Implantation
Genomics to Microbiomics

Ovarian Club X and CoGEN in Asia

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ObGyn Department, School of Medicine, Stanford University, CA, USA
Inmaculada Moreno
Research manager of Igenomix SL
The Endometrial Factor
The puzzle of the endometrial factor

**Histology**
- Noyes et al., 1950
- Coutifaris et al., 2004
- Murray et al., 2007

**Immunohistochemistry**
- Lessey et al.
- Kliman et al.

**Ultrasound**
- Kasius et al., 2014

**Doppler**
- Kupesick et al., 2001

**Hysteroscopy**
- Rambouts et al., 2016

**Omics**
- Diaze et al., 2011, 2013
- Aghajanova, 2012
- Gargett et al., 2009
- Cervello et al., 2010, 2011, 2012
- Santamaria et al., 2016

**Secretomics**
- Van der Gaast et al., 2002, 2009
- Vilella et al., 2013

**Endometrial SC**
- Gargett et al., 2009
- Cervello et al., 2010, 2011, 2012
- Santamaria et al., 2016

**Microbes**
- Franasiak et al., 2015
- Moreno et al., 2016
Transcriptomics
<table>
<thead>
<tr>
<th>YEAR</th>
<th>TITLE</th>
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</thead>
<tbody>
<tr>
<td>2011</td>
<td>A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature</td>
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<td>2012</td>
<td>The genomics of the human endometrium</td>
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<td>2013</td>
<td>The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity</td>
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<td>2013</td>
<td>Profiling the gene signature of endometrial receptivity: clinical results</td>
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<td>2013</td>
<td>The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure</td>
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<td>2014</td>
<td>Impact of final oocyte maturation using gonadotropin-releasing hormone agonist triggering and different luteal support protocols on endometrial gene expression</td>
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<td>2014</td>
<td>The impact of using the combined oral contraceptive pill for cycle scheduling on gene expression related to endometrial receptivity</td>
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<td>2014</td>
<td>What a difference two days make: “personalized” embryo transfer (pET) paradigm: A case report and pilot study</td>
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<td>2014</td>
<td>Scratching beneath ‘The Scratching Case’: systematic reviews and meta-analyses, the back door for evidence-based medicine</td>
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<td>2014</td>
<td>Transcriptomics of the human endometrium</td>
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<td>2014</td>
<td>Deciphering the proteomic signature of human endometrial receptivity</td>
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**JOURNAL**

- Fertility and Sterility
- Biochimica et Biophysica Acta – Molecular Basis Disease
- Human Reproduction
- Int J Dev Biol
<table>
<thead>
<tr>
<th>YEAR</th>
<th>TITLE</th>
<th>JOURNAL</th>
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<tr>
<td>2014</td>
<td>Clinical Management of Endometrial Receptivity</td>
<td>Semin Reprod Med. 32(5):410-4</td>
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<td>2014</td>
<td>Timing the window of implantation by nucleolar channel system prevalence matches the accuracy of the endometrial receptivity array</td>
<td>Fertility and Sterility. 102(5):1477-81</td>
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<td>2015</td>
<td>Human Endometrial Transcriptomics: Implications for Embryonic Implantation</td>
<td>Cold Spring Harb Perspect Med. 5(7):a022996</td>
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<td>2015</td>
<td>Understanding and improving endometrial receptivity</td>
<td>Current Opinion in Obstetrics &amp; Gynecology. 27(3):187-92</td>
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<td>2015</td>
<td>Is endometrial receptivity transcriptomics affected in women with endometriosis? A pilot study</td>
<td>Reproductive BioMedicine Online. 31(5):647-54</td>
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<td>2016</td>
<td>Diagnosis of endometrial-factor infertility: current approaches and new avenues for research</td>
<td>Geburthilfe Frauenheilkd. 76(6):699-703</td>
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<td>2017</td>
<td>Does an increased body mass index affect endometrial gene expression patterns in infertile patients? A functional genomics analysis</td>
<td>Fertility and Sterility. 107(3):740-748.e2</td>
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<td>2017</td>
<td>Endometrial function: facts, urban legends, and an eye to the future</td>
<td>Fertility and Sterility. 108(1):4-8</td>
</tr>
<tr>
<td>2017</td>
<td>Implantation failure of endometrial origin: it is not pathology, but our failure to synchronize the developing embryo with a receptive endometrium</td>
<td>Fertility and Sterility. 108(1):15-18</td>
</tr>
<tr>
<td>2017</td>
<td>Meta-signature of human endometrial receptivity: a meta-analysis and validation study of transcriptomic biomarkers</td>
<td>Scientific Reports. 7(1):10077</td>
</tr>
<tr>
<td>2017</td>
<td>Window of implantation transcriptomic stratification reveals different endometrial subsignatures associated with live birth and biochemical pregnancy</td>
<td>Fertility and Sterility. 108(4):703-710.e3</td>
</tr>
</tbody>
</table>
Endometrial Receptivity Analysis - ERA

**Methodology**

**MAIN STAGES OF THE ASSAY**

1. **MESSAGE RNA (mRNA) IS OBTAINED**
2. **THE QUALITY OF THE EXTRACTED mRNA IS DETERMINED**
3. **RNA IS SEQUENCED TO ANALYZE THE EXPRESSION OF 236 GENES**
4. **THE DATA OBTAINED IS MEASURED AND THE SAMPLE IS CLASSIFIED BY THE COMPUTATIONAL PREDICTOR**
5. **REPORT**

**Displacement of the WOI**

**Personalization of the embryo transfer**

**Endometrium**

<table>
<thead>
<tr>
<th>HRT CYCLE</th>
<th>Pre-Receptive</th>
<th>Receptive</th>
<th>Post-Receptive</th>
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</thead>
<tbody>
<tr>
<td>Standard WOI (ERA® P+5: R)</td>
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<tr>
<td>Delayed WOI (ERA® P+5: PRE)</td>
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<tr>
<td>Advanced WOI (ERA® P+5: POST)</td>
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</tbody>
</table>

**Endometrium's state**

- Pre-receptive
- Receptive
- Post-receptive

**Embryo**

- Day 3: Keep developing
- Day 5: Implantation
ERA results

- **24,500 patients**
- **54 countries**
  - More than 600 clinics
- **Endometrial biopsy**
  - 65% Receptive
  - 35% Non-receptive

- **87.0% Pre-receptive**
- **0.2% Proliferative**
- **12.8% Post-receptive**
Microbiomics
• Human body have 10 times more bacterial than human cells

• Up to 99% of DNA in humans comes from microbial genes (3M vs 23K)

• Between 1-3% of human weight corresponds to bacteria

• Our body contains bacteria, particularly abundant in the skin and digestive tract

• Between 20 and 60% of these bacteria (depending on location) cannot be cultured under standard laboratory conditions

The Human Microbiome Project (http://hmpdacc.org)
González et al., 2014.Cell 158: 690-690.e1
VAGINAL BACTERIA & HUMAN REPRODUCTION

- Up to 40% of patients undergoing IVF treatments present abnormal vaginal microbiota.
- Bacterial vaginosis (BV) is responsible for:
  - 2-fold increase risk of early miscarriage.
  - >5-fold increased risk of late miscarriage.
  - >3-fold increased risk of premature rupture of membranes.
  - Up to 2-fold increased risk of preterm labor.

Sirota et al. 2014. Semin Reprod Med. 32:35-42
Krauss-Silva et al. 2010. Reprod Health 7:14
## Endometrial pathogens and infertility

<table>
<thead>
<tr>
<th>Study</th>
<th>No. patients analyzed</th>
<th>Patients positive for culture (%)</th>
<th>Pathogens isolated (% patients)</th>
<th>IR (%) positive vs. negative</th>
<th>PR (%) positive vs. negative</th>
<th>MR (%) positive vs. negative</th>
<th>References</th>
</tr>
</thead>
</table>
| Egbase et al. (1996) | 110                   | 54 (49.1%)                         | *Escherichia coli* (25.9%)
*Klebsiella pneumoniae* (9.2%)
*Staphylococcus* sp. (7.4%)
*Streptococcus* sp. (61.1%) | NA                           | 29.6 vs. 57.1 (p < 0.005)                          | NA                           | 64                     |
| Fanchin et al. (1998) | 279                   | 143 (51.2%)                        | Anaerobic (5%)
*Enterobacteriaceae* (5%)
*Enterococcus* sp. (3%)
*Escherichia coli* (64%)
*Haemophylus* sp. (2%)
*Staphylococcus* sp. (3%)
*Streptococcus* sp. (8%)
Miscellaneous (10%) | 6.3 vs. 11.8 (p < 0.01) | 16.8 vs. 27.2 (p > 0.02)                          | 29.2 vs. 24.3 (p < 0.04)                          | 62                     |
| Egbase et al. (1999) | 430                   | 297 (69%)                          | *Enterococcus* sp. (59.4%)
*Staphylococcus* sp. (31.2%)
*Escherichia coli* (1.6%)
Mixed growth (7.8%) | 9.3 vs. 21.6 (p < 0.001) | 18.7 vs. 41.3 (p < 0.01)                          | NA                           | 66                     |
| Moore et al. (2000)  | 127                   | 109 (85.8%)                        | Anaerobic Gram positive (45%)
*Enterococcus* (27%)
*Staphylococcus epidermidis* (75%)
*Streptococcus viridans* (6%) | NA                           | NA                           | 7 vs. 88 (p < 0.0006)                       | 65                     |
| Salim et al. (2002)  | 204                   | 129 (63.2%)                        | Anaerobic (29.5%)
*Escherichia coli* (9.3%)
Gram-positive bacteria (35.6%)
Other Gram negative (5.4%) | 8 vs. 17.3 (p = 0.001) | 16.3 vs. 30.7 (p = 0.002)                          | NA                           | 63                     |
| Selman et al. (2007) | 152                   | 133 (87.5%)                        | *Enterobacteriaceae* (65.1%)
*Staphylococcus* sp.(45%)
*Streptococcus* sp. (28.2%)
Other pathogens (18.3%) | NA                           | 22.2 vs. 77.8 (p < 0.001)                          | 17.6 vs. 82.4 (p < 0.001)                          | 61                     |

... is there a specific endometrial microbiota?

And, if this is so...

... could the endometrial microbiota play a role in endometrial receptivity and pregnancy outcomes?
Evidence that the endometrial microbiota has an effect on implantation success or failure

Inmaculada Moreno, PhD; Francisco M. Codoñer, PhD; Felipe Vilella, PhD; Diana Valbuena, MD, PhD; Juan F. Martinez-Blanch, PhD; Jorge Jimenez-Almazán, PhD; Roberto Alonso; Pilar Alamá, MD, PhD; Jose Remohi, MD, PhD; Antonio Pellicer, MD, PhD; Daniel Ramon, PhD; Carlos Simon, MD, PhD

BACKGROUND: Bacterial cells in the human body account for 1–3% of total body weight and are at least equal in number to human cells. Recent research has focused on understanding how the different bacterial communities in the body (e.g., gut, respiratory, skin, and vaginal microbiomes) predispose to health and disease. The microbiota of the reproductive tract has been inferred from the vaginal bacterial communities, and the uterus has been classically considered a sterile cavity. However, while the vaginal microbiota has been investigated in depth, there is a paucity of consistent data regarding the existence of an endometrial microbiota and its possible impact in reproductive function.

OBJECTIVE: This study sought to test the existence of an endometrial microbiota that differs from that in the vagina, assess its hormonal regulation, and analyze the impact of the endometrial microbial community on reproductive outcome in infertile patients undergoing in vitro fertilization.

STUDY DESIGN: To identify the existence of an endometrial microbiota, paired samples of endometrial fluid and vaginal aspirates were obtained simultaneously from 13 fertile women in prereceptive and receptive phases within the same menstrual cycle (total samples analyzed n = 52). To investigate the hormonal regulation of the endometrial microbiota during the acquisition of endometrial receptivity, endometrial fluid was collected at prereceptive and receptive phases within the same cycle from 22 fertile women (n = 44). Finally, the reproductive impact of an altered endometrial microbiota in endometrial fluid was assessed by implantation, ongoing pregnancy, and live birth rates in 35 infertile patients undergoing in vitro fertilization (total samples n = 41) with a receptive endometrium diagnosed using the endometrial receptivity array. Genomic DNA was obtained either from endometrial fluid or vaginal aspirate and sequenced by 454 pyrosequencing of the V3–V5 region of the 16S ribosomal RNA (rRNA) gene; the resulting sequences were taxonomically assigned using QIIME. Data analysis was performed using R packages. The χ² test, Student t test, and analysis of variance were used for statistical analyses.

RESULTS: When bacterial communities from paired endometrial fluid and vaginal aspirate samples within the same subjects were interrogated, different bacterial communities were detected between the uterine cavity and the vagina of some subjects. Based on its composition, the microbiota in the endometrial fluid, comprising up to 191 operational taxonomic units, was defined as a Lactobacillus-dominated microbiota (>90% Lactobacillus spp.) or a non-Lactobacillus-dominated microbiota (<90% Lactobacillus spp. with >10% of other bacteria). Although the endometrial microbiota was not hormonally regulated during the acquisition of endometrial receptivity, the presence of a non-Lactobacillus-dominated microbiota in a receptive endometrium was associated with significant decreases in implantation [60.7% vs 23.1% (P = .02)], pregnancy [70.6% vs 33.3% (P = .03)], ongoing pregnancy [58.8% vs 13.3% (P = .02)], and live birth [58.8% vs 6.7% (P = .002)] rates.

CONCLUSION: Our results demonstrate the existence of an endometrial microbiota that is highly stable during the acquisition of endometrial receptivity. However, pathological modification of its profile is associated with poor reproductive outcomes for in vitro fertilization patients. This finding adds a novel microbiological dimension to the reproductive process.

Key words: assisted reproductive techniques, bacterial pathogens, embryo implantation, endometrial microbiota, endometrial receptivity array
OBJECTIVE & DESIGN

OBJECTIVE: To demonstrate if the presence of bacterial species in the endometrium play a role in endometrial receptivity and pregnancy outcomes.

Patients analyzed: n=35
Total samples analyzed: n=41
Molecular assessment of endometrial microbiota by NGS

METHODS

1. ENDOMETRIAL/VAGINAL ASPIRATION
2. gDNA PURIFICATION
3. BARCODED BACTERIAL 16S rRNA PCR
4. SEQUENCING
5. DATA ANALYSIS & TAXONOMICAL ASSIGNMENT
Endometrial microbiota profile of infertile patients

Classification into endometrial microbiota profiles

METHOD A: Classification and Regression Trees (CART)

2 target classes
- Live birth (LB)
- No Live birth (No LB)

4 variables
- % Lactobacillus
- % Bifidobacterium
- % Gardnerella
- % Streptococcus

Both models presented a similar conclusion in establishing 90% of Lactobacillus as the cut-off value to predict reproductive outcomes

METHOD B: Generalized linear model (GLM)

Logistic regression:
\[ P(LB) = \frac{e^x}{1 + e^x} \]

where:
\[ x = \frac{\ln p}{1 - p} = -2.359 + 2.554 \times (\% \text{Lactobacillus}) \]

Both models presented a similar conclusion in establishing 90% of Lactobacillus as the cut-off value to predict reproductive outcomes.

Endometrial microbiota profile of infertile patients


Endometrial microbiota profile of infertile patients

Samples 1 to 41
LB: Live birth
MISC: Miscarriage
NP: No Pregnant
NoET: No embryo transfer

Lactobacillus
Roseburia
Ruminococcus
Faecalibacterium
Lachnospiraceae
Blautia
Pseudomonas
Escherichia
Bacteroides
[Ruminococcus]
Allobaculum
Lactococcus
Clostridium[Clostridiaceae]
Clostridiales
Bacillus
Propionibacterium
Veillonella
Streptococcus
Bifidobacterium
Gardnerella

<90% Lactobacillus
Non-Lactobacillus-dominated (NLD) Microbiota

<90% Lactobacillus
Non-Lactobacillus-dominated (NLD) Microbiota

Color Key

0 0.4 0.8
Value
NLD endometrial microbiota is associated with poor reproductive outcome

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Subject</th>
<th>Microbial taxa abundance</th>
<th>Endometrial Microbiome</th>
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<tbody>
<tr>
<td>MISC</td>
<td>9</td>
<td></td>
<td>NLD</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>NLD</td>
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<td></td>
<td>13</td>
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<td>LD</td>
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<td>14</td>
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<td></td>
<td>16</td>
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<td></td>
<td>19</td>
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<td>LD</td>
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</tbody>
</table>

MISC: Miscarriage  
NP: No pregnant  
LB: Live Birth  
LD: Lactobacillus-dominated  
NLD: Non Lactobacillus-dominated

Low abundance of *Lactobacillus* in endometrium is associated with poor reproductive IVF outcomes

**Lactobacillus** abundance in EF is a significant variable to predict pregnancy success in IVF patients.

Linear discriminant analysis (LDA) showing mean values of the most abundant operational taxonomic units (OTUs) in the receptive subjects grouped by their reproductive success.
Low abundance of *Lactobacillus* in endometrium is associated with poor reproductive IVF outcomes

<table>
<thead>
<tr>
<th>Characteristics and Outcomes</th>
<th>LDM (n=17)</th>
<th>NLDM (n=15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>40.06±3.47</td>
<td>39.00±5.09</td>
<td>0.49</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.18±5.18</td>
<td>22.45±4.02</td>
<td>0.30</td>
</tr>
<tr>
<td>Previous pregnancies (n)</td>
<td>1.71±2.44</td>
<td>1.53±2.32</td>
<td>0.84</td>
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<tr>
<td>Previous miscarriages (n)</td>
<td>1.53±2.21</td>
<td>1.14±1.56</td>
<td>0.58</td>
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<tr>
<td>Metaphase II oocytes per cycle (n)</td>
<td>11.94±4.27</td>
<td>10.20±4.81</td>
<td>0.28</td>
</tr>
<tr>
<td>Fertilization rate per cycle</td>
<td>157/203 (77.34%)</td>
<td>118/153 (77.12%)</td>
<td>0.62</td>
</tr>
<tr>
<td>Transferred embryos per cycle (n)</td>
<td>1.65±0.49</td>
<td>1.73±0.59</td>
<td>0.65</td>
</tr>
<tr>
<td>Months between EF and transfer (n)</td>
<td>2.82±2.55</td>
<td>1.80±1.08</td>
<td>0.16</td>
</tr>
<tr>
<td>Pregnancy rate per transfer</td>
<td>12/17 (70.6%)</td>
<td>5/15 (33.3%)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Implantation rate per transfer</td>
<td>17/28 (60.7%)</td>
<td>6/26 (23.1%)</td>
<td>0.02*</td>
</tr>
<tr>
<td>Ongoing pregnancy per transfer</td>
<td>10/17 (58.5%)</td>
<td>2/15 (13.3%)</td>
<td>0.02*</td>
</tr>
<tr>
<td>Miscarriage rates (%)</td>
<td>2/10 (16.7%)</td>
<td>3/5 (60.0%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Live birth rate per transfer</td>
<td>10/17 (58.8%)</td>
<td>1§/15 (6.7%)</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

BMI: body mass index; LDM: *Lactobacillus*-dominated microbiota; NLDM: non-*Lactobacillus*-dominated microbiota; *Chi Square (χ² test) and Student’s t-test were performed; *p-value<0.05; §: Voluntary termination of pregnancy.

• The percentage of *Lactobacillus* in EF, is a significant variable to predict reproductive success (cut-off value: 90%).

• Endometrial microbiota profile can be classified as LD or NLD according to the structure and relative abundance of the bacteria identified in EF. This classification enabled the diagnosis of the endometrial microbiological health of IVF patients and its association with their reproductive outcome.

• An NLD microbiota (specially *Gardnerella* and *Streptococcus*) strongly correlates with adverse reproductive outcomes when compared to subjects presenting an LD endometrial microbiota.
Microbiome in human reproduction

The Microbiome in Human Reproduction

- Altered immune milieu
- Decreased implantation
- Increased early pregnancy loss
- Impaired fertilization
- Altered embryo migration
- Impaired folliculogenesis
- Altered gonadotropin response
- Asthenospermia
- Oligospermia
- Leukospermia
- Increased late pregnancy loss
- Intraamniotic infection
- Preterm birth
- Intrauterine growth restriction
- Puerperal infection neonatal colonization

Franasiak and Scott. 2015. Fertil Steril 104:1341-6
Conclusions

- The existence of non-*Lactobacillus* bacteria in the endometrium negatively impacts reproductive function and should be considered as an emerging cause of implantation failure and pregnancy loss.

- The results presented herein expand the evaluation of endometrial receptivity not only at the morphological and molecular levels but also at the microbiological viewpoint.


**FUTURE PERSPECTIVES**

**Main goals:**

1. To develop a non-invasive diagnosis tool for the assessment of endometrial factor including endometrial receptivity and endometrial microbiome.

2. To test the efficacy of *Lactobacillus* probiotics to improve reproductive outcomes in patients with NLD endometrial microbiota.
**Development of a non-invasive diagnosis tool for the simultaneous analysis of endometrial receptivity and microbiota to improve reproductive outcomes in infertile patients (niERA-MICROBIOME)**

<table>
<thead>
<tr>
<th>PI Study/Coordinator</th>
<th>Prof. Carlos Simón / Inmaculada Moreno / Felipe Vilella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Support</td>
<td>Diana Valbuena / Carlos Gómez</td>
</tr>
<tr>
<td>Site IRB approval</td>
<td>From <strong>July 17th, 2017</strong> to July 17th, 2018</td>
</tr>
</tbody>
</table>

**Study Design**

Competitive, multi-center, international biomedical research study, analytical observational of cohorts, prospective, longitudinal, open, non-randomized and single-group assignment. The results analyzed will be those of their ART.

**Study Population**

ART patients, with indication of diagnosis of endometrial receptivity by ERA that will receive embryo transfer with frozen blastocyst stage embryos (day 5/day 6).
niERA-Microbiome - PROSPECTIVE STUDY

Participant sites worldwide

Active sites

Procreatec & Fertia (Spain)
Oak Clinic Sumiyoshi (Japan)
New Hope Fertility (Mexico)
IVF Florida, Dominion Fertility, MCRM Fertility & RMA CT (USA)
Pregna (Argentina)
Alpha Fertility Center (Malaysia)
Prof. Carlos Simon’s Lab

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