New Bio markers of ovarian reserve: AMH or nucleic acids-what it is the best?

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ART/PGD Department
Arnaud de Villeneuve hospital
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Montpellier-34295, France
# Excessive Age-Related Decline in Functional Ovarian Reserve in Infertile Women: Prospective Cohort of 15,500 Women

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<table>
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<tr>
<th>Age Bands, y</th>
<th>No. of Donors</th>
<th>AFC in Oocyte Donors, Median (IQR)</th>
<th>No. of Infertility Patients</th>
<th>AFC in Infertility Patients, Median (IQR)</th>
<th>P Value</th>
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<td>≤25</td>
<td>3100</td>
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<td>26–27</td>
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39%
Conventional bio markers of OR

Hormonal Tests of Ovarian Reserve (OR)
(d3 of natural cycle)

- Day 3 FSH Levels
- Day 3 Estradiol Levels
- Inhibin B Levels
- AMH Levels

Sonographic Evaluation of OR

- Antral follicle counts (AFC)
- Ovarian volume (not recommended by ASRM, 2015)
- Doppler imaging techniques
Conventional bio markers of OR

- Clinical:
  - age: oocyte quality++

- Biological at d3:
  - FSH/LH/ E2
  - AMH
  - Ovarian volume

- US:
  - AFC

AMH predict OR

Kwee, 2008 FS
ASRM Practice Committee Opinion, 2015

- Basal FSH most commonly used, but AFC and AMH promising due to less variability
- Single basal FSH has limited utility due to variability
- Basal E2 has little value alone
- Very low AMH has high specificity for DOR
- Low AFC has high specificity for DOR
- Pregnancy rates are not improved by waiting for normal FSH (in women with previously elevated level)
- No proven “battery” of tests has been shown to reliably predict DOR
Why new OR bio markers?

- **AMH= good quantitative bio marker of OR**
  - Can not be used alone (age, ovulatory status, AFC, BMI....)

- **AMH= bad qualitative bio marker to predict conception**
  - Low AMH=elevated FSH

- **Age= best criteria for oocyte quality**

- **Normal AMH= non prognosis value**

  - AMH may be the better predictor
  - AMH needs stable assays with reference standards

  - AFC is still an adequate predictor and is easily available
  - AFC needs stricter guidelines to limit intra and interobserver variability
Use of combining Biomarkers to establish OR status

BMI

Age

Ethnicity

Smoking
Applying Genomics medicine to ART

- Predicting Ovarian Stress (OS) and OR
- Predicting Ovarian Responses IVF/ICSI
- Selection Viable Embryo
- IW Identification
- NIPT diagnosis
Cell-free nucleic acids as non-invasive biomarkers of gynecological cancers, ovarian, endometrial and obstetric disorders and fetal aneuploidy

S. Traver1, S. Assou2, E. Scalici1,2, D. Haouzi1, T. Al-Edani1,2, S. Belloccì, and S. Hamamah1,4*
Nucleic acids quantification: cfDNA and microRNAs

Follicle

Oocyte cumulus cells

Endometrium

Blood vessel

Follicular fluid

Serum
Cell-free DNA

- Double-stranded molecules
- Short or long fragments
- Apoptotic or necrotic origin
- No identified physiological role

Circulating microRNAs

- Single-stranded molecules
- 19-25 nucleotides
- Two mechanisms of release
- Degradation of target mRNA and translation inhibition

Schwarzenbach et al., Nature Reviews Clinical Oncology, 2014
Biology of cell-free DNA

- **Small double-stranded DNA molecules**
- **Short** (70-200 base pairs) or **long fragments** (up to 21kb) in bloodstream
- DNA release from **apoptotic, necrotic and/or inflammatory** process
1. Serum collection
   At day 3 of menstrual cycle

2. Cell-free DNA extraction
   (from serum samples)

3. Cell-free DNA quantification by ALU-qPCR

4. Associations with:
   - Ovarian reserve status
   - Ovarian response to stimulation
Cell-free DNA in serum on D3

Cell free DNA level at day 3 of menstrual cycle

- According to patient’s age
- Related to ovarian reserve status
- Prediction of ovarian response to stimulation
- Comparison with AMH

WO 2015197858 A1: Methods employing circulating DNA and miRNA as biomarkers for female infertility.
Cell-free DNA level in serum according to patient’s age

- By including only patients with normal OR (N=40)
- Linear Regression $R^2=0.27$, $p=0.001$
- By using cut off of 38 years: **Significant higher cfDNA levels for women with ≥38 years** compared to women with < 38 years (p=0.03)

Significant and positive association between cell-free DNA levels in serum at day 3 and patient’s age
• cfDNA level in serum was significantly higher in women with LFOR compared with normal OR (p=0.045).

• cfDNA level in serum tended to be higher in PCOS patients compared with normal OR (p=0.05).
Cell-free DNA level in serum at day 3 related to ovarian reserve status

Prediction of LOR (n=38) according to cfDNA at D3

- AUC = 0.71 (0.59-0.81)
- P < 0.001
- Se = 68%
- Sp = 73%

Prediction of PCOS (n=30) according to cfDNA at D3

- AUC = 0.81 (0.69-0.90)
- P = 0.01
- Se = 70%
- Sp = 68%

OR status based on AMH level and AFC at day 3.
cfDNA level was significantly higher in serum samples from women with AMH ≤1 ng/ml or ≤ 2 ng/ml compared to those with AMH>1 ng/ml or > 2 ng/ml, respectively (p=0.004)
Comparison of cell-free DNA levels between women with normal OR, and PCOS

A

B

C

Infertility length (years)
Summary

cfDNA in blood constitute promising tools for infertile patients

- to estimate OR in case of discrepancy between AMH and AFC
- High cfDNA levels in serum could be significantly related to ovarian disorders
- High cfDNA levels in serum could significantly predict a poor ovarian response
- Clinical applications of cfDNA levels in serum for ovarian response prediction
- New predictive and powerful biomarker of ovarian response efficiency.
Circulating microRNAs related to OR

MicroRNAs expression at D3
Circulating microRNAs

- Single-stranded molecules
- 19-25 nucleotides
- Two mechanisms of release
- Degradation of target mRNA and translation inhibition

Schwarzenbach et al., Nature Reviews Clinical Oncology, 2014
Circulating nucleic acids: new source of diagnostic/prognostic biomarkers for many diseases

- Circulating nucleic acids
- Diagnostic biomarkers
- Prognostic tools
- Therapeutic targets

Cancer, gynecological and pregnancy disorders, fetal aneuploidy.

Cell-free nucleic acids as non-invasive biomarkers of gynecological cancers, ovarian, endometrial and obstetric disorders and fetal aneuploidy

S. Traver¹, S. Assou¹,², E. Scalici¹,², D. Haouzi¹, T. Al-Edani¹,², S. Bello³, and S. Hamamah¹,²,⁴,*
<table>
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<tr>
<th>Gynecological pathology</th>
<th>Increased miRNAs</th>
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<th>Decreased miRNAs</th>
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<td>Ovarian cancer</td>
<td>miR-205</td>
<td>Zheng et al. (2013)</td>
<td>let-7f</td>
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<td>miR-103</td>
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<td>Murri et al. (2013)</td>
<td>miR-132 and miR-320</td>
<td>Sang et al. (2013)</td>
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<td>Yang et al. (2012)</td>
<td>let-7c and miR-144</td>
<td>Yang et al. (2012)</td>
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</table>

PCOS, polycystic ovary syndrome; POF, premature ovarian failure.

*a* in follicular fluid.
Let-7b microRNA expression in serum was higher in women (age $\geq 38$ y) compared with those less than 38 (y) old.

At day 3 of natural cycle

$\rightarrow$ Ovarian reserve decrease is associated with high Let-7b level in serum at day 3 of natural cycle

Hamamah team, unpublished data
Comparison microRNA expression profiles and LH at d3 to assess ovarian function

**MiR-30d, miR-191** and **miR-320a** expression decreased significantly in women with high LH levels (>5IU/l) compared to those with normal LH levels (between 3-5 IU/l) (p<0.03; p<0.02; p<0.02, respectively)
Circulating nucleic acids, new biomarkers for OR evaluation and COS responses

‘Compagnon Tests’

Ovary

Ovarian follicle: Granulosa cells Cumulus cells

Bloodstream

Communication through Blood-follicle barrier

Follicular fluid

Biological fluids

Analysis of microRNA expressions
Quantification of cell-free DNA

New Biomarkers for ovarian OS, OR and COS responses

Scalici...; Hamamah et al.2014, 2015
Associations between intra-follicular cell-free DNA levels and follicle size

For all follicles (n=100):
- 8-12mm follicles: High cell-free DNA levels
- 13-18mm follicles: Low cell-free DNA levels
- >18mm follicles: Significant and negative correlation between intra-follicular cfDNA levels and follicle size

For follicles containing oocytes (MI, MII) (n=76):
- 8-12mm follicles: *p=0.024
- 13-18mm follicles: Low cell-free DNA levels
- >18mm follicles: Significant and negative correlation between intra-follicular cfDNA levels and follicle size

Relationship with dynamic and functional follicle state.

Scalici...Hamamah et al., HR 2014
Significant associations between intra-follicular cfDNA levels and ovarian response to COS

- **P=0.008**
  - Nber of days of stimulation
  - ≤ 10
  - > 10

- **P=0.01**
  - Total dose of gonadotropins (IU)
  - < 3000
  - ≥ 3000

- **P=0.045**
  - Number of oocytes
  - ≤ 6
  - > 6

*Use not more than 300 IU*

Traver...Hamamah et al., Plos One 2015
Cell-free DNA levels in serum at D3 and COS

- By including patients undergoing IVF/ICSI procedure (N=31)
- Linear Regression
  $R^2=0.15$, $p=0.03$
- Logistic regression: Prediction of retrieved oocytes by using mutivariate model including AMH and cfDNA levels at day 3

CfDNA levels at day 3 can predict significantly the nbr of retrieved oocyte independently of AMH levels.
Cell-free DNA versus AMH levels in serum at D3 for prediction of oocyte collection

- Patients undergoing IVF/ICSI procedure

Cut off of 6 oocytes

Cell-free DNA
- AUC=0.79 (0.60-0.91)
- P=0.001
- Se=73%
- Sp=82%

AMH
- AUC=0.73 (0.54-0.87)
- P=0.02
- Se=67%
- Sp=81%

CfDNA levels at D3 can predict significantly a poor ovarian response (<6 oocytes) to stimulation with higher Se and Sp than of AMH levels.
ROC curve to evaluate the predictive value of follicular fluid cfDNA level for clinical pregnancy outcome in a multivariate model (including the rank of IVF/ICSI attempts and the number of embryos):

area under the curve = 0.73 [0.66–0.87], sensitivity = 60%, specificity = 88%.

Traver - Hamamah et al 2015
High cell-free DNA levels in follicles: Why?

**PCOS:**
- Increase of apoptosis processes in follicles due to hyperinsulinaemia (Ni et al., 2015)
- Defect of follicular maturation: Increased number of small pre-antral follicles (Scalici et al., 2014)

**Low ovarian reserve:**
- Accelerated apoptosis process (Sadraie et al, 2000; Vital Reyes et al, 2001; Guan et al, 2016)
- Effect of strong and long stimulation by gonadotropins (>10 days and/or ≥ 3000IU of r-FSH) (Liu et al, 2011)

- According to clinical practice: **Ovarian retrieval including follicles with small size** in order to optimize the total number of collected oocytes (Scalici et al, 2014)
Cell-free DNA in follicular fluid, diagnostic and predictive biomarker in IVF process

- Ovarian reserve disorders
  - Follicular maturity abnormalities
  - Increased or accelerated apoptosis process

- Long infertility length
  - Increased stress in infertile couples

- Long and strong stimulation
  - Poor ovarian reserve and/or effect of stimulation treatment by gonadotropin (r-FSH)

- Poor ovarian response
  - Poor quality of follicular micro-environment

- Poor embryo quality
  - Negative effect on early embryo development (Scalici et al., 2014)

- Clinical pregnancy prediction
  - Negative effect on conception

Scalici – Hamamah et al., HR 2014
Scalici - Hamamah et al., Plos One 2015
Circulating microRNAs in follicular fluid, powerful tools to explore in vitro fertilization process

E. Scalici\textsuperscript{1,2,3}, S. Traver\textsuperscript{1}, T. Mullet\textsuperscript{1,2}, N. Molinari\textsuperscript{1}, A. Ferrières\textsuperscript{3}, C. Brunet\textsuperscript{3}, S. Belloc\textsuperscript{5} & S. Hamamah\textsuperscript{1,2,3}
Figure 4. (A) ROC analysis to evaluate FF let-7b expression predictive value for blastocyst formation. (B) ROC analysis to evaluate FF let-7b expression predictive value for expanded blastocyst development. These analyses included only the group of women with normal ovarian reserve (n = 91).
Figure 5. (A) ROC analysis to evaluate FF miR-29a expression predictive value for clinical pregnancy outcome. (B) Comparison of the ROC curves showing the predictive value of FF miR-29a expression and top quality embryo proportion for clinical pregnancy outcome. These analyses included only the group of women with normal ovarian reserve (n = 91).
Figure 6. Schematic model showing that miRNA expression profiling in FF samples provides powerful tools to efficiently discriminate women with polycystic ovary syndrome (PCOS) and to predict IVF outcomes. The expression of some FF miRNAs varies according to the gonadotropin treatment. HP-hMG, highly purified human menopausal gonadotropin; r-FSH, recombinant follicle-stimulating hormone.
Summary

cfDNA and miRNA expression profiling in human FF might provide biomarkers to efficiently discriminate women with PCOS and to predict blastocyst development and clinical pregnancy outcomes.

these new potential biomarkers could be used in the daily practice to improve personalized IVF strategies and to identify new therapeutic targets in female infertility management.
Conclusions

cfDNA and microRNAs in blood constitute promising tools for infertile patients:

- to establish OR in case of discrepancy between AMH and AFC
- to quantify ovarian stress during COS
- to predict patient ovarian stimulation response
- to administrate a protocol « à la carte » for each patient
- to propose new strategies for fertility preservation
ART ‘à la Carte’

• to explore ovarian reserve
• to optimize IVF outcome

We need more Science!
Inserm U 1203
Développement Embryonnaire Précoce Humain et Pluripotence