>TVS Sensitiv/Specif</th>
<th>MRI Sensitiv/Specif</th>
</tr>
</thead>
<tbody>
<tr>
<td>USLs</td>
<td>78.3% / 66.7%</td>
<td>84.4% / 88.9%</td>
</tr>
<tr>
<td>Vagina</td>
<td>46.7% / 95%</td>
<td>80% / 85.5%</td>
</tr>
<tr>
<td>RV septum</td>
<td>9% / 98.7%</td>
<td>54.5% / 98.7%</td>
</tr>
<tr>
<td>Intestine</td>
<td>93.6% / 100%</td>
<td>87.3% / 93.1%</td>
</tr>
</tbody>
</table>
NIH Definition biomarker

• A characteristic that is
  - objectively measured and
  - evaluated as an indicator of
    - a normal biologic process,
    - a pathogenic process, or
    - a pharmacologic responses to a therapeutic intervention

(Woodcock, 2010)
Possible biomarker application in endometriosis

- **Early diagnosis** in symptomatic patients (pain, infertility)
- **Identification of individuals at risk** for disease prevention (adolescents with therapy resistant pelvic pain?)
- Potential **drug target**
- Potential **marker for response** after endometriosis surgery or medical treatment
- **Monitoring recurrence** or progression
- **Identification of clinically relevant subpopulations** with different etiologies, or with different susceptibility to treatment
Pitfalls of biomarker development (Palmer and Barnhart, 2013)

• Lack of standardization regarding tissue collection, storage, clinical phenotyping,..
• Degradation of biomarker during collection, transport, storage, marker instability
• Assay imprecision
• Bias in selected subjects for study
• Association only present in subgroups
• Confounding variables (age, ethnicity, comorbidities)
• Marker does not precede disease
World Endometriosis Research Foundation

- Endometriosis Phenome and Biobanking Harmonization Project (EPHECT)
- 4 papers submitted in 2014 for publication
  - Clinical phenome
  - Surgical data
  - Body fluid collection
  - Body tissue collection
Ideal non-invasive test for endometriosis: for whom?

• Symptomatic patients with subfertility and/or pain and without US evidence of endometriosis
• Identify patients who might benefit from a laparoscopic surgery for endometriosis or for other causes of subfertility or pain that can be treated surgically (D’Hooghe et al, 2006)
• 100% sensitivity, even if specificity only 50%
• Do not miss patients with endometriosis, since surgery may double their MFR and improve their pain (ESHRE Guidelines 2005 and 2013)
Non-invasive or semi-invasive test

- Noninvasive: urine, saliva,
- Minimally invasive: blood, ultrasound
- Semi-invasive: endometrial biopsy
- Methods of analysis:
  - Known biomarkers: ELISA, multiplex ELISA,…
  - New biomarkers: mRNA microarray, miRNA, proteomics, metabolomics
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1. Pathogenesis of endometriosis: increased glycoproteins, adhesion and inflammation as target for endometriosis biomarkers
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3. **Peripheral blood biomarkers**
4. Endometrial biomarkers: microarray/proteomics
5. Endometrial biomarkers: nerve fibers
Non-invasive blood test
The current state-of-the-art

• Promising studies (May et al, 2010), but:
  ➢ Not carried out in an independent validation set
  ➢ Mostly focused on single biomarkers
  ➢ Lack of multivariate statistical approach

• No reliable non-invasive test available for the diagnosis of endometriosis:
  CA-125, CA-19-9 (low sensitivity)
## Diagnosis of endometriosis
### Panel of BM

<table>
<thead>
<tr>
<th>Predictive model</th>
<th>Study population</th>
<th>Study design</th>
<th>Validation phase</th>
<th>Results</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-125, EM leucocytes, length of menses</td>
<td>Controls: 195 Stage I-IV: 173</td>
<td>Luteal Phase, Laparoscopy, Log. Regression model.</td>
<td>Internal by bootstrapping</td>
<td>61% / 95%</td>
<td>Gagne et al., 2003</td>
</tr>
<tr>
<td>Ca-125, CA 19-9, IL-6</td>
<td>Controls: 35 Stage I-IV: 45</td>
<td>All phases, Laparoscopy, Univariate</td>
<td>N/A</td>
<td>38% /80% (one of BM+)</td>
<td>Somigliana et al., 2004</td>
</tr>
<tr>
<td>CCR1mRNA, CA-125, MCP-1</td>
<td>Controls: 49 Stage I-IV: 102</td>
<td>Follicular phase, Laparoscopy, Univariate</td>
<td>N/A</td>
<td>92.2% /81.6% (one of BM+)</td>
<td>Agic et al., 2008</td>
</tr>
<tr>
<td>CA-125, MCP-1, leptin, MIF</td>
<td>Controls: 78 Stage II-IV: 63</td>
<td>Follic/Unknown, Laparoscopy, Classification tree analysis</td>
<td>Internal self-validation procedures</td>
<td>100% / 40%</td>
<td>Seeber et al., 2008</td>
</tr>
<tr>
<td>IL-6, IL-8, CA-125, hsCRP, TNF-α, CA 19-9</td>
<td>Controls: 93 Stage I-IV: 201</td>
<td>Menst/Follic/Lut, Laparoscopy, Multivariate LR/LSSVM</td>
<td>N/A</td>
<td>Stage I-II: 87-92% / 60-71% Stage III-IV: 100% / 84%</td>
<td>Mihalyi et al., 2010</td>
</tr>
</tbody>
</table>
Evaluation of a panel of 28 biomarkers for the non-invasive diagnosis of endometriosis

A. Vodolazkaia¹,², Y. El-Aalamat³,⁴, D. Popovic³,⁴, A. Mihalyi¹,², X. Bossuyt⁵, C.M. Kyama¹,²,⁶, A. Fassbender¹,², A. Bokor¹,²,⁷, D. Schols⁸, D. Huskens⁸, C. Meuleman¹, K. Peeraer¹, C. Tomassetti¹, O. Gevaert³,⁴, E. Waelkens⁹,¹⁰, A. Kasran¹¹, B. De Moor³,⁴, and T.M. D’Hooghe¹,¹²,*
Hypothesis and Methods (Vodolazkaia et al, 2012)

• Non-invasive test with high sensitivity (80%) for women with US-negative endometriosis based on panel of selected plasma biomarkers

• High sensitivity (80% or more) required
  - to avoid false negatives
  - in order not to miss any symptomatic women with endometriosis
  - who might benefit from surgery for endometriosis-associated infertility or pain (ESHRE guidelines, 2005 and 2013)
NOVELTY (Vodolazkaia et al, 2012)

- Large sample size (n=296)
- Symptomatic patients without US evidence of endo
- Laparoscopy confirmed (cases, n=175) or excluded (controls, n=121) endometriosis
- Large panel of tested biomarkers (n=28)
- Validation design: independent training/test sets
- Based on QUADAS criteria (Quality Assessment of Diagnostic Accuracy Studies) (Whiting et al., 2003; May et al., 2010):
  - Different Cycle Phases
  - Controls with endometriosis associated symptoms, but laparoscopically nl pelvis
<table>
<thead>
<tr>
<th></th>
<th>Question</th>
<th>Answer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Were patients and controls recruited from women with symptoms consistent with endometriosis?</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>Were selection criteria clearly described? Did the study describe time frame, consecutive recruitment, inclusion/exclusion criteria?</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>Was the time period between the diagnosis and biomarker test short enough to avoid a change in disease status?</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>Were controls surgically verified (not to have endometriosis)?</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Were the methods for testing sufficiently explained?</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>Were the biomarker test results interpreted in a blinded fashion?</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>Was the diagnosis of endometriosis made without knowledge of the biomarker test results?</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>Were uninterpretable/intermediate test results reported?</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>Were withdrawals from the study explained?</td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>Were samples collected at a consistent phase of the cycle, or results corrected for cycle phase?</td>
<td>Yes</td>
</tr>
<tr>
<td>11</td>
<td>Were samples collected from women with a particular stage(s) of disease, or results corrected for stage?</td>
<td>Yes</td>
</tr>
</tbody>
</table>
## Study population: Training set

<table>
<thead>
<tr>
<th>Phase of cycle</th>
<th>Control</th>
<th>Stage I-II</th>
<th>Stage III-IV</th>
<th>Total per phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual</td>
<td>17</td>
<td>20</td>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>Follicular</td>
<td>30</td>
<td>42</td>
<td>8</td>
<td>80</td>
</tr>
<tr>
<td>Luteal</td>
<td>34</td>
<td>37</td>
<td>7</td>
<td>78</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>81</strong></td>
<td><strong>99</strong></td>
<td><strong>18</strong></td>
<td><strong>198</strong></td>
</tr>
</tbody>
</table>
Study population: **Test set**

<table>
<thead>
<tr>
<th>Phase of cycle</th>
<th>Control</th>
<th>Stage I-II</th>
<th>Stage III-IV</th>
<th>Total per phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual</td>
<td>10</td>
<td>12</td>
<td>5</td>
<td>27</td>
</tr>
<tr>
<td>Follicular</td>
<td>16</td>
<td>19</td>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>Luteal</td>
<td>14</td>
<td>16</td>
<td>3</td>
<td>33</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>40</strong></td>
<td><strong>47</strong></td>
<td><strong>11</strong></td>
<td><strong>98</strong></td>
</tr>
</tbody>
</table>
Overview of selected 28 biomarkers (literature search)

<table>
<thead>
<tr>
<th>Group</th>
<th>Biomarkers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoprotein markers</td>
<td>CA-125, CA 19-9; Follistatin</td>
<td>Mol et al., 1998; Matalliotakis et al., 1998; Agic et al., 2008; Kurdoglu et al., 2009; Florio et al., 2009</td>
</tr>
<tr>
<td>Inflammatory markers</td>
<td>IL-1beta, IL-6, IL-8, IL-17, IL-21, RANTES, TNF-alpha, IFN-gamma, MCP-1, MIF, CRP, OPN</td>
<td>Pizzo et al., 2002; Mihalyi et al., 2008; 2010; Khorram et al., 1993; Abrao et al., 1997; Morin et al., 2005</td>
</tr>
<tr>
<td>Non-inflammatory markers</td>
<td>IL-4, IL-10, Annexin V</td>
<td>Antsiferova et al, 2005, Kyama et al., 2011</td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>sICAM-1, VCAM-1</td>
<td>Barrier and Sharpe-Timms, 2002</td>
</tr>
<tr>
<td>Angiogenic and Growth factors</td>
<td>VEGF, NGF, FGF-2, Leptin, IGF-BP3, glycodelin (PP-14), M-CSF, HGF</td>
<td>Matalliotakis et al., 2003; Kim et al, 2000; Telimaa et al., 1989; Zong et al., 2003</td>
</tr>
</tbody>
</table>
Multivariate statistical analysis

- Multivariate logistic regression (MLR)
- Least Squares Support Vector Machines (LS-SVM)
- Diagnostic performance of a panel of biomarkers:
  - Selection of the diagnostic model based on the highest AUC (training set)
  - Validation of selected model on an independent test set (Robin et al., 2009)
Multivariate analysis
Selected Models for prediction of US-negative endometriosis

<table>
<thead>
<tr>
<th></th>
<th>Cycle phase</th>
<th>AUC Train. set</th>
<th>AUC Test set</th>
<th>Sensit /Specif Training set</th>
<th>Sensit /Specif Test set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin V, VEGF, CA-125, sICAM-1,</td>
<td>Menstr</td>
<td>0.79</td>
<td>0.79</td>
<td>81% / 77%</td>
<td>82% / 75%</td>
</tr>
<tr>
<td>Multivariate Logistic regression</td>
<td>Menstr</td>
<td>0.86</td>
<td>0.80</td>
<td>86% / 68%</td>
<td>82% / 75%</td>
</tr>
<tr>
<td>LSSVM</td>
<td>Menstr</td>
<td>0.86</td>
<td>0.80</td>
<td>86% / 68%</td>
<td>82% / 75%</td>
</tr>
</tbody>
</table>
## Multivariate analysis

### Selected Models for prediction of US-negative endometriosis

<table>
<thead>
<tr>
<th>Annexin V, VEGF, CA-125, Glycodelin</th>
<th>Cycle phase</th>
<th>AUC Train. set</th>
<th>AUC Test set</th>
<th>Sensit / Specif Training set</th>
<th>Sensit / Specif Test set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multivariate Logistic regression</td>
<td>Menstr</td>
<td>0.81</td>
<td>0.78</td>
<td>81% / 81%</td>
<td>82% / 75%</td>
</tr>
<tr>
<td>LSSVM</td>
<td>Menstr</td>
<td>0.85</td>
<td>0.84</td>
<td>90% / 68%</td>
<td>82% / 62%</td>
</tr>
</tbody>
</table>
Conclusions (Vodolazkaia et al, 2012)

- Important step in the development of a higher sensitivity non-invasive test for US-negative endometriosis
- 4-Biomarker panel during menstrual phase has better diagnostic performance than any single BM: (Annexin V, VEGF, CA-125, sICAM-1/ glycodelin)
- Sensitivity of 81%-90%
- Specificity of 62-81%
- Confirmed in an independent test set but extra validation in preoperative patients needed (prospectively collected data set (LUFC → Multicenter)
- Extra value of additional biomarkers?
- Importance of non-inflammatory markers?
Proteomics

The study of the protein library
Why Proteomic Analysis in endometriosis research?

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
What is Protein Chip SELDI Technology

Retentate Chromatography + Mass Spectrometry

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

H50/H4 hydrophobic

CM10- Anionic surface

Q10- Cationic surface

IMAC-30-Metal affinity surface
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.
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
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 (Hb in EM; Albumin and IgG in plasma): experimental
• Less resolution if MW >20kDa
• Expensive
• Protein/Peptide Identification: extra step
Future studies SELDI TOF MS (Fassbender et al, 2013)

<table>
<thead>
<tr>
<th>Assay improvement</th>
<th>Sample Population</th>
<th>Protein/peptide Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>• More chip surfaces</td>
<td>• Large sample size</td>
<td>• MALDI-TOF/TOF MS</td>
</tr>
<tr>
<td>• Intra- and Interassay variability</td>
<td>• Control for cycle phase</td>
<td>• Confirmation tests using ELISA, IH, Western Blots,..</td>
</tr>
<tr>
<td>• Use and validation of depletion methods</td>
<td>• Need for training and test set (validation in mono- and multicenter context)</td>
<td>• Development of novel markers (?) nonID profiles) as possible diagnostic test</td>
</tr>
<tr>
<td>• Need for standardization of technique</td>
<td>• Advanced bio-informatics</td>
<td></td>
</tr>
</tbody>
</table>
Proteomics Analysis of Plasma for Early Diagnosis of Endometriosis

Amelie Fassbender, PhD, Etienne Waelkens, MD, PhD, Nico Verbeeck, MSc, Cleophas M. Kyama, PhD, Attila Bokor, MD, PhD, Alexandra Vodolazkaia, MD, Raf Van de Plas, PhD, Christel Meuleman, MD, PhD, Karen Peeraer, MD, Carla Tomassetti, MD, Olivier Gevaert, PhD, Fabian Ojeda, PhD, Bart De Moor, PhD, and Thomas D’Hooghe, MD, PhD
## Sample distribution

<table>
<thead>
<tr>
<th>PATIENTS</th>
<th>Cycle phase</th>
<th>Controls</th>
<th>Disease</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stage I-II</td>
<td>Stage III-IV</td>
</tr>
<tr>
<td></td>
<td>Menstrual</td>
<td>23</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Luteal</td>
<td>33</td>
<td>33</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Follicular</td>
<td>33</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>89</td>
<td>89</td>
<td>76</td>
</tr>
</tbody>
</table>
Blood proteome

<table>
<thead>
<tr>
<th>Albumin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td></td>
</tr>
<tr>
<td>α2-macroglobulin</td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td></td>
</tr>
<tr>
<td>α1-AT</td>
<td></td>
</tr>
<tr>
<td>C3 Comp</td>
<td>10%</td>
</tr>
</tbody>
</table>

Deep Proteome
Large number of Low abundance proteins

| Apolipo-A1       |     |
| Apolipo-B        |     |
| AGP              |     |
| Lipoprotein A    |     |
| Factor H         |     |
| Ceruloplasmin    |     |
| C4-Complt        |     |
| Compl.Factor B   |     |
| Pre-albumin      |     |
| C9-Complt        |     |
| C19-Complt       |     |
| C8-Complt        |     |

10%
Depletion by proteominer
## Results

<table>
<thead>
<tr>
<th>Groups</th>
<th>Potential plasma biomarkers (m/z)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual phase Stage I-II vs. control</td>
<td>↓4898, ↓5715, ↓8328, ↑9926, ↑14698</td>
<td>75%</td>
<td>86%</td>
<td>83.6%</td>
<td>78.3%</td>
</tr>
<tr>
<td>Luteal phase Stage III-IV vs. controls</td>
<td>↑3192, ↑4315, ↑2189, ↑4373, ↓7457</td>
<td>98%</td>
<td>81%</td>
<td>74.4%</td>
<td>98.6%</td>
</tr>
<tr>
<td>Ultrasound negative CM10SPA data Menstrual phase Stage I-IV vs. controls</td>
<td>↑2058, ↑2455, ↑3883, ↑14694, ↑42065</td>
<td>88%</td>
<td>84%</td>
<td>75.2%</td>
<td>92%</td>
</tr>
</tbody>
</table>

2189 m/z identified as fibrinogen beta chain peptide
Conclusion (Fassbender et al, 2012)

• ↓fibrinogen beta chain in PB due to high consumption of fibrinogen beta chain?

• ↑production of fibrin in the peritoneal fluid
  – facilitating adhesion
  – attachment of endometrial fragments
Learning objectives

At the conclusion of this presentation, participants should understand:

1. Pathogenesis of endometriosis: increased glycoproteins, adhesion and inflammation as target for endometriosis biomarkers
2. Which women would benefit from a noninvasive test for endometriosis.
3. Peripheral blood biomarkers
4. **Endometrial biomarkers:** microarray/proteomics
5. Endometrial biomarkers: nerve fibers
Semi-invasive diagnosis of endometriosis via endometrial biomarkers

- Transcervical endometrial biopsy
- Outpatient clinic
- Pipelle/Novak

- ? Dependent on cycle phase
- ? Dependent on biopsy technique
Endometrial alterations in endometriosis: a systematic review of putative biomarkers

K.E. May, J. Villar, S. Kirtley, S.H. Kennedy, and C.M. Becker

Cytokines
Immunology
Steroids and Hormones
Growth Factors
Cell Adhesion and Extracellular Matrix
Tissue Remodelling
Angiogenesis
Apoptosis and Cell Cycle Control
Reactive Oxygen and Nitrogen Species
Genetic Studies
Proteomics
Histology
Other
Menstrual Effluent and Uterine Fluid
Important considerations

• EM Compartment: whole EM, epithelial, glandular, stromal
• Cycle phase: menstrual, proliferative, secretory
• Endometriosis stage (ASRM)
• Sample size
• Technology used: IH, mRNA, ELISA,
• Reproducibility
Abbreviation code per paper, as used in following slides

- +: increased endo > co
- -: decreased endo < co
- =: similar endo = co
- Red: at least 2 confirmatory studies
- ND: not detectable endo and/or co
- M menstrual; P proliferative;
  S secretory phase EM
EM in women with endometriosis vs controls
(May et al 2011)

- **INFLAMM. CYTOKINES:**
  IL-1 R type II: -, -, -, -,-,- (6); IL-8: +, +, - (endothelium)

- **OXIDATIVE STRESS AND COX-2:** COX-2: + (P, epith), + (S, Stroma), =, + (P)

- **IMMUNOLOGY:** Endometrial IgG: +, +?, =

- **STEROIDS: PRO LOCAL E2 PRODUCTION**
  Aromatase : +, +, +, +, =,ND, ND, ND

- **TISSUE REMODELLING:**
  MMP-2: =, +, +, +, +; MMP3: +, +, +, +, =;
  MMP9: =, +, +, +, +; Urokinase: +, +, + (S)

- **ANGIOGENESIS:**
  VEGF: + gland/ = stroma(S), + (S), + (S), + (S), = (S), S, S, S, S
  VEGF receptor -1 and -2: - (stroma)/- (Gland, S), - (stroma)/- (gland, S), =
  Angiopoetin-1: +, + (S); Angiopoetin-2: + (S), +(S)
  Microvessel density: =, + (stroma), + (S), + (S)
EM in women with endometriosis vs controls (May et al 2011)

• APOPTOSIS AND CELL CYCLE CONTROL:
  **LESS APOPTOSIS, MORE PROLIFERATION**
  TUNEL stained N apoptotic cells: -, -, -, -, -, + (early S)/- (late S)
  Bcl-2 (anti-apoptosis): =, =, =, +, +, +
  Ki67 (prolif marker): =, =, =, +, +, +
  Telomerase activity (prolif marker): =, +, + (S), +(S)
  PCNA-1 (prolif marker): +, +, +, =
  Pak-1 (cell survival): +, + (S)
  Phosphorylated ERK1/2: (prolif marker) +, + (S)

• GLYCOPROTEINS: Menstrual fluid: CA125: +, +

EM DIFFERENCES: CAUSE OR EFFECT?
MOST LIKELY EFFECT BASED ON BABOON DATA (FAZLEABAS GROUP, 2013)
Guidelines for the design, analysis and interpretation of ‘omics’ data: focus on human endometrium

Signe Altmäe, Francisco J. Esteban, Anneli Stavreus-Evers, Carlos Simón, Linda Giudice, Bruce A. Lessey, Jose A. Horcajadas, Nick S. Macklon, Thomas D’Hooghe, Cristina Campoy, Bart C. Fauser, Lois A. Salamonsen, and Andres Salumets
### Table 1: Points to consider for adequate study design and ‘good-reporting-practice’ in studies of human endometrium.

**Experimental design**
- Set the study hypothesis
- Define study type (e.g. prospective, retrospective)
- Precisely define phenotype of participants
- Carefully select and describe controls
- Calculate sample size and power
- Provide adequate participant data (age, cycle characteristics, BMI, race/ethnicity, parity, obstetric and gynaecological history including family history of gynaecological complications/pathologies, hormonal profiles and other measured markers, medication including contraceptives)
- Assess endometrial phase (histology, biomarkers)
- Assess environmental exposure (tobacco, alcohol, drugs, nutritional status, socioeconomic status, education, psychological stress)
- Identify risk factors and possible confounders
- Design patient informed consent with the potential for possible implications

**Sample collection and preparation**
- Define and record sampling conditions (biopsy location, time)
- Provide detailed protocol for sample processing and storage
- Add biological duplicates for replication purposes (e.g. repeated sampling)
- Avoid pooling of samples
- Assess sample quality and quantity

**Sample analysis**
- Provide detailed protocol for ‘omics’ technology to be applied
- Consider technical duplicates
- Define statistical methods, databases to be utilized for data analysis

**Data validation**
- Validate results using alternative technologies (quantitative PCR, western blot, immunohistochemistry, \textit{in situ} hybridization, etc.)

**Data presentation**
- Upload raw ‘omics’ data and detailed sample/analysis data to public database (e.g. GEO, ArrayExpress)
- Address limitations/ strengths of the study
mRNA Microarray: 
Quantify gene expression in EM

www.affymetrix.com
Combined mRNA microarray and proteomic analysis of eutopic endometrium of women with and without endometriosis

A. Fassbender¹, N. Verbeeck²,₃,₄, D. Börnigen²,₄, C.M. Kyama¹,⁵, A. Bokor¹,⁶, A. Vodolazkaia¹, K. Peeraer¹, C. Tomassetti¹, C. Meuleman¹, O. Gevaert²,⁷, R. Van de Plas⁸, F. Ojeda²,₃, B. De Moor²,₄, Y. Moreau²,₄, E. Waelkens³,⁹, and T.M. D’Hooghe¹,¹⁰,*
# Microarray results

<table>
<thead>
<tr>
<th>Study</th>
<th>Stages</th>
<th>Cycle phases</th>
<th>Microarray platform</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microarray study n=49</td>
<td>•Stage I-II (n=16)</td>
<td>•Early luteal phase (n=27)</td>
<td>Affymetrix</td>
<td>No genes differentially expressed in disease vs</td>
</tr>
<tr>
<td></td>
<td>•Stage III-IV (n=15)</td>
<td>•Menstrual phase (n=22)</td>
<td></td>
<td>controls</td>
</tr>
<tr>
<td></td>
<td>•Control (n=18)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Microarray results

<table>
<thead>
<tr>
<th>Cycle phase</th>
<th>Control</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual vs. luteal</td>
<td>621↑</td>
<td>454↑</td>
</tr>
<tr>
<td></td>
<td>466↓</td>
<td>471↓</td>
</tr>
<tr>
<td>Total</td>
<td>1087</td>
<td>925</td>
</tr>
</tbody>
</table>

### UPREGULATED
- wound healing
- blood coagulation
- hemostasis
- chemotaxis
- extracellular matrix

### DOWNREGULATED
- carboxylic acid metabolic
- oxoacid metabolic
- cellular amino acid catabolic
<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample number</th>
<th>Cycle phase</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fassbender et al., 2012</td>
<td>n=49 minimal-mild (n=16) moderate-severe (n=15) control (n=18)</td>
<td>Early luteal phase (n=27) Menstrual phase (n=22)</td>
<td>Endo vs control No genes differentially expressed</td>
</tr>
<tr>
<td>Sherwin et al. 2008</td>
<td>n=16 eutopic EM minimal-mild (n=5) moderate-severe (n=5) controls (n=6)</td>
<td>Late luteal phase (day 23-26)</td>
<td>Endo vs control 8 genes upregulated &gt;1.75 fold (p&lt;0.001) and 1 gene down-regulated</td>
</tr>
<tr>
<td>Burney et al. 2007</td>
<td>n=37 moderate-severe (n=21) controls (n=16)</td>
<td>Follicular (n=6) (day 8-14) Early Luteal (n=6) (day 15-18) Mid luteal (n=9) (day 19-23) Late luteal phase (day 24-28)</td>
<td>Early luteal phase 87 transcripts were altered more than 4fold such as FOXO1A, MIG6, CYP26A1</td>
</tr>
<tr>
<td>Reference</td>
<td>Sample number</td>
<td>Cycle phase</td>
<td>Results Endo vs control</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Matsuzaki et al., 2005</td>
<td>n= 24 minimal-severe (n-12) controls (n=12)</td>
<td>Late follicular (n=6)</td>
<td>No gene was differentially expressed in a constant manner in eutopic endometrium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Early, mid, late luteal (n=18) (day not mentioned)</td>
<td></td>
</tr>
<tr>
<td>Absenger et al., 2004</td>
<td>Endometriosis (n=43) controls=48</td>
<td>Follicular or luteal phase (day not mentioned)</td>
<td>95&gt;1.5 fold  ↓64 ↑31 during luteal and follicular phase  ↑Cyr61 in the luteal phase</td>
</tr>
<tr>
<td>Kao et al. 2003</td>
<td>n=20 mild-moderate (n=8) control (n=12)</td>
<td>Mid luteal phase (n=20) (LH8 to 10, the LH surge)</td>
<td>↑91 and ↓115 more than 2 fold</td>
</tr>
</tbody>
</table>
Sherwin et al., 2008 (endo; late luteal phase)

• Fibronectin 1 (FN1 / 2335): upregulated
  • Role in cell adhesion
  • growth
  • migration
  • differentiation
  • wound healing
  • embryonic development
Burney et al., 2007 (endo; early luteal phase)

- FOXOA1: downregulated
  - ↓ endometriosis in early luteal phase
  - progesterone-regulated transcription factor
  - cell cycle control
  - role in the incomplete transitioning of the endometrium from the proliferative-to early luteal phase
Endometrial proteomic analysis

• Based on important biological differences in eutopic endometrium from women with and without endometriosis
• Aimed at identifying new proteins as biomarkers
• Proteomics by 2D–gel analysis
• Proteomics by SELDI-TOF analysis
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 $M_r$

(b)

(a) Decreasing pI
**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)
Identify differentially expressed proteins

2D Gel

Excise spot of interest, destain, digest, extract peptides

MALDI-TOF-MS

Spot onto surface and mass analyze

Search spectra against protein databases

Protein ID

MALDI TOF MS identification of proteins from 2DE

Identify differentially expressed proteins
# 2DE/MALDI TOF MS results in endometriosis research

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample Size</th>
<th>Technique</th>
<th>Results</th>
</tr>
</thead>
</table>
| 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                                                                                           |
<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample Size</th>
<th>Technique</th>
<th>Results</th>
</tr>
</thead>
</table>
| 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  
Validated proteins  
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 |
Proteomics by Protein Chip SELDI TOF

Retentate Chromatography + Mass Spectrometry

An extremely powerful tool for the HTP analysis of proteins and peptides
<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample Size</th>
<th>Surface</th>
<th>Results</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fassbender et al., 2010</td>
<td>eutopic EM Stage I-II (n=5) Stage III-IV (n=5) Controls (n=6)</td>
<td>CM10 CM</td>
<td>32 peaks differentially expressed proteins in EM endo versus controls No relation with same sample mRNA array data</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kyama et al., 2006</td>
<td>Stage II (n=3) Paired eutopic EM &amp; perit &amp; perit endo lesion Controls (n=3) Eutopic EM</td>
<td>CM10 CM</td>
<td><strong>Transgelin</strong> 22-23kDa Upregulated in ectopic EM when compared to normal peritoneum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kyama et al., 2010</td>
<td>Eutopic EM Stage I-II (n=9) + StIII-IV (n=10) Controls (n=10)</td>
<td>Q10 CM</td>
<td>•<strong>T-Plastin</strong> 90.675 •<strong>Annexin 5</strong> 39.956</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
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

• Also identified as one of the 5 relevant plasma biomarkers

(menstrual phase; Vodolazkaia et al, 2012)
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)
Combined mRNA microarray and proteomic analysis of eutopic endometrium of women with and without endometriosis

## Endometrium Proteomics Study (Fassbender et al, 2012)

<table>
<thead>
<tr>
<th>PATIENTS</th>
<th>Cycle phase</th>
<th>Controls</th>
<th>Disease</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stage I-II</td>
<td>Stage III-IV</td>
</tr>
<tr>
<td>Menstrual</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Early Luteal (18-21 days)</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>16</td>
<td>15</td>
<td>31</td>
</tr>
</tbody>
</table>
EM Proteomic analysis

<table>
<thead>
<tr>
<th>Groups (luteal phase)</th>
<th>Potential endometrium biomarkers (m/z)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMAC CHCA Stage I-II</td>
<td>↑2071; ↑2166; ↑2228; ↑3649; ↑40367</td>
<td>94%</td>
<td>100%</td>
<td>100%</td>
<td>93.5%</td>
</tr>
<tr>
<td>Stage III-IV</td>
<td>↓13552; ↑39889; ↑42108</td>
<td>92%</td>
<td>84%</td>
<td>70.8%</td>
<td>94.3%</td>
</tr>
<tr>
<td>IMAC CHCA Stage I-IV</td>
<td>↑2072; ↑2973; ↑3623; ↑3680; ↑21133</td>
<td>91%</td>
<td>81%</td>
<td>87.9%</td>
<td>84.8%</td>
</tr>
</tbody>
</table>

Basis for semi-invasive test for endo
Learning objectives

At the conclusion of this presentation, participants should understand:

1. Pathogenesis of endometriosis: increased glycoproteins, adhesion and inflammation as target for endometriosis biomarkers
2. Which women would benefit from a noninvasive test for endometriosis.
3. Peripheral blood biomarkers
4. Endometrial biomarkers: microarray/proteomics
5. Endometrial biomarkers: nerve fibers
Semi-invasive diagnosis
Nerve fibers on endometrial biopsy

- Fraser group (Australia)
  - Tokushige et al, 2006: proof of concept
  - AL Jefout et al, 2007: pilot trial
  - Al Jefout et al, 2009: double blind study

Endo (n=65), controls (n=35)

PGP 9.5 IHC

Sensitivity 83%/Specificity 98%

Caveat: technique of EM sampling/IHC!!!
- To test that small diameter nerve fibers are present in a density in endometrium from endo when compared to controls

- Difference can lead to development of a semi-invasive diagnostic method
POTENTIAL ENDOMETRIAL NEURAL MARKERS

Neural transmitters

- **SP**-substance P
- **VIP**-vasoactive intestinal polypeptide

Neural proteins

- **PGP9.5**-protein gene product 9.5
- **NF**-neurofilament protein
- **NPY**-neuropeptide Y
- **CGRP**-calcitonin gene-related protein
MATERIALS AND METHODS

- Secretory phase endometrium samples (n=40)
- Laparoscopically confirmed pelvic status
  - Endo=minimal–mild endometriosis (n=20)
  - Control= women with a normal pelvis (n=20)
- IHC to localise neural markers for sensory C, Aδ, adrenergic and cholinergic nerve fibers
POSITIVELY STAINED NF IN FUNCTIONAL LAYER OF ENDOMETRIUM
NF DENSITY IN ENDO VS CONTROLS

NF Density 14 x higher in endometrium from patients with minimal–mild endometriosis (1.96±2.73, nf/mm²±SD) when compared to women with a normal pelvis (0.14±0.46, p<0.0001).
ROC_LSSVM : 3 BEST FEATURES

LSSVM-least squares support vector machine

Top 3 AUC: PGP 9.5
SP
VIP

<table>
<thead>
<tr>
<th>Number of neural markers</th>
<th>AUC (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.56 (0.10)</td>
</tr>
<tr>
<td>2</td>
<td>0.84 (0.07)</td>
</tr>
<tr>
<td>3</td>
<td><strong>0.98 (0.02)</strong></td>
</tr>
<tr>
<td>4</td>
<td>0.94 (0.05)</td>
</tr>
<tr>
<td>5</td>
<td>0.96 (0.03)</td>
</tr>
<tr>
<td>6 (all)</td>
<td>0.94 (0.04)</td>
</tr>
</tbody>
</table>

AUC: Area under the ROC curve
SE: standard error
CONCLUSION

- Combined analysis of neural markers PGP9.5, VIP, SP could predict presence of minimal–mild endometriosis with 95% sensitivity, 100% specificity, 97.5% accuracy
- Need for validation in set up with 30% endo prevalence
Diagnosis of endometrial nerve fibers in women with endometriosis

Fatemeh Aghaey Meibody · Abolfazl Mehdizadeh Kashi · Ali Zare Mirzaie · Marjan Ghajarie Bani Amam · Afsane Shariati Behbahani · Bita Zolali · Laily Najafi
STUDY DESIGN

- Women with endometriosis \((n = 12)\)
- Without endometriosis \((n = 15)\)
- PGP 9.5 and NF (blinded assessment)

Nerve fibers were detected in all endometrial biopsies from all women with endometriosis but detected only in three women without endometriosis \((p < 0.001)\).

PGP 9.5 AUC = 0.961
Original Article

Is the Detection of Endometrial Nerve Fibers Useful in the Diagnosis of Endometriosis?

STUDY DESIGN

• 68 patients prospectively collected before laparoscopic inspection (Perth, Australia) (21 endo, 47 controls)
• Absent hormonal therapy: 17/21 endo, 27/47 co
• Metal Curette, multiple small EM tissue fragments, 
  “satisfactory” if at least on LPF “well oriented mucosa”
• Neural marker PGP 9.5
RESULTS

Endometrial functional layer nerve fibres:

- 15 (22%) biopsies overall
- 9/47 (19%) cases with histologically confirmed peritoneal endometriosis
- 6/21 (29% cases) without endometriosis

BUT:

- no subanalysis for patients without hormonal treatment, ? EM tissue quality
Expression of neuronal markers in the endometrium of women with and those without endometriosis

T.A. Newman\textsuperscript{1,\textdagger}, J.L. Bailey\textsuperscript{2,\textdagger}, L.J. Stocker\textsuperscript{2,3}, Y.L. Woo\textsuperscript{4}, N.S. Macklon\textsuperscript{2,3}, and Y.C. Cheong\textsuperscript{2,3,*}

\textbf{STUDY DESIGN, SIZE, DURATION:} This study included 45 women undergoing laparoscopic examination for the diagnosis of endometriosis. Endometrial samples were analysed by western blot for the expression of neuronal and neurotrophic markers, PGP9.5, VR\textsubscript{1} and NGFp75.

\textbf{PARTICIPANTS/MATERIALS, SETTINGS, METHODS:} Endometrial pipelle biopsies were obtained from patients with ($n = 20$, study group) and without ($n = 25$, control group) endometriosis. Tissue was analysed by immunohistochemistry and western blot analysis for the expression of pan-neuronal marker, PGP9.5, sensory nociceptive marker, TPVR\textsubscript{1}, and low-affinity neurotrophic growth factor receptor, NGFR\textsubscript{p75}.

\textbf{MAIN RESULTS AND THE ROLE OF CHANCE:} PGP9.5, NGFp75 and VR\textsubscript{1} were expressed in the endometrium of women, independent of the presence of endometriosis. Furthermore, the expression level of PGP9.5, VR\textsubscript{1} and NGFp75 did not alter between the two cohorts of women.
Combination of non-invasive and semi-invasive tests for diagnosis of minimal to mild endometriosis

Ibrahim Abd Elgafor el Sharkwy

**Table 3** Sensitivity, specificity, PPV, and NPV for Serum level of IL-6 alone and combined with endometrial biopsy

<table>
<thead>
<tr>
<th></th>
<th>Serum level of IL-6 (%)</th>
<th>Endometrial biopsy combined with Serum level of IL-6 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>89.5</td>
<td>100</td>
</tr>
<tr>
<td>Specificity</td>
<td>82.5</td>
<td>92.5</td>
</tr>
<tr>
<td>PPV</td>
<td>83</td>
<td>92.7</td>
</tr>
<tr>
<td>NPV</td>
<td>89</td>
<td>100</td>
</tr>
</tbody>
</table>
1. Pathologists and gynecologists considering this diagnostic approach should carefully consider the methodological factors that may influence its reliability.

2. Prospective study needed in patient population with 30% prevalence of endometriosis.

3. Important: Quality of EM tissue and IHC (background staining!)
General Conclusion: Biomarkers in endometriosis

Early diagnosis with high sensitivity in symptomatic women with US negative endometriosis

1. Blood tests: minimally invasive
   - 28 biomarker panel: external validation (menstrual)
   - Proteomics: ID peaks, ? Reproducibility

2. Endometrial tests: semi-invasive
   - Nerve fiber: external validation
   - Proteomics: ID peaks, ? Reproducibility

3. Integrate clinical data with biomarker in IT model?