Institute for Preventive and Social Medicine

Does higher level of serum folate and lower Ca/Mg ratio before ovarian stimulation worsen outcomes of assisted reproductive technologies in normogonadotropic women?

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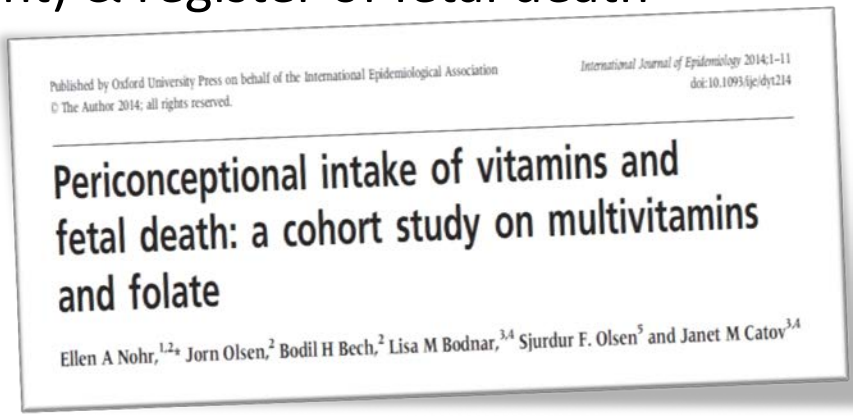
Disclosure

- I have nothing to disclosure
- Mikhail Polzikov is an employee of IVFarma LLC, the parental study NCT03088137 was financed by IVFarma LLC
- Dmitry Blinov serves as a medical affairs consultant to Sanofi, Merck and Dr. Reddy's

Why focus on folate and micronutrients in women?

- Inadequate maternal folate status has been linked to spontaneous abortion and low birth weight (Molloy et al, 2008) and neural tube defects (Hibbard et al, 1965)
- WHO, 2012 recommended dose of 400 mkg/day, including food folate from a diet and/or a supplement
- (Nohr, Olsen et al. 2014) , n=35 914, Denmark
  - Telephone interview (use of supplement) & register of fetal death
  - Any multivitamin use was associated with a increased risk of fetal death [HR 1.23 (1.06–1.42)]
  - However, no association was found between folate-only use & fetal death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Periconceptional intake of vitamins and fetal death: a cohort study on multivitamins and folate

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- Gaskins et al, 2015, using data EARTH study, found that higher serum concentrations of folate and vitamin B-12 before ART treatment were associated with higher live birth rates among a population exposed to folic acid fortification

Study aim

Materials and methods

- To investigate the association of baseline serum micronutrients level before IVF with outcomes of ART in a single ovarian stimulation GnRH-antagonist protocol and fresh embryo transfer
  - using data obtained from a multicenter, randomized, embryologist-blinded, parallel-group, therapeutic equivalence study of follitropin alpha biosimilar
  - ClinicalTrials.gov Identifier: NCT03088137; Barakhoeva, Vovk et al. 2019

Predictors – baseline serum micronutrients level
Folate
Magnesium
Vitamin B-12
Calcium
Calcium/Magnesium Ratio

Outcomes
Total oocytes yield
Total number of matured oocytes
Total number of fertilized oocytes
Biochemical pregnancy
Clinical pregnancy
Live birth rate

Study subjects

Materials and methods

- Women seeking fertility treatment at 3 large Moscow IVF clinics

114 recruited (2017-2018) satisfied to inclusion criteria:

- Ages 20-35 years
- Regular menstrual cycle duration: 21 to 35 days
- Established causes of infertility (tubal and/or male factors)
- First or second attempt at IVF/ICSI
- $18 \leq BMI \leq 30$ kg/m^2
- FSH 10<IU/l and estradiol level <50 pg/ml (cycle day 2-5)
- AMH≥1.0 ng/ml
- $4 \leq AFC \leq 15$

3 excluded

- **2** contraindications to ART
- **1** severe male factor

111 invited

1 refused to participate

110 enrolled and followed up till fertilization (2017-2018)

2 dropped out due to family reason

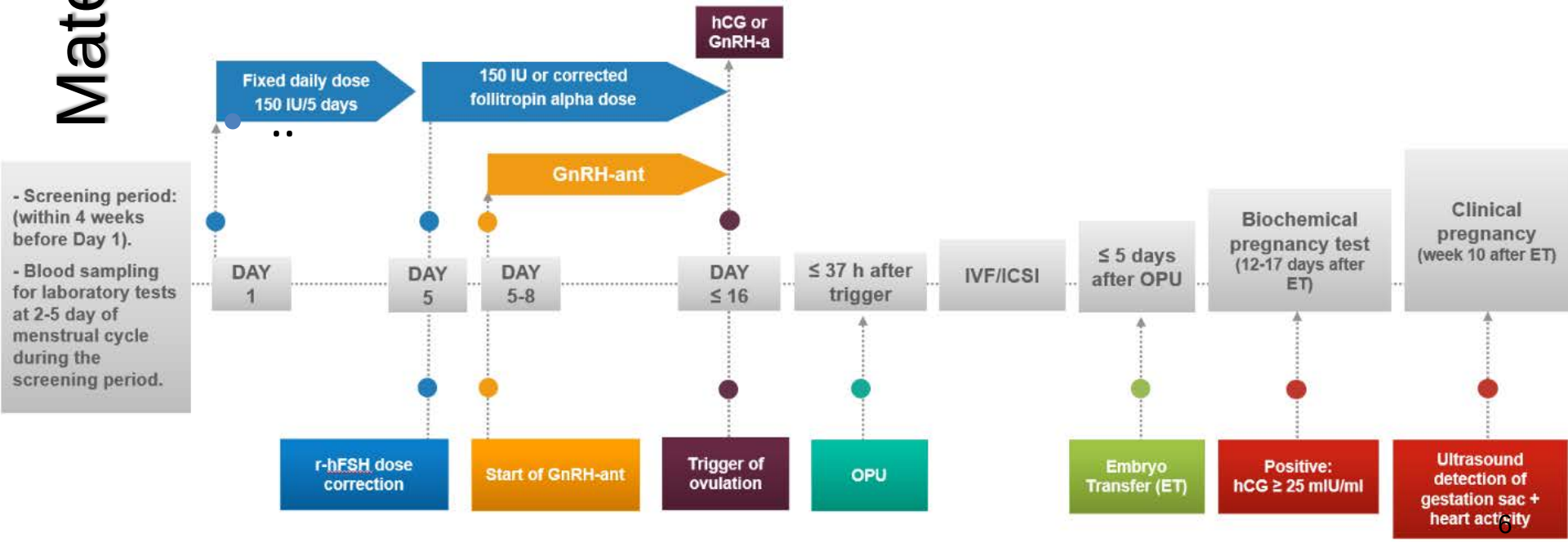
108 followed up till expected delivery (2017-2019)

98 fresh embryo transfer, 1 women – 1 cycle

Materials and methods

Flowchart of the study design

- Single fresh ovarian stimulation using GnRH-antagonist protocol
- Fixed daily dose of recombinant FSH for 5 days
- Biochemical pregnancy test – 12-17 day after embryo transfer
- Clinical pregnancy – 10 week after transfer



Materials and methods

Methods

- Baseline serum
- Serum folate and vitamin B12 were measured by microparticle chemiluminescent immunoassay analyzer Architect i2000 (Abbott, USA) with LOD 0.5 ng/mL and LOQ 1.5 ng/mL for folate, 125 pg/mL and 150 pg/mL for B12, respectively.
  - For folate - upper limit of detection was 49 ng/ml, 9 women ≥49
- Serum Ca and Mg were measured by AAS (atomic absorption spectrometry) using an AA-7000 spectrometer (Shimadzu, Japan), all samples above LOD, mg/L
- Ca/Mg ratio was calculated using values measured in mg/L

Strategy of associations search

Table. *Spearman's correlation matrix of micronutrients and ART outcomes*

Baseline serum micronutrients level	Total oocytes yield	M2 oocytes	Fertilization	Biochemical pregnancy	Clinical pregnancy	Live birth
Folate	-0,13	-0,07	-0,12	-0,05	-0,14	-0,14
B12	0,02	-0,01	0,01	0,01	-0,01	-0,02
Calcium	**-0,22***	-0,11	-0,17	**0,2***	0,15	0,11
Magnesium	**-0,21***	**-0,24***	**-0,26***	-0,13	-0,15	**-0,19***
Ca/Mg ratio	0,05	0,13	0,12	**0,24***	**0,25***	**0,25***

*p<0.05

- Based on univariate regression and correlation analysis
  - Baseline **B12** level was **not associated** with any ART outcomes
  - **Negative** association between **magnesium** and **ART outcomes**
  - **Negative** association (not significant) between **folate** and **ART outcomes**
  - **Multidirectional** association between **calcium** and **ART outcomes**
  - **Positive** association between **Ca/Mg ratio** and **ART outcomes**
- Specific focus on **folates**

Materials and methods

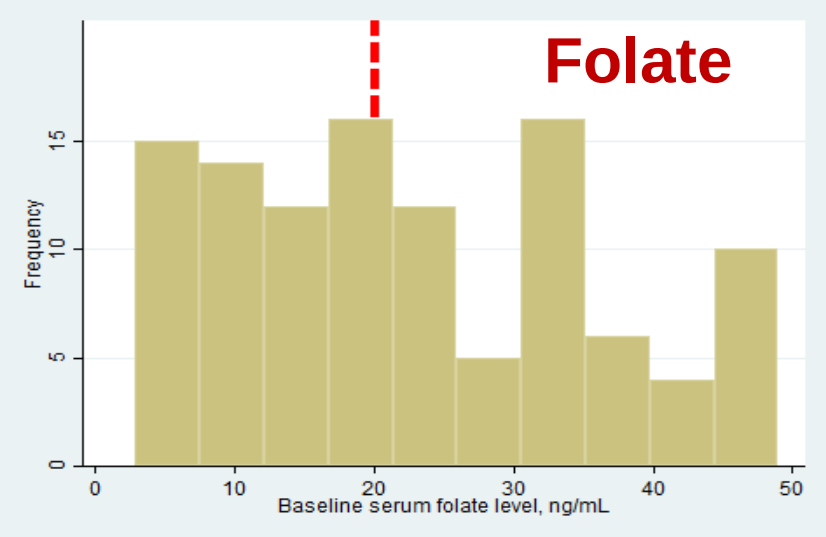
Multivariate regression models

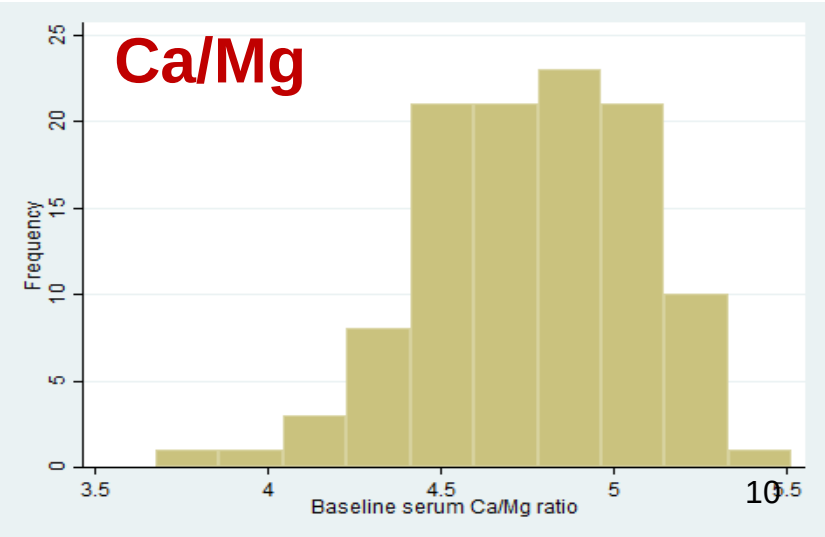
- Generalized linear models
  - A Poisson distribution and log link function - for oocyte counts
  - Binomial distribution and log link function - for pregnancies
- Covariates in “core” model (30+)
  - Age; BMI; infertility duration, infertility cause (male /female factor); number of IVF/ICSI attempt (1 or 2);
  - Type of follitropin alpha (original or biosimilar); duration of FSH stimulation and FSH dose; ovulation trigger (GnRH-a or hCG); embryo transfer day;
  - Baseline serum hormone level (AMH, FSH, LH, estradiol, prolactin, testosterone) and baseline red blood cell count;
  - Baseline hemostasis factors level (APTT, antithrombin, international normalized ratio, prothrombin)
- For each outcome, we fit a full multivariable model including all covariates with univariate $p \leq 0.20$ for that outcome, and reduced to a final model retaining covariates with $p < 0.05$-0.10
- Variables were retained in the final model if they changed the regression coefficient for the primary predictor by 10%.

Study results

Baseline serum micronutrients level

- Median serum folate concentration - 20.55 ng/ml (interquartile range, IQR: 10.8, 32.9 ng/ml)
- Median serum magnesium – 19.4 mg/L (IQR: 18.7, 20.7)
- Median serum calcium – 94 mg/L (IQR: 91.2, 96.4)
- Median serum Ca/Mg ratio - 4.78 mg/L (IQR: 4.55, 5.02)
- Median serum B12 – 350 pg/mL (IQR: 239, 471)
- 110 women were classified into quartiles based on serum micronutrients concentration





Characteristics of 110 women

Study results

	Total group	Serum folate, ng/mL		Ratio Ca/Mg	
		Q1 (<10.8)	Q4 (≥33,0)	Q1 (<4.56)	Q4 (≥5.03)
No. of subjects	110	27	26	28	27
Age, years	30.7 ± 2.8	30.5 ± 3.0	31.0 ± 3.1	31.1 ± 2.8	29.9 ± 2.9
BMI, kg/m^2	22.15±2.87	22.6 ± 3.1	22.3 ± 3.0	21.4 ± 2.6	23.0 ± 3.32
Infertility duration, months	41.6 ± 28.2	42.4 ± 29.9	42.0 ± 23.0	38.3 ± 25.8	37.7 ± 26.7
Infertility diagnosis, n (%)					
Female tubal factor	39 (35.5)	12 (44.4)	6 (23.1)	10 (35.7%)	11 (40.7%)
Male factor	48 (43.6)	13 (48.1)	12 (46.2)	10 (35.7%)	12 (44.4%)
Tubal + male factors	23 (20.9)	2 (7.4)	8 (30.8)	8 (28.6%)	4 (14.8%)
IVF/ICSI attempt					
1st, n (%)	73 (66.4)	18 (66.7)	18 (69.2)	22 (78.6%)	15 (55.6%)
2nd, n (%)	37 (33.6)	9 (33.3)	8 (30.8)	6 (21.4%)	12 (44.4%)
Type of follitropin alpha					
Original, n (%)	55 (50.0%)	15 (55.6%)	12 (46.15%)	11 (39.3%)	16 (59.3%)
Biosimilar, n (%)	55 (50.0%)	12 (44.4%)	14 (53.85%)	17 (60.7%)	11 (40.7%)
FSH dose, IU	1525 ± 260	1492 ± 194	1551±336	1507±238	1501±257
Duration of stimulation, days	9.74 ± 1.05	9.63 ± 0.74	9.89 ± 1.48	9.61 ± 0.99	9.78 ± 1.15
Ovulation trigger					
GnRH-a. n (%)	18 (16.4%)	6 (22.2%)	3 (11.5%)	5 (17.9%)	4 (14.8%)
hCG, n (%)	92 (83.6%)	21 (77.8%)	23 (88.5%)	23 (82.1%)	23 (85.2%)
Embryo transfer day, n (%)					
No embryos transferred	12 (10.9)	2 (7.4)	3 (11.5)	5 (17.9%)	3 (11.1%)
Day 3	20 (18.2)	6 (22.2)	3 (11.5)	4 (14.3%)	6 (22.2%)
Day 5	78 (70.9)	19 (70.4)	20 (79.9)	19 (67.8%)	18 (66.7%)

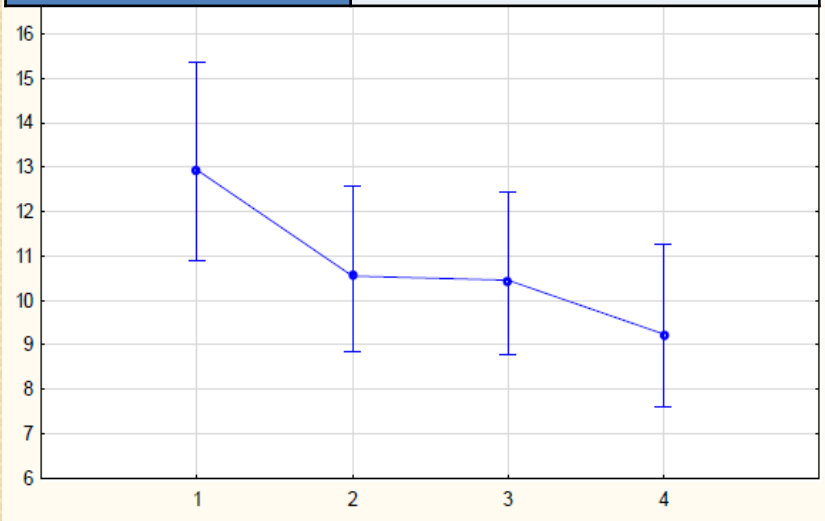
Baseline serum folate, Ca/Mg ratio and total oocyte yield

Study results

- Oocytes retrieved: 11.9 ± 6.8

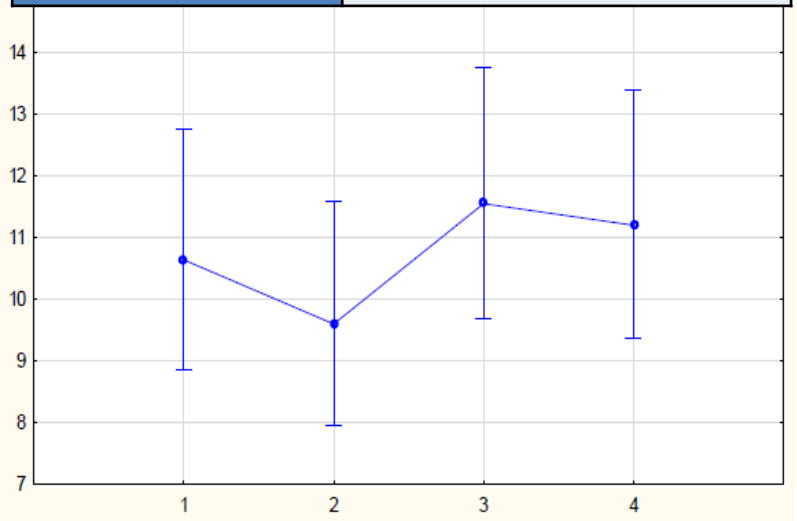
Folate

Quartile (min–max), ng/mL	Total oocyte yield	
	n	Adj mean (95% CI)
Q1 (2.9 – 10.7)	27	12.9 (10.9 – 15.4)
Q2 (10.8 – 20.5)	28	10.6 (8.8 – 12.6)
Q3 (20.6 – 32.9)	29	10.5 (8.8 – 12.4)
Q4 (≥ 33.0)	26	9.2 (7.6 – 11.3)
P-trend	0.006	



Ca/Mg

Quartile (min-max), mg/L	Total oocyte yield	
	n	Adj mean (95% CI)
Q1 (3.68 – 4.55)	28	10.6 (8.9 – 12.8)
Q2 (4.56 – 4.78)	27	9.60 (8.0 – 11.6)
Q3 (4.79 – 5.02)	28	11.6 (9.7 – 13.8)
Q4 (≥ 5.03)	27	11.2 (9.4 – 13.4)
P-trend	0.88	



- GLM with Poisson distribution and log link function
- adjustment for BMI (β-est=-0.04;p=0.01), baseline serum AMH (β-est=0.06;p=0.000); APTT (β-est=-0.02;p=0.01), duration of stimulation (β-est=0.29;p=0.003) and FSH dose (β-est=-0.001;p=0.002); and IVF/ICSI attempts: 0;1; (β-est=-0.12;p=0.01)

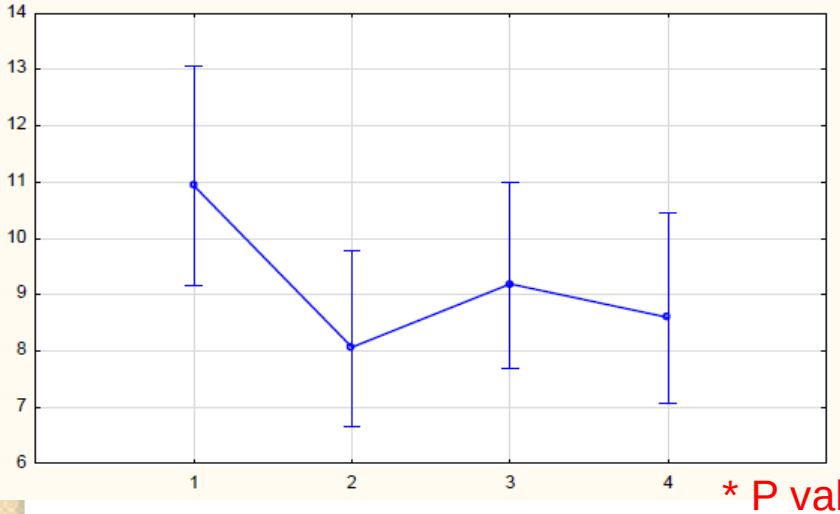
Baseline serum folate, Ca/Mg ratio and total number of matured oocytes

Study results

- MII stage: 9.8 ± 5.9

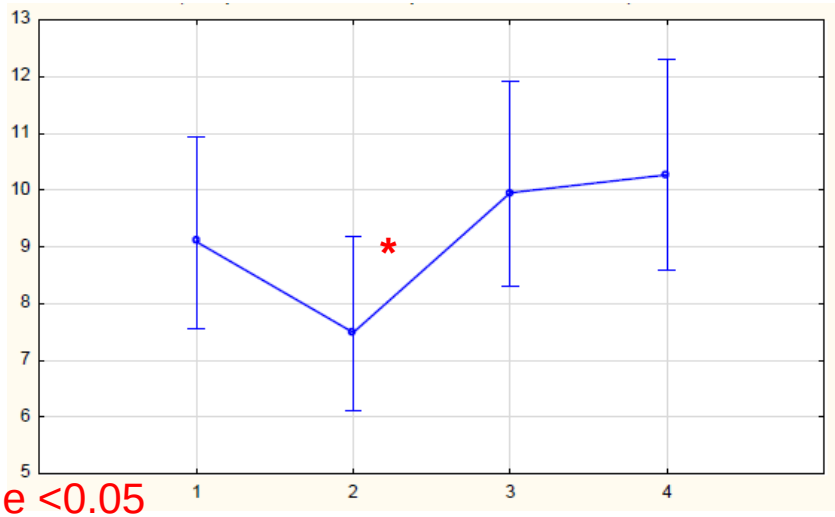
Folate

Quartile (min–max), ng/mL	MII oocytes	
	n	Adj mean (95% CI)
Q1 (2.9 – 10.7)	27	10.9 (9.2 – 13.1)
Q2 (10.8 – 20.5)	28	8.1 (6.6 – 9.8)
Q3 (20.6 – 32.9)	29	9.2 (7.7 – 11.0)
Q4 (≥ 33.0)	26	8.6 (7.1 – 10.5)
P-trend	0.13	



Ca/Mg

Quartile (min-max), mg/L	MII oocytes	
	n	Adj mean (95% CI)
Q1 (3.68 – 4.55)	28	9.1 (7.6 – 10.9)
Q2 (4.56 – 4.78)	27	7.5 (6.1 – 9.2)*
Q3 (4.79 – 5.02)	28	10.0 (8.3 – 11.9)
Q4 (≥ 5.03)	27	10.3 (8.6 – 12.3)
P-trend	0.16	



* P value <0.05

- GLM with Poisson distribution and log link function
- adjustment for BMI (β-est=-0.04;p=0.01), baseline serum AMH (β-est=0.06;p=0.000) and APTT (β-est=-0.02;p=0.01), duration of stimulation (β-est=0.23;p=0.02) and FSH dose (β-est=-0.001;p=0.02)

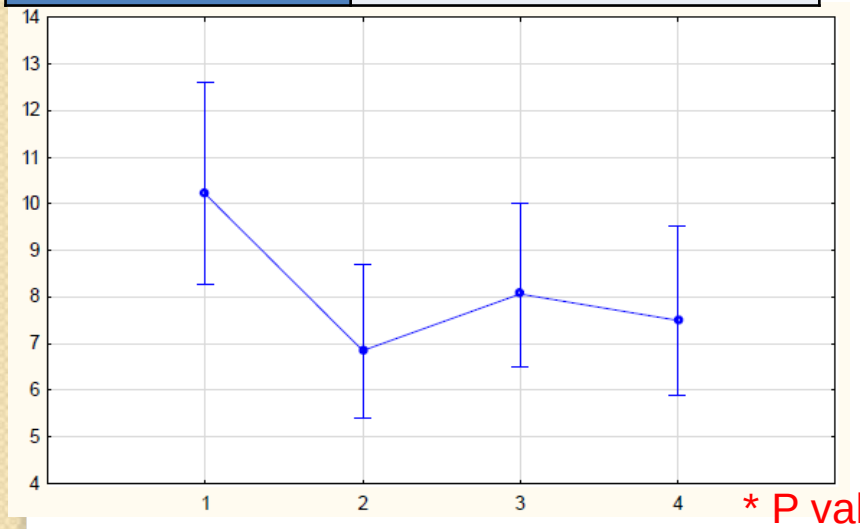
Baseline serum folate, Ca/Mg ratio and total number of fertilized oocytes

Study results

- Zygotes with 2PN: 8.7 ± 6.0

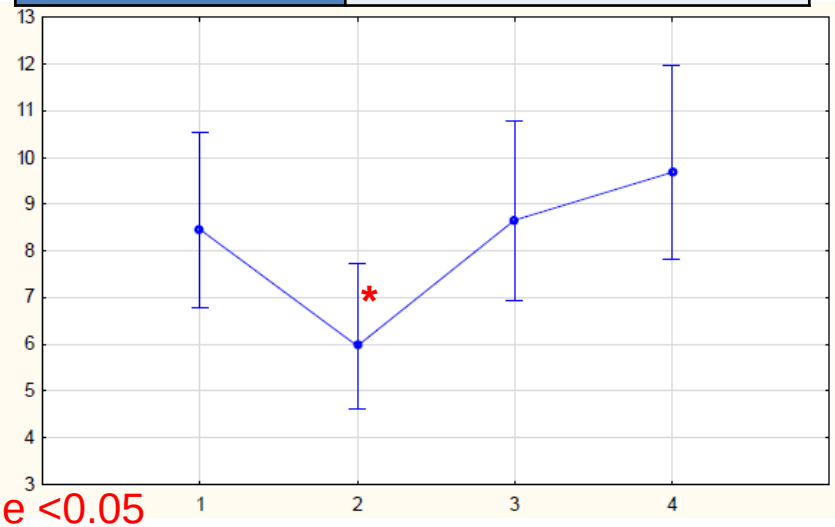
Folate

Quartile (min–max), ng/mL	Fertilization	
	n	Adj mean (95% CI)
Q1 (2.9 – 10.7)	27	10.2 (8.36 – 12.6)
Q2 (10.8 – 20.5)	28	6.8 (5.4 – 8.70)
Q3 (20.6 – 32.9)	29	8.1 (6.5 – 10.0)
Q4 (≥ 33.0)	26	7.5 (5.9 – 9.5)
P-trend	0.16	



Ca/Mg

Quartile (min-max), mg/L	Fertilization	
	n	Adj mean (95% CI)
Q1 (3.68 – 4.55)	28	8.5 (6.8 – 10.5)
Q2 (4.56 – 4.78)	27	6.0 (4.6 – 7.7)*
Q3 (4.79 – 5.02)	28	8.7 (6.9 – 10.8)
Q4 (≥ 5.03)	27	9.7 (7.8 – 12.0)
P-trend	0.15	



* P value <0.05

- GLM with Poisson distribution and log link function
- adjustment for BMI (β-est=-0.05;p=0.02), baseline serum AMH (β-est=0.06;p=0.000) and APTT (β-est=-0.03;p=0.02), duration of stimulation (β-est=0.28;p=0.02) and FSH dose (β-est=-0.001;p=0.07)

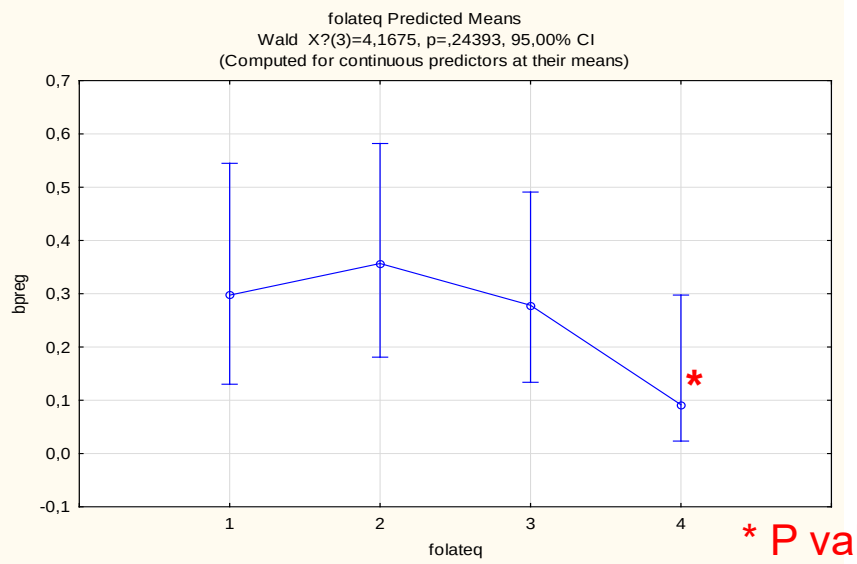
Baseline serum folate, Ca/Mg ratio and biochemical pregnancy

Study results

- hCG≥25 mIU/ml, 12-17 days after ET – 35 of 98 ET – 35.7%

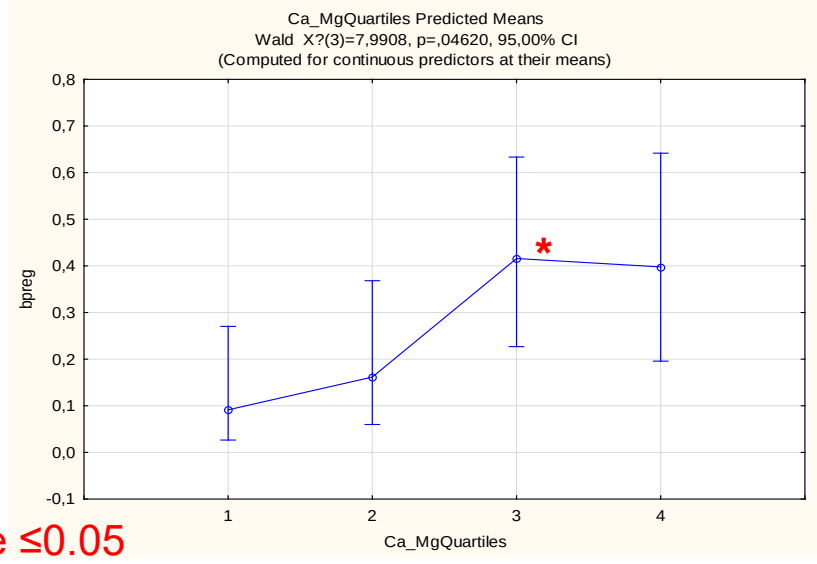
Folate

Quartile (min–max), ng/mL	Biochemical pregnancy	
	Cases (%)	OR (95% CI)
Q1 (2.9 – 10.7)	8/27 (29.6)	1.0 (ref)
Q2 (10.8 – 20.5)	11/28 (39.3)	1.3 (0.3, 5.2)
Q3 (20.6 – 32.9)	11/29 (37.9)	0.9 (0.2, 3.6)
Q4 (≥ 33.0)	**5/24 (20.8)**	**0.2 (0.04, 1.4)***
P-trend	0.08	



Ca/Mg

Quartile (min–max), mg/L	Biochemical pregnancy	
	Cases (%)	OR (95% CI)
Q1 (3.68 – 4.55)	4/27 (14.8)	1.0 (ref)
Q2 (4.56 – 4.78)	7/27 (25.9)	1.9 (0.4, 9.5)
Q3 (4.79 – 5.02)	**12/27 (44.4)**	**7.1 (1.5, 34.6)***
Q4 (≥ 5.03)	12/27 (44.4)	6.6 (1.3, 33.0)
P-trend	**0.000**	



* P value ≤0.05

- GLM with binomial distribution and log link function
- adjustment for red blood cells (β-est=2.27;p=0.004*)*, baseline serum FSH (β-est=0.45;p=0.003) and antithrombin (β-est=-0.09;p=0.004)

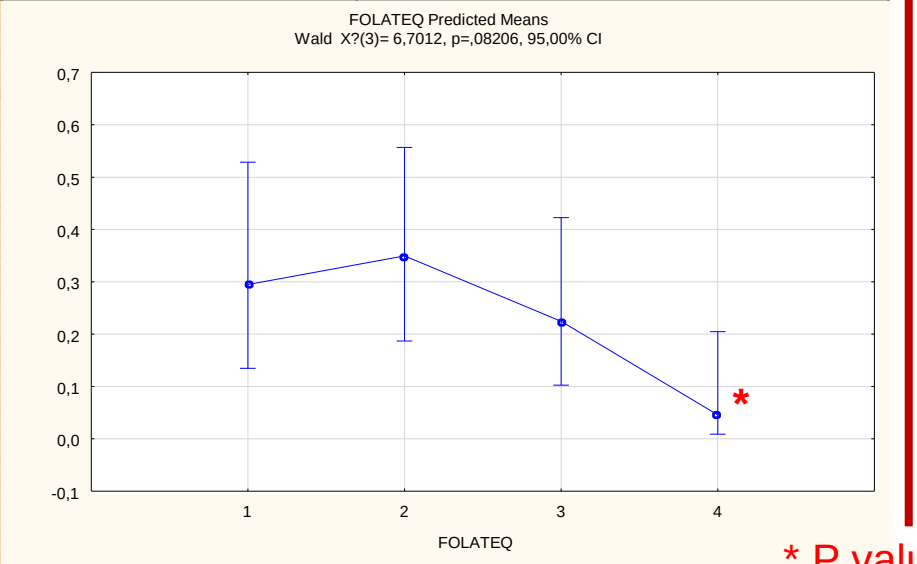
Baseline serum folate, Ca/Mg ratio and clinical pregnancy

Study results

- US detection of gestational sac, 10 wks after ET– 29 of 98 ET – 29.6%

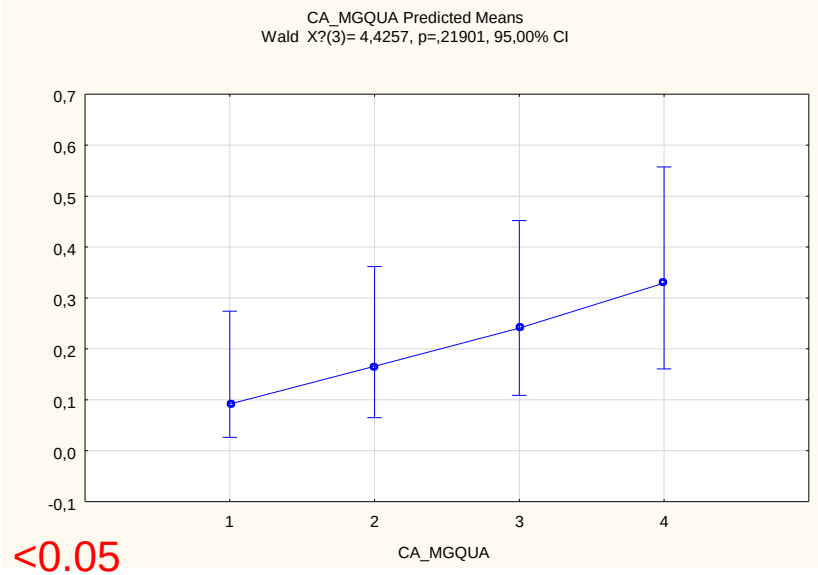
Folate

Quartile (min–max), ng/mL	Clinical pregnancy	
	Cases (%)	OR (95% CI)
Q1 (2.9 – 10.7)	8/27 (29.6)	1.00 (ref)
Q2 (10.8 – 20.5)	10/28 (35.7)	1.3 (0.4, 4.7)
Q3 (20.6 – 32.9)	9/29 (31.0)	0.7 (0.2, 2.6)
Q4 (≥ 33.0)	**2/24 (8.3)**	**0.1 (0.02, 0.8)***
P-trend	**0.000**	



Ca/Mg

Quartile (min–max), mg/L	Clinical pregnancy	
	Cases (%)	OR (95% CI)
Q1 (3.68 – 4.55)	3/27 (11.1)	1.0 (ref)
Q2 (4.56 – 4.78)	6/27 (22.2)	2.0 (0.4, 9.9)
Q3 (4.79 – 5.02)	9/27 (33.3)	3.1 (0.7, 14.9)
Q4 (≥ 5.03)	11/27 (40.7)	4.9 (1.0, 23.1)
P-trend	**0.000**	



* P value <0.05

- GLM with binomial distribution and log link function
- adjustment for red blood cells (β-est=1.71;p=0.02*)* and baseline serum FSH (β-est=0.32;p=0.03)

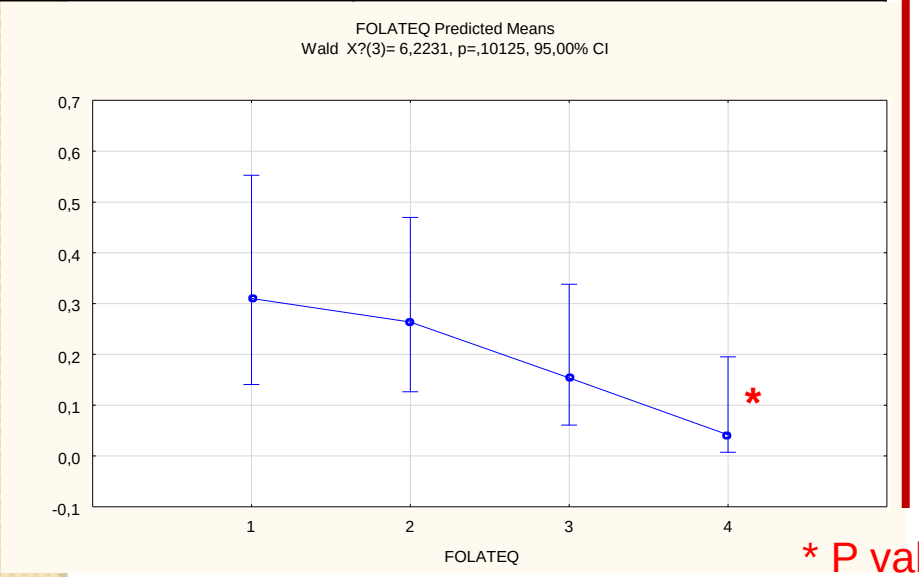
Baseline serum folate, Ca/Mg ratio and live birth

Study results

- 25 of 98 embryo transfer – 25.5%

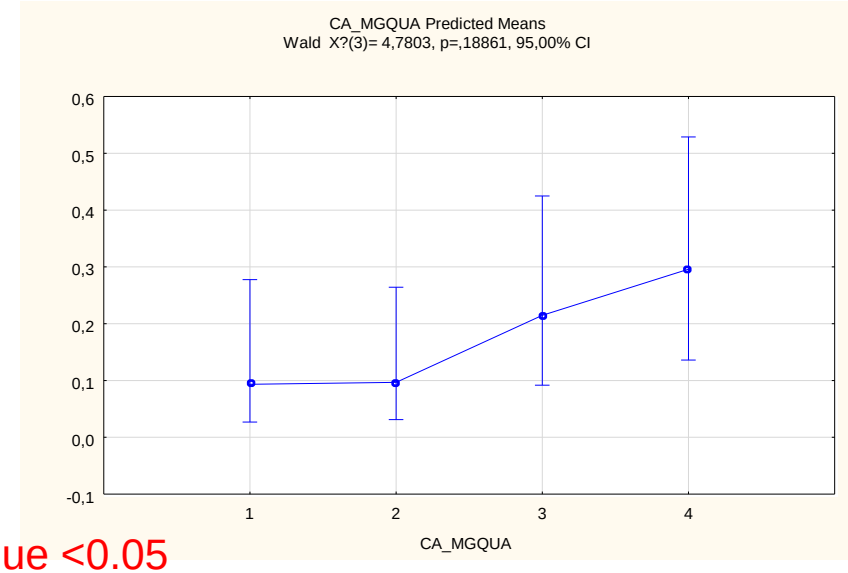
Folate

Quartile (min–max), ng/mL	Live birth	
	Cases (%)	OR (95% CI)
Q1 (2.9 – 10.7)	8/27 (29.6)	1.00 (ref)
Q2 (10.8 – 20.5)	8/28 (28.6)	0.8 (0.2, 3.1)
Q3 (20.6 – 32.9)	7/29 (24.1)	0.4 (0.1, 1.7)
Q4 (≥ 33.0)	2/24 (8.3)	0.1 (0.01, 0.7)*
P-trend	0.000	



Ca/Mg

Quartile (min–max), mg/L	Live birth	
	Cases (%)	OR (95% CI)
Q1 (3.68 – 4.55)	3/27 (11.1)	1.0 (ref)
Q2 (4.56 – 4.78)	4/27 (14.8)	1.0 (0.19, 5.7)
Q3 (4.79 – 5.02)	8/27 (29.6)	2.7 (0.5, 12.9)
Q4 (≥ 5.03)	10/27 (37.0)	4.1 (0.8, 19.9)
P-trend	0.000	



* P value <0.05

- GLM with binomial distribution and log link function
- adjustment for red blood cells (β-est=1.77;p=0.03*)* and baseline serum FSH (β-est=0.36;p=0.02)

Discussion

Strengths and limitations

- Strengths:
  - Single IVF protocol with fresh embryo transfer
  - Women aged 20-35 years without endocrine pathologies/endometriosis/PCOS/obesity
  - Many controlled factors as potential confounders
  - Prospective design
- Limitations:
  - 3 accredited clinical laboratories for lab tests of folate, B12, Ca, Mg
  - Due to sample limitation the level of folates was not measured in the red blood cells
  - No assessment of diet as well as multivitamin and supplement consumption
  - Small sample size

Conclusions

- In multivariable models, among non-obese, normogonadotropic women aged 20-35 years
  - baseline higher serum folate concentration (>33.0 ng/mL) was associated with worse ART outcomes including biochemical, clinical pregnancies and live birth rate
  - baseline lower serum Ca/Mg ratio was associated with worse clinical ART outcomes in dose-dependent manner
- Our findings might be useful for choose of safe dosage of folate supplementation for both fertile women and women undergoing infertility treatment
- Further pre-conception large-scale studies with known micro- and macronutrient status of both parents, including daily doses, serum folate, Ca, Mg, homocysteine and hormone levels are needed

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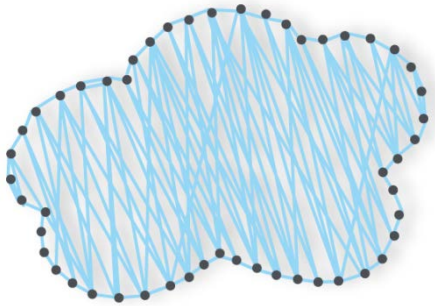

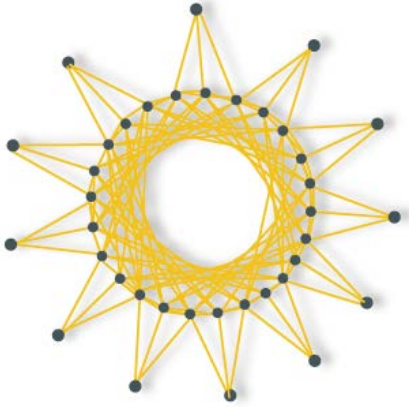

Thank you for attention!

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